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CONTENTS

Childhood Brain Lesions: 15 years Experience of King Abdulaziz University Hospital (1995-2010)

Hessa M. AlJhdali and Awatif A. Jamal

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Abstract: Pediatric age brain lesions can be of neoplastic and non-neoplastic nature, the latter include: congenital malformations, inflammatory processes, vascular and cystic lesions. One of most concerning brain pathology in childhood age is CNS tumors. Malignant brain tumors are the second most common type of pediatric cancer after leukemia. Cancer of the brain and central nervous system comprised 17% of malignancies in children younger than 20 years of age . In Saudi Arabia childhood CNS cancer accounted 11.3% of all childhood cancers. The current study presented the experience of King Abdulaziz University Hospital regarding Childhood Brain Lesions diagnosed over 15 years period (1995 to 2010) considering frequency, morphological pattern and the demographic data (age distribution and gender) of these lesions and further compared the findings with the national and international experience. A retrospective study conducted using a computerized search of the archives of Pathology Department at King Abdulaziz University Hospital in Jeddah; from 1995 tell 2010 to retrieve all the brain cases inclusive of all brain regions. In 15 years period 71 cases (25.1%) out of total brain lesions (283 cases) were childhood brain lesions. Non-neoplastic lesions were 40.8% and neoplastic lesions were 59.2%. Congenital malformations (23.9%) were the commonest non-neoplastic brain lesions, while neuroepithelial tumors ranked first among neoplastic lesions and accounted for 25.4% of childhood brain lesions (CBL) in the study. The astrocytic tumors comprised the majority of the glial tumors (94.4%) with mean age of 8.3 years and M: F ratio 1.4:1. The pilocytic astrocytoma represented 64.7% of all astrocytic tumors. The second malignant tumor was embryonal tumors (medulloblastoma) and accounted for 18.3 % of CBL with male predominance. In conclusion, a single institute experience was reported revealing that primary CNS tumors were the commonest brain lesions in the pediatric age. Furthermore, in concurrence with the national and international experience, astrocytic tumors ranked as first primary CNS tumor of childhood age, followed by medulloblastoma.
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Keywords: Pediatric; brain; lesion; neoplastic; congenital malformation; inflammatory; medulloblastoma

1. Introduction

Pediatric age brain lesions can be of neoplastic and non-neoplastic nature and include benign or malignant, primary or secondary neoplasms, as well as congenital malformation, inflammatory or parasitic mass, different types of cysts and vascular lesions [1-2]. One of most concerning brain pathology in the childhood pediatric age group is brain tumor because children with CNS cancer do not share the favorable prognosis of those with many other common pediatric neoplasms [3]. Cancer of the brain and central nervous system comprise 17% of malignancies in children younger than 20 years of age and the leading cause of cancer death among children [4]. Malignant brain tumors are the second most common type of pediatric cancer after leukemia and it is the most common solid tumor [3-4]. The annual incidence rate of all primary non-malignant and malignant brain and central nervous system tumors is 16.5 cases per 1000,000 person/ year, and in pediatric age, the annual incidence rate for primary non–malignant and malignant brain and central nervous system tumors is 4.5 cases per 100,000 person/ years [5]. In the United state approximately 2200 children younger than the age of 20 are diagnosed with a brain tumor each year [3].

Brain tumor represents 22% of the total childhood cancers (< 15 years of age) in Europe, and account for 16% of total childhood cancer in Latin America, 4% in Africa and 19% in East Asia [6]. Studies from the Central and Eastern provinces of Saudi Arabia reported lower incidence of childhood primary CNS tumors. **Akhtar & Reyes** [7] showed that Lymphomas (Hodgkin's disease accounted for 34%, Non-Hodgkin's Lymphoma accounted for 32%) was the commonest tumors among the children under 14 years, leukemias accounted for 19.35% and brain tumors ranked third and accounted for 6.45% of pediatric age tumors. **Ibrahim** [8] documented a lower rate of CNS neoplasms in the Eastern province. The total cases of primary CNS tumors identified per annum were 43 cases, the incidence of primary CNS neoplasms was 3.1/100,000 of the total population and it was 2.9/100,000 in Saudis. Saudi Cancer Registry 2007 [4], reported that childhood cancers accounted for 7% of all cancers among Saudis and brain (CNS) cancer accounted for 11.3% of all childhood cancers and it is second cause of childhood cancer after Leukemia which accounted for 34.6% in Saudi children. The incidence of childhood primary CNS neoplasms in Saudi Arabia is far less than the

incidence reported in North America & Europe, but it is similar to that reported for Chinese and black Americans and it is less than the one reported by the Ashkenazi or Safari Jews [8].

Gurney *et al.* [3] reported that the most frequent encountered brain tumor is astrocytomas which accounted for 52% of childhood brain tumors, followed by cerebellar PNET (medulloblastoma) which accounted for 21%, ependymomas accounted for 9%, and other gliomas accounted for 15%.

Ansari & Al-Hilli [10] showed that the incidence of malignant brain and spinal cord neoplasms in Bahrain is very low and might be attributed to the small number of Bahraini population, inefficient registration of cancers, and the lack of routine hospital autopsies. Amongst the Bahraini population, astrocytomas were the most common tumor seen in children and comprised 25% of malignant primary CNS tumors. Medulloblastoma ranked as second malignant primary CNS tumor and accounted for 16.9% of all primary CNS neoplasms. Astrocytomas and medulloblastoma were the commonest primary malignant CNS neoplasms in both adults and children.

Jamjoom[1] revealed that neuroepihelial tumors were the most common intracranial neoplasm (39.7% of the total) and they constituted 73% of all brain tumors below the age of 15 years. **Memon *et al.*** [11] & **Zakrzewski *et al.*** [12] supported the fact that astrocytoma is the most frequent childhood tumor. However, institutional based studies carried in Pakistan by **Ahmed *et al.*** [13] documented predominance of medulloblastoma and paucity of astrocytoma. Furthermore, **Nasir *et al.*** [14] study reported that medulloblastoms did account for 33.3% and astrocytoma accounted for 24.7% of all primary brain tumors among children.

Cerebral neoplasms are heterogeneous tumors in regard to histology and clinical course; there are several classification systems to describe CNS tumors. The World Health Organization Classification System [15] and the American Collage of Pathologists update [16] were used in the current study to classify CNS neoplasms.

The objective of the current study to report all Childhood brain lesions diagnosed at King Abdulaziz University Hospital over a period of 15years (from 1995 to 2010), including the frequency, the histo-pathological entities encountered, the age distribution and the gender of the patients. Furthermore, it compared the study findings with national and international experience.

2. Materials and Methods

A retrospective study performed using a computerized search of the archives of Histopathology Department at King Abdulaziz University Hospital in Jeddah from 1995 tell 2010 to retrieve all the brain cases inclusive of all brain regions. The data was collected using appropriate morphological SNOMED codes (Systematized Nomenclature of Medicine) obtaining the following information, receiving date of the specimen, Hospital identification number, demographic information (age & gender), clinical diagnosis, topography and morphology information. Data double checked, exported to Microsoft Excel program and SPSS program (version14) for analysis. Out of the 283 cases of brain lesions retrieved from the archives between 1995- 2010, Seventy one cases (71) represented the study group of childhood brain lesions, the cut off age range used to represent the pediatric age group in the present study is from 0 day to 18 years.

The pathological reports and available H&E sections as well as the available performed immune-histochemical stained sections, were collected and revised by the pathologist. Detailed radiological imaging as MRI images or both CT and MRI imaging studies information before and after treatment were available from the Radiology Department for the majority of the cases. Detailed Clinical data and follow-up data of the patients were beyond the scope of the study, except for the concise and brief information attached to the pathology request.

3. Results

The current retrospective study is based on histo-pathological review, associated with radiological correlation, of total brain lesions received during 1995 – 2010 at the Department of Histopathology at King Abdulaziz University (KAUH).

A total of 283 brain surgical cases were received and were 152 (53.7%) male cases and 131 (46.3%) female cases. M: F ratio was 1.2:1 and the age ranged between 0day-99 years (mean age 33.5 years). The cases were divided into two age groups, Adult brain lesions and Childhood brain lesions (Table 1).

71 cases out of 283 cases of total brain lesions were childhood brain lesions and accounted for 25.1% of the total brain lesions with mean age of 6.9 years and M: F ratio of 1.6:1. The frequency of various histological childhood brain lesions in the study were: congenital malformations accounted for 23.9%, infection and inflammatory lesions accounted 12.7%, benign lesions accounted for 4.2% and primary brain tumors accounted for 59. 2%. The total primary brain tumors were neuroepithelial tumors accounted for 25.4%, and non-neuroepithelial tumors accounted for 33.7% (Figure 1). The most common childhood brain tumors diagnosed were the neuroepithelial tumors 18 cases accounted for 25.4% and 13 cases of embryonal tumors and accounted for 18.3% of total brain lesions. Mesenchymal and meningeal tumors were 5 cases and accounted for 7% of benign tumors and sellar region tumors and germ cell tumors each accounted for 4.2% (Figure 1). Embryonal tumors and tumors of the

mesenchymal and meninges, showed male predominance.

Table 1. The Percent of Total Childhood Brain Lesions out of Total Brain Lesions (1995-2010) at KAUH.

Total Brain Lesions= 283 case	Age Range	M:F Ratio	% of Total Brain Lesions-283
Adult Brain Lesions= 212 cases	**19-99 yrs (Mean= 45.3yrs)**	**1.1 M: 1 F**	**74.9%**
Childhood Brain Lesions= 71 cases	**0day-18yrs (Mean= 6.97yrs)**	**1.6 M: 1 F**	**25,1%**

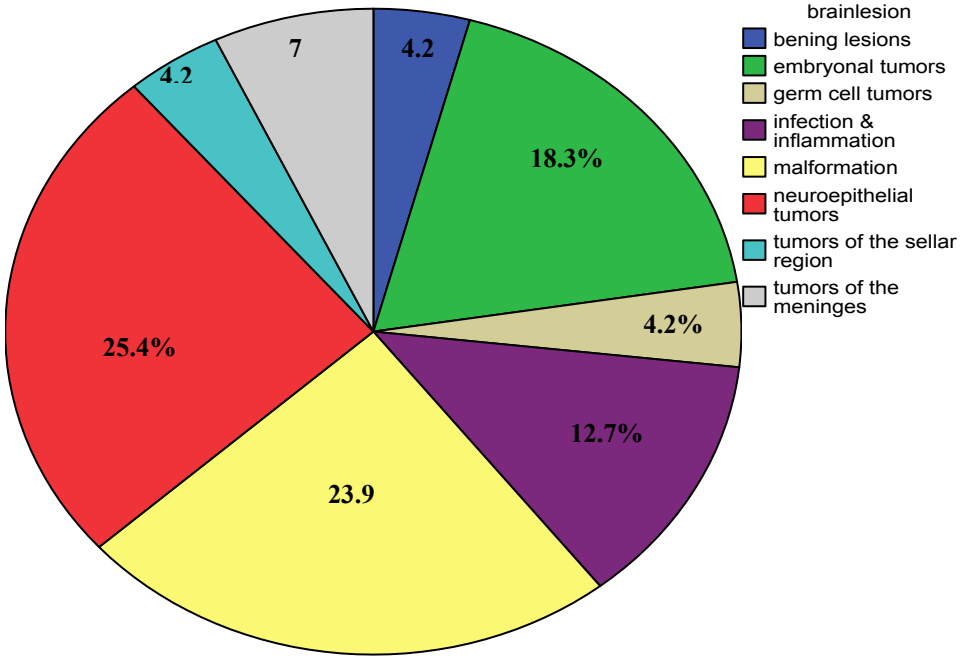

Figure 1. Frequency of the Various Histological Childhood Brain Lesions (1995-2010) at KAUH

The Glial astrocytic tumor (94.4%) accounted for the majority of the neuroepithelial tumors, 17 cases were diagnosed with age ranging between 1- 18 years (mean age 7.9 years) and M: F ratio of 1.4: 1. All grades of Glial astrocytic tumors according to WHO 2007 were identified in this study [15-16]. The pilocytic astrocytoma (Grade I) represented 64.7% of all astrocytic tumors with mean age of 8 years old and M: F ratio of 1.2:1 (Table 2). Clinically these neoplasms occurred mainly in the posterior fossa or cerebellum and histologically composed of fibrillary glial tissue with highly vascularized tumor exhibiting compact and loose areas. The compact areas show bipolar piloid cells with long-hair like processes associated with Rosenthal fibers. Microcytic areas and eosinphilic granular bodies admixed with protoplasmic astrocytes represented the loss textured areas. The Grade II neoplasms accounted for 17.6% with age distribution between 5-12 years old. These neoplasms occurred in various lobes mainly frontal and temporal and showed the classical diffuse neoplastic astrocytic cells proliferation in a fibrillary background with the cells exhibiting mild pleomorphism and hyperchromasia. Grade III the anaplastic glioma occurred in a 10 years old child and accounted for 5.9%, histologically the neoplasm expressed higher morphological atypia and cellularity. Grade IV encountered in 2 cases histologically showed high grade cytological atypia with bizarre nuclei, frequent abnormal mitosis, variation of appearance from one filed to another associated with the classical necrosis and increased glomoriloed blood vessels proliferation.

Congenital malformations was the second encountered brain lesions (10 encephalocele, 4 meningocele and 3 myelomeningocele); this group represented the youngest age group (mean age 2.8 years). The next category was infection and inflammation forming inflammatory masses within the brain and prompting a biopsy for histo-pathological diagnosis and accounted for 12.7% of all brain lesions (4 cases were abscesses, 2 granulation tissue, 1 aspergillosis, 1 edema with necrosis, 1 chronic non-specific inflammation). Almost equal M: F ratio was found and the mean age was 11.2 years. Embryonal tumors were third in line (12 cases medulloblastoma and 1 case neuroblastoma) and observed in males only with mean age of 5.3 years; no female cases identified in the study. The available pathological- radiological

correlations of the medulloblastoma cases were obtained. Data about the tumor site, tumor size, the presence of metastasis or residual tumor after treatment was collected. Majority of the cases were reported as classic medulloblastoma (83.3%) displaying anaplastic features, including increased nuclear size, marked cytological pleomorphism, numerous mitoses and pseudo-rossites. In five cases the tumor was located in the midline of the cerebellum, one case showed evidence of dorsolumbar spine metastasis, one presented with residual tumor after surgical resection and two of the patients deceased after diagnosis (Table 3).

Table 2. Percentages of Various Grads of Neuroepithelial Lesions and Age Range

Neuroepithelial Tumors= 18 cases	Age Range
Astrocytic tumors = 17 (94.4%)	**(Mean= 7.9yrs)**
Grade I = 11 cases (64.7%)	**1 – 18 yrs**
Grade II = 3 cases (17.6%)	**5 – 12 yrs**
Grade III = 1 case (5.9%)	**10 yrs**
Grade IV = 2 cases (11.8%)	**9 – 10 yrs**
Ependymal Tumors = 1 (5.6%)	**1yrs**

Table 3. Embryonal Tumors at KAUH (1995-2010).

Medulloblastoma (MB)= 12 cases	Age Range	Size Range of The Tumor	Site	Metastasis
Classic MB = 10 **Desmoplastic MB = 1** **Neuroblastic MB = 1**	**2-10 yrs** **Mean= 5.7yrs**	**3-6.6 cm** **Mean= 4.3cm**	**Midline= 5** **RT cerebellum= 4** **Disseminated=1** **Not available = 2**	**1 (8.3%)** **Dorsal lumbar spine**

There were 5 cases of meningeal/ mesenchymal tumors according to the WHO classification (4 mesenchymal tumors and one meningioma). All were diagnosed in males with mean age of 10.2 years. The meningioma case was anaplastic type grade III and occurred in a 15 years old male. The other mesenchymal tumors included 2 haemangiomas, one lipoma and one myxoma. The tumors identified in the sellar region were 3 cases, 1 case was craniophrangioma and two were pituitary adenomas. The germ cell tumors were also 3 cases (2 mature teratoma and one mixed germ cell tumor) with mean age of 5.6 years and M: F ratio of 1: 2. The last category comprised 3 cases of benign lesions including two vascular lesions and one benign meningoepithelial cyst (Table 4).

4. Discussion

In Saudi Arabia the experience of childhood brain lesions in general and brain tumors in particular is not extensively documented. Unfortunately many of the epidemiological studies that focused on brain tumors are confounded by selection bias conferred by sole reliance on tertiary referral centers and tertiary referral hospitals in the various regions of the kingdom. No integrated unified Brain Cancer Registry is instituted. However, the small number of the reported studies attempted to give mainly an estimate of the frequency and incidence of brain tumors in this pediatric age group, the frequent histological type, the age range distribution and gender.

Brain lesions in general can be caused by injury, infectious diseases, exposure to certain chemicals or ionizing radiation, problems with the immune system, and other factors [3]. Although different environmental exposure was proposed to be related to the development of childhood brain tumors; many investigators claim that childhood brain tumors reflect the inherent risk associated with the complex process of normal development rather than a response to an external toxic insult [4-17].

The current study over a period of 15 years estimated the frequency of brain lesions and highlighted the institute experience with the pediatric age group brain lesions (non-neoplastic and neoplastic lesions). 40.8% were non-neoplastic and 59.2% were neoplastic lesions. Congenital malformations were the commonest non-neoplastic brain lesions while astrocytoma was the most frequent tumor in the pediatric age group.

Only few studies included neoplastic and non-neoplastic brain lesions, **Jamjoom** [1] conducted a study on 212 brain cases diagnosed at King Khalid University Hospital in Riyadh over a period of 5 years and reported that the non-neoplastic brain lesions represented only 13% of the total brain lesions and that neoplastic brain lesions represented 87% of all brain lesions. Abscess, granulomas, gliosis and mucocele

were among the non-neoplastic brain lesions and represented 50%, 42.8%, 3.6% and 3.6% respectively of the total intracranial space occupying non-neoplastic lesions. Neuroepithelial tumors ranked first neoplastic lesions and comprised 39.7% of the total intracranial neoplasms and 73% of all brain tumors seen below the age of 15 years [1].

Table 4. Childhood Brain Lesions at KAUH (1995-2010).

Brain Lesions (Frequencies)	Age Range	Gender Ratio
Congenital Malformations = 17 cases **Encephalocele = 10** **Meningocele = 4** **Meningomyelocele = 3**	**0 day – 15 yrs (Mean= 2.8yrs)**	**1 M : 1.1 F**
Infection and Inflammation = 9 cases **Abscess = 4** **Granulation tissue = 2** **Aspergillosis = 1** **Edema with Necrosis = 1** **Chronic Non-specific Inflammation = 1**	**1-18 yrs (Mean= 11.2yrs)**	**1 M : 1.3 F**
Benign Lesions = 3 cases **Vascular Lesion = 2** **Benign Meningioepithelial Cyst = 1**	**3-16 yrs (Mean= 7.7yrs)**	**1 M : 2 F**
Neuroepithelial Tumors = 18 cases	**1-18 yrs (Mean= 7.9yrs)**	**1.6 M : 1 F**
Non- Neuroepithelial Tumors = 24 cases **Embryonal Tumors = 13** **Tumors of the Mesenchem & Meninges = 5** **Tumors of the Sellar Region = 3** **Germ Cell Tumors = 3**	**1 day-18yrs (Mean= 7.5yrs)**	**5 M: 1 F**

Table 5. Morphological Distribution of Childhood Brain Tumors, Comparison of Current Study and other Published Studies

References	Location	Period of Study	Total (n) of all tumor cases	Most common Pediatric Tumor
Current study	Saudi Arabia, Jeddah	1995 – 2010	42	Astrocytic tumors 40.5%
Rickert *et al.*, 2001	Germany	2001	340	Astrocytic tumors 37.6%
Zakrzewski *et al.*, 2003	Poland	1990 – 2003	216	Astrocytic tumors 41.5%
Rosemberg *et al.*, 2005	Brazilian	1974 – 2003	1195	Astrocytic tumors 32%
Ahmad *et al.*, 2007	Karachi, Pakistan	1989 – 1998	81	Astrocytic tumors 34.6%
Nasir *et al.*, 2010	Islamabad, Pakistan	Jan1998 – July2010	231	Medulloblastoma 33.3%
Saavedra *et al.*, 2011	Puerto Rico	2002 – 2007	136	Astrocytic tumors 31%

The current study showed higher percentage of non-neoplastic lesion in comparison with **Jamjoom** [1] experience, this might be attributed to the study design, number of cases, and the study period, furthermore, it might reflect advancing in the imaging techniques and diagnostic expertise as well as treatment modalities. However, the study consensus with the generated experience from the various regions of the kingdom, and it demonstrated that neuroepithelial tumors, mainly glial tumors, are the most frequent brain tumors encountered in the pediatric age group and accounted for 25.3 % of all childhood brain lesions with slightly higher frequency in male and M: F ratio of 1.4:1. Pilocytic astrocytoma was the leading tumor in this age group in the study and represented 64.7% of all astrocytic tumors with M: F ratio of 1.2:1. The second most frequent tumor was embryonal tumor (medulloblastoma) which accounted for 18.3 % and showed male predominance. The current study findings compatible with **Gurney *et al.*** [3], were they reported male predominance of the childhood brain tumors. However, many national and international studies showed male predominance of both astrocytic and medulloblastoma tumors [12, 13, 18, 19].

The literature displayed a dispute between astrocytoma and medulloblastoma tumors as which compete for the highest frequency. The current study

confirm the previous experience which revealed preponderance of astrocytoma as was reported by **Richert *et al.*** [18] were he reported that astrocytoma comprising 37.6%, followed by medulloblastoma 17.7%. Similar reports by **Zakrzewski *et al.*** [12], **Rosemberg *et al.*** [19], **Memon *et al.*** [11], and **Ahmad *et al.*** [13] were their experience revealed the prevalence of astrocytic tumors as the leading childhood brain tumor followed by medulloblastoma. Furthermore, they reported that Pilocytic astrocytomas are the most frequent grade encountered among the Glial tumors confirming the finding of the current study. Furthermore, **Ansari & Al-Hilli** [10] observed that among the Bahraini population, astrocytic tumors are common in children and representing 25% of primary CNS tumors but with predominance of diffuse fibrillary astrocytoma. Medulloblastoma ranked as second malignant primary CNS tumor and accounted for 16.9% of all primary CNS neoplasms.

In contrast to the current study finding, Saudi Cancer Registry had meduloblastoma as the first leading childhood brain tumor in their series [9, 14]. **Nasir *et al.*** [14] also reported medulloblastoma to be the first ranking children brain tumors under the age of 14 years with a mean age of 6.2 years and they accounted for 33.3% of their cases. Astrocytoma was the second most dominant type accounted for 24.7%, with a mean age of 6.7 years. This discrepancy in findings between studies could be attributed to variation in the ethnicity, race, and selection bias and to the size of the study population. Furthermore, it might be related to the selected cutoff age range of childhood age, which varies between studies especially if we consider the various peak of occurrence of some brain tumors.

Classical type medulloblastoma was the dominant type in our study and other studies with mean age of occurrence 5.7 years [14, 20, 21]. **Rodriguez *et al.*** [22] reported that 73% of the medulloblastoma cases were classical type, 45% occur in cerebellar hemisphere.

The number of cases presented in the current study is smaller in comparison to the reported national and international studies [12, 13, 14, 19, 23] (Table 5). This might be attributed to ineffective case registration, or difficulty in access the established health care system like the University center, or it may be due to limitation of the diagnostic facilities (such as radio-imaging techniques or histopathological diagnostic experts) in early years. Another plausible explanation could be the low ethnic- racial predisposition in the Eastern region of the Kingdome, or a lower prevalence of the risk factors resulting in lower brain lesions / tumors frequency/incidence in the Eastern region. This latter proposal is detected in South Africa population where the incidence of intracranial neoplasm's in whites was higher than in blacks [4].

5. Conclusion

This is single institute study presenting the King Abdulaziz University Hospital experience with childhood brain lesions over a period of 15 years. The results were consistent with national and international experience regarding the childhood neoplastic and non-neoplastic brain lesions. The findings call for a large scale nationwide study to determine the incidence and prevalence of the childhood brain tumors and provide better categorization of this important pediatric pathology among Saudi population.

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Propolis versus Daktarin® in mucosal wound healing

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Abstract: The aim of this study was to compare the effect of propolis versus daktarin on mucosal wound healing. Fifty two albino rats were randomly divided into three groups; G 1 (propolis), G 2 (daktarin) and control group. Following the induction of a surgical mucosal wound in the labial mucosa by means of a 1-mm punch-biopsy instrument, biopsy specimens were taken on days 1, 3, 7 and 14 from groups of sacrificed rats and stained with haematoxylin-eosin stains, Mallory's trichrome stain as well as CD68 immunohistochemical stain. Data were analyzed statistically. Histological evaluation of each specimen was done and scoring criteria were used to compare the healing status of wounds. There was no statistical significance between different groups, on day 1. However there was statistically significant difference between G1 and both G2 and control group on day 3. On day 7, statistically significant difference was found between G1 and control group, but there was no statistically significant difference between G1 and G2. Conclusion: Propolis has an enhancing effect on the healing of oral mucosal wounds compared to daktarin.

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Key words: Propolis; Daktarin®; Labial mucosa; CD68 Immunohistochemical stain.

1. Introduction:

Oral mucosal wound or mouth ulcers are sores or open lesions in the mouth which are caused by various disorders [(1)]. Wounds are not just a physical hindrance due to blood loss or tissue damage, but they may threaten the individual survival by development of infection and sepsis due to invasion of micro-organisms or contaminants. Mucosal wounds occur frequently, and the healing of the mucosa is important in most surgical outcomes. And although wound healing in the oral mucosa is improved by sound surgical principles, yet it is also mediated by biologic processes beyond the surgeon's control [(2)]. It should also be noted that ulcers and/or erosions can be the final common manifestation, often clinically indistinguishable, of a wide and complex spectrum of conditions including traumatic lesions, infectious, vesiculo-bullous, neoplastic and gastrointestinal diseases [(3)].

The immediate imperative of the body is to close the wound and prevent establishment of infection [(4)]. Clinically mucosal wound healing in oral cavity occurs by 5 to 7 days [(5, 6)]. It achieves this objective by the means of a rapid and robust inflammatory response, with recruitment of neutrophils, macrophages and lymphocytes to the wound site. This is followed by fibroplasia, ECM synthesis and reorganization [(4)]. This is especially important in the oral cavity which is colonized and contaminated with numerous micro-organisms [(4)].

Thus it is crucial for the oral mucosa to heal healthily and quickly.

Propolis is a golden-dark brown resinous substance that worker bees gather and pack on their hind legs from the sap of trees, shrubs and flower blossoms, the resinous substance of propolis is then carried back to their colony combined with beeswax then used by the bees as a sealant and sterilant in and around the hive. Propolis has a protecting role for the bee colony. Beehives have been found to be more sterile than most modern day hospitals [(7)].

Hundreds of publications have appeared in the last 40 years describing the biological and health enhancing properties of propolis[(8)]. Propolis has been documented to have many positive medical effects in many fields including an antibacterial, antiviral and antifungal effect [(9-11)]. Also propolis was found to have an effect against parasites [(12,13)] as an antiulcer (stomach, skin, buccal) [(14,15)]as well as an antioxidant[(10)] . Researches have segregated and tested single substances in propolis; however, it is likely that the presence of a large number of products in propolis may produce a synergistic effect greater than the sum of the effects of individual components [(16)]. Studies evaluating the efficacy of isolated constituents have demonstrated minimal effectiveness compared to the natural compound[(17)]. Similarly **Ahn *et al.*,** [(8)] stated that the health

enhancing effects are found in the ethanol extractable part of propolis called balsam.

In spite of the big compositional differences of the different propolis types depending on its botanical origin, it is astonishing that the biological effects of the different propolis types are very similar. Antibacterial activity has been demonstrated against both, gram positive and gram negative bacteria, both aerobic and anaerobic types [18].

Moreover, in vitro antiviral activity of propolis has been attributed to a synergistic action of both flavonoid and flavanol components in propolis[19]. Daktarin® oral gel contains the active ingredient miconazole. Miconazole is an antifungal medicine used to treat infections with fungi and yeasts [20]. Miconazole also has some antibacterial action and kills certain bacteria that may also be present in the infection [21, 22].

However many side effects had been associated with its use in some patients, and the most commonly reported side effects include: nausea, vomiting, diarrhea, allergic reactions and hepatitis [23]. Since daktarin® is widely used in the oral cavity as compared to propolis, it has been the aim of the present study to evaluate the efficiency of propolis in healing oral mucosal ulcers in comparison with daktarin®.

2. Materials and Methods

This study was undertaken in the department of Oral Histology and the department of Oral Pathology, Faculty of Oral and Dental Medicine, Cairo University, Egypt, based on an ethical approved protocol.

Fifty two 3-months old male rats (albino rats) were selected, with an initial weight ranging between 220 and 240 grams. The rats were kept in housing cages (polyethylene, 16×40×30 cm), six animals per cage, with standardized food and water, under a light/dark cycle of 12 h. The cages were kept in a room which had a constant temperature of 25±1°C. In order to prevent the animals from coming in contact with their feces and/or urine, a permeable metal floor was installed in the cages, separating the rats from the lower part of the cage.

All surgical procedures were performed under general anesthesia, by intramuscular administration of 0.1 ml of ketamine hydrochloride (SIGMATEC Company) combined with 0.05 ml of xylazine hydrochloride (ADWIA Company), per 100 g body weight of the animal. After anesthesia, the labial mucosa was antiseptically cleaned with 2% chlorhexidine then a surgical mucosal wound was made in the labial mucosa of all animals by means of a 1-mm punch-biopsy instrument (Acu-Punch, Acuderm Inc., Ft. Lauderdale, FL, USA). The wounds were done so that their depths would include the submucosa.

The same investigator performed all the surgical procedures.

The animals were randomly divided into three groups as follows; group1 (G1; n=20) was treated with propolis applied to the wound site on the labial mucosa three times daily, group2 (G2; n=20) was treated with daktarin applied to the wound site on the labial mucosa three times daily, and group 3 (G3; n=12) was left to heal spontaneously and served as the control group.

Within each group, the rats were subdivided such as 5 rats of G1, 5 rats of G2 and 3 rats of G3 were consecutively sacrificed on days 1, 3, 7 and 14.

The type of propolis used in this study was Bee Propolis extract (Honey paste) (Y.S. Organic Bee Farms 2774N. 4351 Rd. Sheridan, IL 60551 USA). While the Daktarin® used was miconazole nitrate (Janssen Cilag. Pharm. N.V., Turnhoutseweg 30, B-2340 Beerse, Belgium)

Biopsy samples were fixed with 10% formalin and embedded in paraffin for histological examination. Specimens were cut into 5-µm sections and stained with:

1. Haematoxylin-eosin (HE) stains.
2. Mallory's trichrome stain used for collagen fibers identification.

For immunohistochemical procedure: Sections of 4-µm thickness were mounted over optiplus slides. These slides were electrically charged. The sections were de-paraffinized and rehydrated, rinsed in PBS and incubated in PBS containing H_2O_2 for ten minutes. The primary antibody used was the monoclonal mouse anti-human CD68, clone KP1 (code N1577 Dako). The tissue sections were incubated with the primary antibody over night in moist chamber at 4˚C then rinsed with PBS, 3 times 2 minutes each. The sections were labeled with a streptavidin- biotin method using Dako- LAB vision Catalog CA94539). The sections were visualized with freshly prepared solution containing 3, 3 diaminobenzidin DAB the sections were finally counterstained with methylgreen and viewed by light microscope.

The cellular staining pattern of anti-CD68, is granular cytoplasmic of variable intensity. The expression of macrophages was determined by counting the CD68-positive stained cells in 5 fields of magnification 400. The means and standard deviations were recorded for each group, and one-way ANOVA was used to compare the differences of

the means of the groups and determine significant differences as well as Paired Student's t-Test to compare between each two groups at every time point.

The number of CD68-positive cells was expressed as [(number of +ve cells)/per 400 x visual field]

Slides were coded and microscopically examined to be evaluated for the histological parameters of wound healing [24, 25] including the amount of granulation tissue, inflammatory infiltrate collagen fiber deposition as well as endothelial cells in each wound.

Histological evaluation:

Scoring criteria were adopted after **Sultana *et al.,*** [26] to compare the healing status of wounds in an ascending order for specific points as follows: Amount of granulation tissue (Profound - 1, Moderate - 2, scanty - 3, absent - 4). Inflammatory infiltrate (Profound - 1, Moderate - 2, few - 3), Collagen fiber orientation (Vertical - 1, Mixed- 2, Horizontal - 3), Amount of early collagen (Profound - 1, Moderate - 2, Minimal - 3, Absent - 4). Amount of mature collagen was (Profound - 1, Moderate - 2, Minimal - 3). In addition, dilated blood capillaries and endothelial cells proliferation was added as a scoring criterion as follows (Profound proliferation - 1, Moderate proliferation- 2, capillary dilatation only - 3).

Total healing score of each case was calculated by adding the score of individual criteria. Lower scores indicated poorer wound healing. While higher scores pointed to a better healing process.

Healing status was graded as follows:

Good (16 - 19), fair (12 - 15) and poor (08 - 11) [26].

3.Results

Histological examination of the groups sacrificed on day one:

The control group showed moderate inflammatory cell infiltration (neutrophils and macrophages) in the control cases. The cells with positive expression of CD68 (CD68+ macrophages) in control group were [(9.80 ± 3.70)/per 400 x visual field].

Endothelial cells were noticeably dilated.

No excessive granulation tissue was seen as well as no collagen fiber deposition as seen both histologically and by Mallory's trichrome stain. (Fig.1)

Group 1 treated with propolis showed few inflammatory cell infiltrations (neutrophils and macrophages) in all cases. (Fig.2) CD68+ macrophages were [(6.00 ± 2.24)/per 400 x visual field]

Endothelial cells showed normal number and architecture with the exception of one case which revealed dilated blood vessels.

No excessive granulation tissue was seen as well as no collagen fiber deposition.

Group 2 treated with daktarin showed moderate inflammatory cell infiltration (neutrophils and macrophages) in 80% of cases and mild inflammatory cell infiltration in 20%. CD68+ macrophages in group 2 were [(8.40 ± 3.78)/per 400 x visual field]

Using ANOVA test and Paired Student's t-Test the difference in mean macrophage count (CD68+ macrophages) did not show any statistical significance between different groups, $P > 0.05$ (Tables I-IV and histogram I)

All cases revealed dilated blood vessels and 40% of them their dilatation was extreme.

No excessive granulation tissue was seen as well as no collagen fiber deposition

Histological examination of the groups sacrificed on day three:

The control group showed profound inflammatory cell infiltration (neutrophils and macrophages) in control cases. (Fig. 3) The cells with positive expression of CD68 (CD68+ macrophages) in control group were [(22.0 ± 6.82)/per 400 x visual field] .

Endothelial cells showed proliferation and attempts of excessive blood vessel formation.

Excessive granulation tissue was seen as well as collagen fiber deposition in a mesh like pattern.

Interrupted epithelization covered the granulation tissue.

Group 1 treated with propolis showed a few inflammatory cell infiltrations (neutrophils and macrophages) in 4 cases (Fig. 4) while 1 case still showed moderate inflammatory cell infiltration. CD68+ macrophages in group 1 were [(10.8± 4.09)/per 400 x visual field]

Endothelial cells showed normal number and architecture.

No excessive granulation tissue was seen. A small amount of well organized horizontally oriented collagen fibrils were seen.

Evidence of early epithelization was seen in all cases of group 1

Group 2 treated with daktarin® showed profound inflammatory cell infiltration (neutrophils and macrophages) in one of cases, moderate inflammatory cell infiltration in 3 of the cases and few inflammatory cell infiltration in one case. The cells with positive expression of CD68 (CD68+

macrophages) in group 2 were [(24.8± 5.50)/per 400 x visual field]

Using ANOVA test, there was a highly significant difference in mean macrophage count (CD 68+) between different groups, (*P*=0.004). (Table V and histogram II). Using Paired Student's t-Test CD68+ macrophages in group 1 were significantly lower than those in both control group and group 2, *P* <0. 01. However, no statistical significance was found between G2 and control group *P*>0.05 (Tables VI-VIII)

All cases revealed dilated blood vessels but no endothelial proliferation. A moderate amount of granulation tissue was seen as well as collagen fibrils deposition. Some of the collagen fibrils showed disorganization while some were well organized.

Evidence of early epithelization was seen in all cases of group 2 except the one showing profound inflammatory cell infiltration. (Fig. 5)

Histological examination of the groups sacrificed on day seven:

The control group showed a marked decrease in inflammatory cell infiltration with only sporadic chronic inflammatory cells seen in the examined fields. The cells with positive expression of CD68 were [(15.2± 5.31)/per 400 x visual field].

A slight increase in the number of blood vessels than normal was seen, as well as more than group 1 and group 2.

Granulation tissue still persisted under an incompletely epithelized wound surface which showed re-epithelization delay compared to the other two groups. Collagen bundles showed some organization, yet with delayed collagen build up and delay in scar maturation compared to the other groups. Collagen showed mainly a wavy or longitudinal organization of the fibers (Figs. 8 & 9) .

Group 1 treated with propolis showed almost no inflammatory cells infiltration with normal number and architecture of the blood vessels. CD68+ macrophages were [(5.40 ± 2.07)/per 400 x visual field] (Fig. 6).

No excessive granulation tissue was seen. And the collagen bundles showed complete organization. Complete epithelization was seen (Fig. 10) .

Group 2 treated with daktarin showed very few inflammatory cells in 2 cases and few inflammatory cells in 3 cases. The cells with positive expression of CD68 were [(8.60 ± 1.67)/per 400 x visual field] (Fig. **7**).

Using ANOVA test, there was a highly significant difference in mean macrophage count (CD 68+) between different groups, (*P*=0.004). (Table IX and histogram III). Using Paired Student's t-Test CD68+ macrophages in group 1 were significantly lower than those in both control group and group 2, *P* <0. 01. Moreover, there was a statistical significance between G2 and control group ($p<0.05$and >0.01) (Tables X-XII)

All cases revealed normal vascularity. Granulation tissue was scanty and showed complete epithelization. Collagen fibers showed good organization, with few areas showing a mixed horizontally oriented and mesh like pattern of collagen organization (Fig.11)

Histological examination o f the groups sacrificed on day fourteen:

All three groups including the control group, group 1 and group 2 showed clearance of all inflammatory cells and normal vascularity. No granulation tissue was seen. And the collagen bundles showed complete organization. Complete epithelization was seen. No histopathological differences were seen between the different groups.

The progression of healing was assessed histologically on the basis of individual scoring criteria adopted after **Sultana *et al.,*** [26] used on days 1, 3, 7 and 14. The results showed that wounds healed progressively with time.

On day 1 healing status was poor for all examined cases. On day 3 healing status was poor for all control group, as well as 40% of G1 and 80% of G2. However, 60% of G1 and 20% of G2 showed fair healing. On day 7; healing status was fair for all control group, as well as 40% of G1 and 80% of G2, while 60% of G1 and 20% of G2 showed good healing. On day 14, healing status was fair for 50% of control group, as well as 40% of G2. But, healing was good for 100% of G1 and 60% of G2 as well as 50% of control group.

From the above findings it can be summarized that the peak of inflammatory cells infiltration was seen at the control group not treated by either propolis or daktarin, with declining cell numbers in all groups after one week. The group treated with propolis showed a noticeable low inflammatory cell infiltrate at all time points along the study. The cells with positive expression of CD68 (CD68+ macrophages) were significantly lower than both control group and group 2 at day 3 (*P* <0. 01), but was significantly lower compared to control group only on day 7. Inflammatory cells completely cleared by day 14 for all groups.

All cases showed dilated blood vessels with variable intensities on day 1, however only the control group showed endothelial cell proliferation on day 3 which persisted till day 7 but with a fewer number.

Granulation tissue was noticeably found in the early stages of wound healing in both the control

group and group 2; more prominently in the control group. Granulation tissue decreased by day 7, but remained more in the control group compared to group 1 and group 2. No granulation tissue was found in any of the groups by day 14.

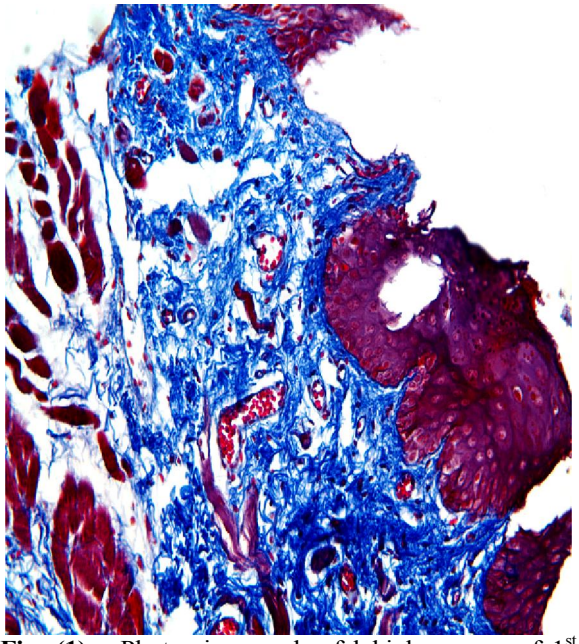


Fig. (1): Photomicrograph of labial mucosa of 1st postoperative day in control group (G 3) showing wound site and underlying disorganized collagen (Mallory trichrome x 100).

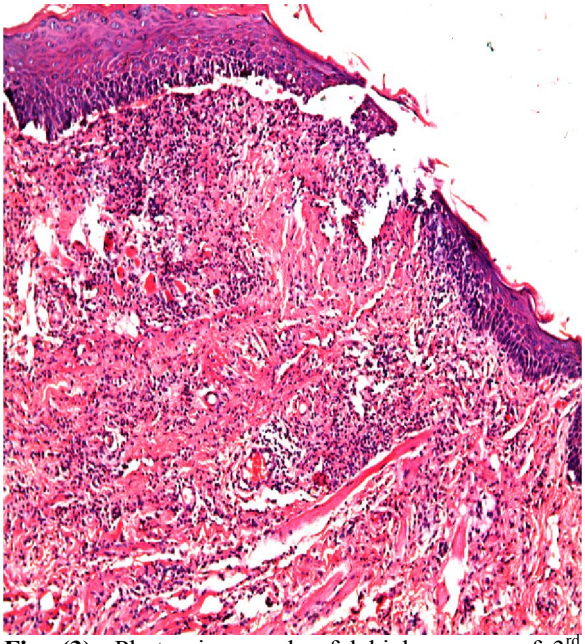


Fig. (3): Photomicrograph of labial mucosa of 3rd postoperative day in control group showing severe inflammatory cell infiltrate. (H & E stain x 100).

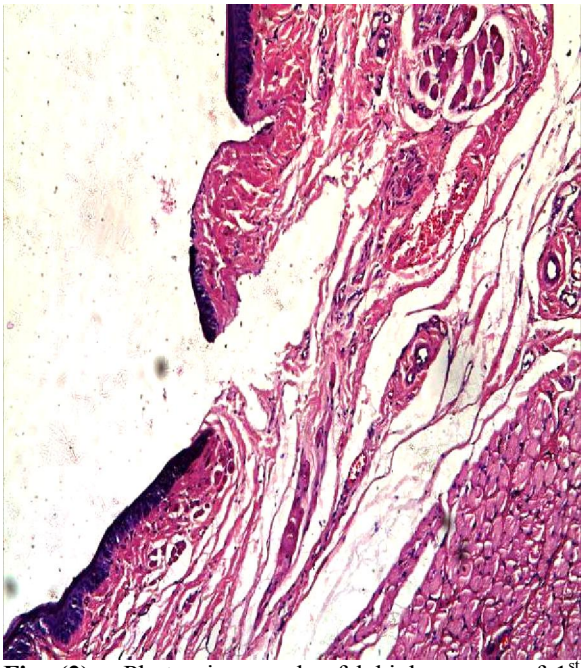


Fig. (2): Photomicrograph of labial mucosa of 1st postoperative day in propolis group showing wound site with few inflammatory cell infiltration. (H&E stain x 100).

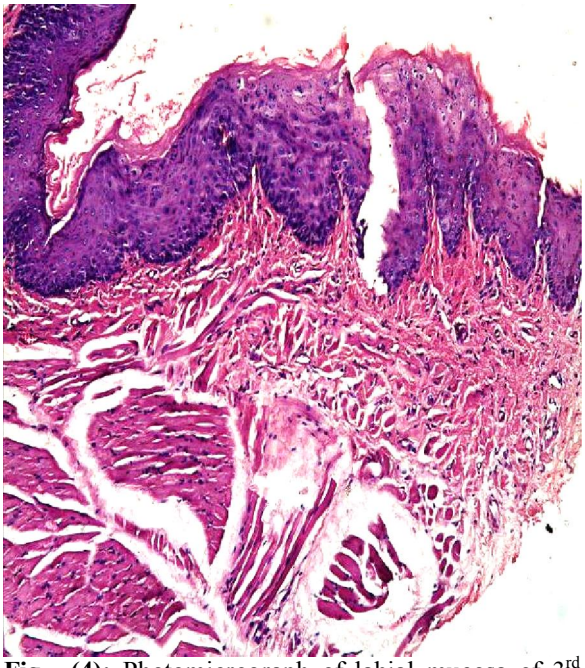


Fig. (4): Photomicrograph of labial mucosa of 3rd postoperative day showing mild inflammatory cell infiltrate in G1. (H & E stain x 100).

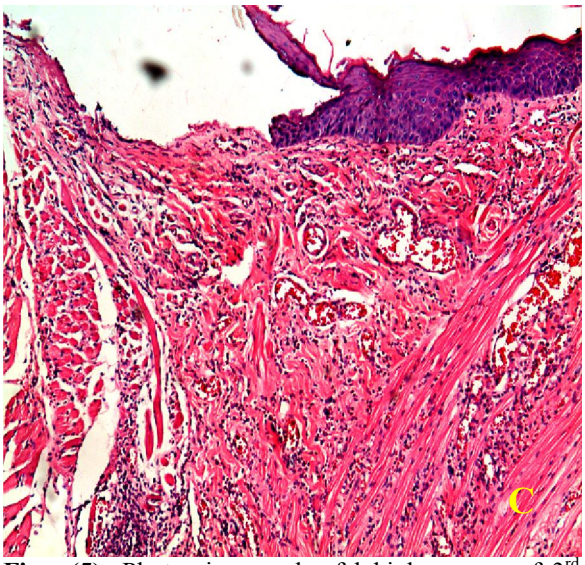


Fig. (5): Photomicrograph of labial mucosa of 3rd postoperative day showing profound inflammatory cell infiltrate and dilated blood vessels in one of the lesions in G2. Note the lack of epithelization with severe inflammatory response. (H & E stain x 100).

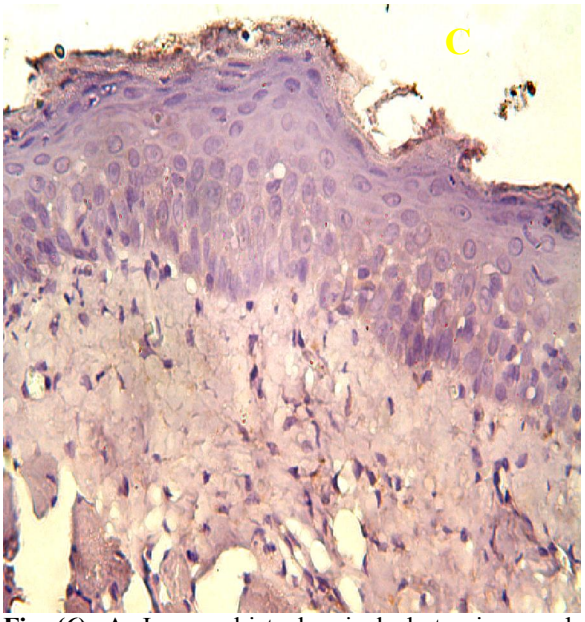


Fig. (6): A: Immunohistochemical photomicrograph of labial mucosa of 7th postoperative day in G1. (CD68 x 400).

Collagen fiber deposition was present in all groups starting in the early stage of wound healing (3-day). Collagen appeared bluish when examined by Mallory's trichrome stain. There was a quantitative increase in collagen synthesis which increased with time in subsequent groups. Collagen fibers orientation ranged from a mesh like organization in the control group to horizontally oriented fibrils in group 1. Mixed orientation was seen in group 2. Collagen fibrils showed organization and maturation on day 7 in all cases, although collagen in the control group showed some delay in scar maturation compared to the other groups. Collagen fibers showed maturation and horizontal organization in all cases on day 14.

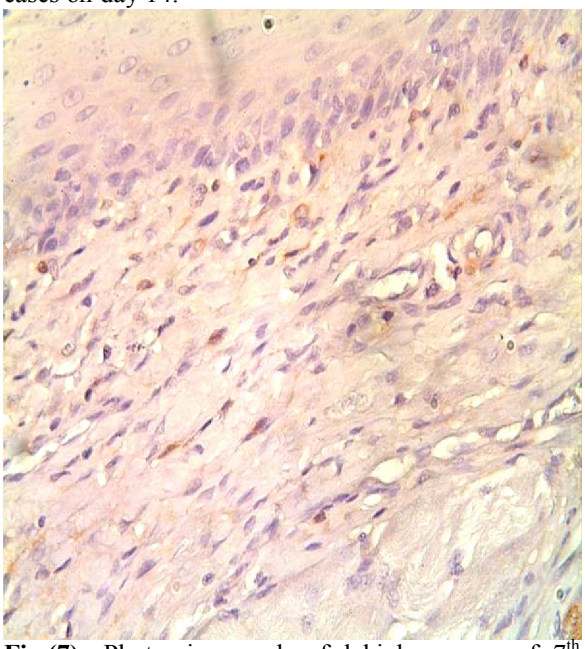


Fig.(7): Photomicrograph of labial mucosa of 7th postoperative day showing mild inflammatory cell infiltrate in G2. (CD68 x 400)

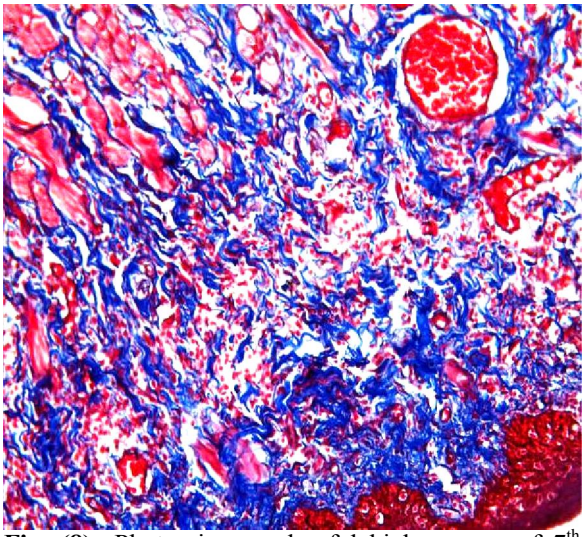


Fig. (8): Photomicrograph of labial mucosa of 7th postoperative day showing short wavy, longitudinally arranged collagen bundles in control group. (Mallory's Trichrome stain x 200)

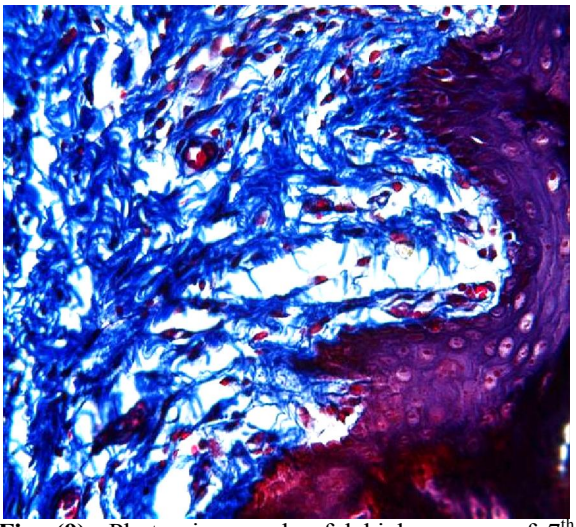


Fig. (9): Photomicrograph of labial mucosa of 7th postoperative day showing longitudinally arranged collagen bundles in control group. (Mallory's Trichrome stain x 400)

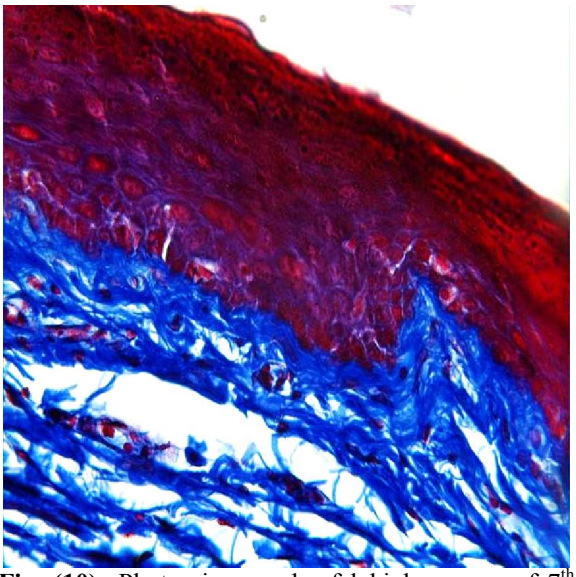


Fig. (10): Photomicrograph of labial mucosa of 7th postoperative day showing well organized horizontally oriented collagen deposition in G1. An indication of good healing (Mallory's trichrome stain x 400)

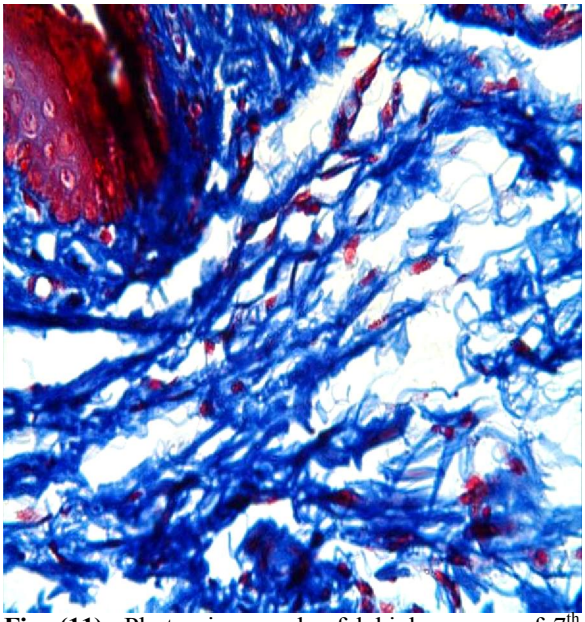


Fig. (11): Photomicrograph of labial mucosa of 7th postoperative day showing a mixed horizontally oriented and mesh like pattern of collagen organization in G2 (Mallory's Trichrome stain x 400)

Histologically assessed scoring criteria showed that healing progressed with time in all groups. G1 treated with propolis showed advanced healing when compared with the other two groups at all time points. Early epithelization was seen starting day 3 in all groups, however, cases showing profound inflammatory cells infiltration did not show epithelization in this early stage. On day 7, all examined cases showed epithelization.

Table I: difference in mean macrophage count (CD 68) between different groups after 1day using ANOVA statistical test:

Group	Mean macrophage count (CD 68)		
	M±SD	F-Value	*p*-Value
Control	9.80 ± 3.70	1.679	0.228
Propolis	6.00 ± 2.24		
Daktarin®	8.40 ± 3.78		

No significant difference, (p>0.05).

Table II: Difference in mean macrophage count (CD 68) between Control and Propolis groups after 1day using Paired Student's t-Test

Group	Mean macrophage count (CD 68)		
	M±SD	t-Value	*p*-Value
Control	9.80 ± 3.70	1.9649	0.0850
Propolis	6.00 ± 2.24		

Not significant difference, (p>0.05).

Table III: Difference in mean macrophage count (CD 68) between Control and Daktarin® groups after 1day using Paired Student's t-Test

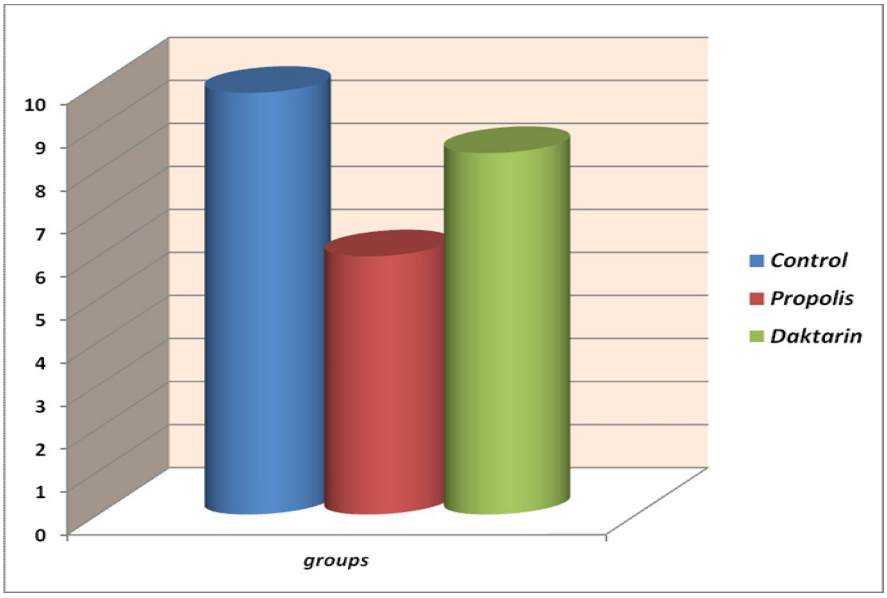
Group	Mean macrophage count (CD 68)		
	M±SD	t-Value	*p*-Value
Control	9.80 ± 3.70	0.5916	0.5704
Daktarin®	8.40 ± 3.78		

Not significant difference, (p>0.05).

Table IV: Difference in mean macrophage count (CD 68) between Propolis and Daktarin® groups after 1day using Paired Student's t-Test

Group	Mean macrophage count (CD 68)		
	M±SD	t-Value	*p*-Value
Propolis	6.00 ± 2.24	1.2216	0.2566
Daktarin®	8.40 ± 3.78		

Not significant difference, (p>0.05).



Histogram I: Showing difference in macrophage count (CD 68) between different groups after 1day.

Table V: Difference in mean macrophage count (CD 68) between different groups after 3days using ANOVA statistical test:

Group	Mean macrophage count (CD 68)		
	M±SD	F-Value	*p*-Value
Control	22.0 ± 6.82	8.814	0.004**
Propolis	10.8± 4.09		
Daktarin®	24.8± 5.50		

** High significant difference, (p<0.01).

Table VI: Difference in mean macrophage count (CD 68) between Control and Propolis groups after 3days using Paired Student's t-Test

Group	Mean macrophage count (CD 68)		
	M±SD	t-Value	*p*-Value
Control	22.0 ± 6.82	3.6003	0.0070**
Propolis	10.8± 4.09		

** High significant difference, (p<0.01).

Table VII: Difference in mean macrophage count (CD 68) between Control and Daktarin® groups after 3days using Paired Student's t-Test

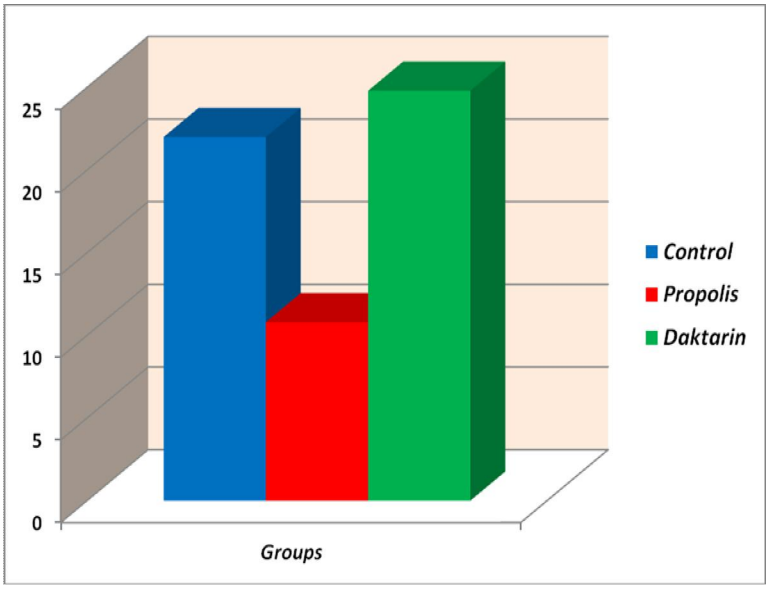
Group	Mean macrophage count (CD 68)		
	M±SD	t-Value	*p*-Value
Control	22.0 ± 6.82	0.7149	0.4950
Daktarin®	24.8± 5.50		

Not significant difference, (p>0.05).

Table VIII: Difference in mean macrophage count (CD 68) between Propolis and Daktarin® groups after 3days using Paired Student's t-Test

Group	Mean macrophage count (CD 68)		
	M±SD	t-Value	*p*-Value
Propolis	10.8± 4.09	5.0936	0.0009**
Daktarin®	24.8± 5.50		

** High significant difference, (p<0.01).



Histogram II: Showing difference in macrophage count (CD 68) between different groups after 3days.

Table IX: Difference in mean macrophage count (CD 68) between different groups after 7days using ANOVA statistical test:

group	Mean macrophage count (CD 68)		
	M±SD	F-Value	*p*-Value
Control	15.2± 5.31	10.61	0.002**
Propolis	5.40 ± 2.07		
Daktarin®	8.60 ± 1.67		

** High significant difference, (p<0.01).

Table X: Difference in mean macrophage count (CD 68) between Control and Propolis groups after 7days using Paired Student's t-Test

Group	Mean macrophage count (CD 68)		
	M±SD	t-Value	*p*-Value
Control	15.2± 5.31	4.7012	0.0015**
Propolis	5.40 ± 2.07		

** High significant difference, (p<0.01).

Table XI: Difference in mean macrophage count (CD 68) between Control and Daktarin® groups after 7days using Paired Student's t-Test

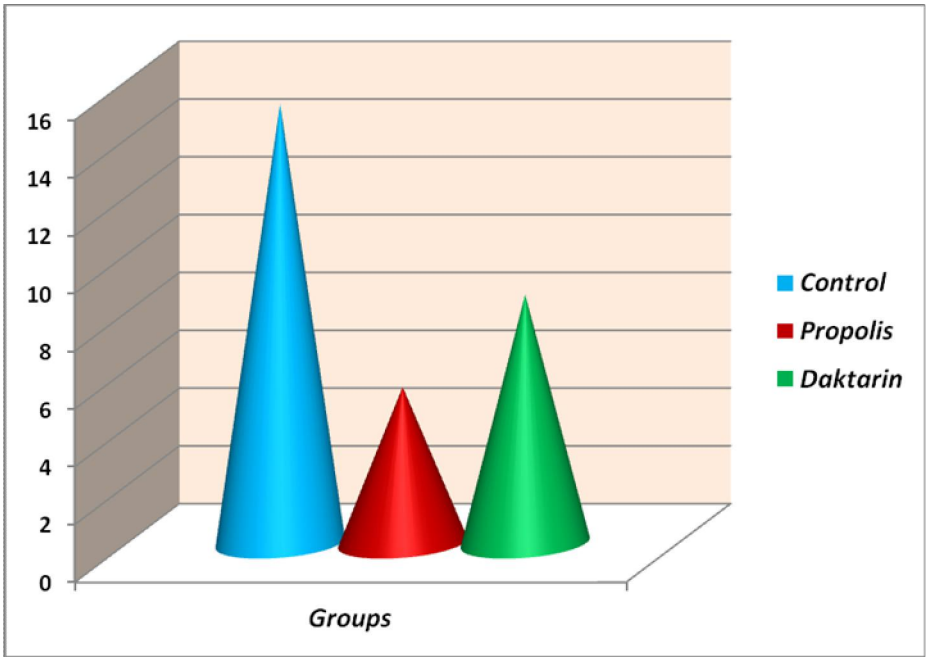
Group	Mean macrophage count (CD 68)		
	M±SD	t-Value	*p*-Value
Control	15.2± 5.31	3.5109	0.0080**
Daktarin®	8.60 ± 1.67		

** High significant difference, (p<0.01).

Table XII: Difference in mean macrophage count (CD 68) between Propolis and Daktarin® groups after 7days using Paired Student's t-Test

Group	Mean macrophage count (CD 68)		
	M±SD	t-Value	*p*-Value
Propolis	5.40 ± 2.07	2.6854	0.0277*
Daktarin®	8.60 ± 1.67		

Significant difference, (p<0.05and >0.01).



Histogram III: Showing difference in macrophage count (CD 68) between different groups after 7days.

4. Discussion:

We chose to study wound healing by second intention because it is a clinical condition that is frequently encountered in traumatic oral ulcers and by the oral surgeons. An experimental time period of 14 days was chosen because most wounds even if infected would show complete healing by the end of this time period.

A chief strength of this study was that all of the wounds were made under the same experimental conditions and were standardized for size, depth and site. The choice of male rats also cancelled the effect of sex hormones on wound healing. Sex hormones likely modulate oral mucosal wound healing.

Studies in rats are of low cost and provide useful information that could be difficult to obtain in

humans. In studies with humans, it is difficult to eliminate biases in relation to their behavioral variables, and standardize and maintain the same living conditions during the entire experiment. Thus, the use of rats in this work produced simple information but still capable of encouraging further researches in this area of knowledge.

In the present study, surgical mucosal wounds were made in the labial mucosa of all animals by means of a 1-mm punch-biopsy instrument before being removed with a scalpel from the rat's labial mucosa. This method is very useful for creating uniform ulcer diameters.

Since reduced wound inflammation is associated with improved tissue repair [27, 28], we examined inflammatory cell infiltration in the oral mucosal wounds of different groups. Whereas macrophages infiltration was found in all the studied specimens peaking in day 3 for all groups, the cells with positive expression of CD68 (CD68+ macrophages) were significantly lower than both control group and the group treated with daktarin at day 3 (P <0. 01), but was significantly lower only in comparison to control group on day 7

Neutrophils and macrophage infiltration is the most prominent feature of the innate response, but they are also a double edged sword. They are aggressive against microbes, but they cause major collateral damage by releasing a corrosive cocktail of protease enzymes and active oxygen species [4].

Even though crucial for antibacterial defense, neutrophils and macrophages can become an unwelcome, damaging presence in a wound, if they stay in residence for too long [4].

Hence, lower inflammatory responses have been associated with faster wound healing [29, 30].

There was a decrease in CD68 positive cells in the group treated with propolis at all time points along the study. This was of a statistical significance when compared to both control group and group 2 on day 3, (P <0. 01)

However, on day 7, the group treated with propolis showed significantly lower macrophage count compared to the control group, P<0. 01, but did not show statistical significance compared to group 2.

The finding of reduced inflammatory cell infiltration in oral wounds treated by propolis is in keeping with the accelerated repair. The anti-inflammatory activity of propolis has been reviewed by **Almeida and Menezes,** [31]. Propolis has inhibitory effects on mieloperoxidase activity, NADPH-oxidase ornithine decarboxilase, tirosine-protein-kinase, and hyaluronidase from guinea pig mast cells. This anti-inflammatory activity can be explained by the presence of active flavonoids and cinnamic acid derivatives. The former includes acacetin, quercetin, and naringenin the latter includes caffeic acid phenyl ester (CAPE) and caffeic acid (CA)[31]. On the other hand, **Santos *et al.,*** [32] observed that propolis propolis gel and Daktarin showed complete clinical remission of palatal edema and erythema and concluded that the efficacy of propolis was comparable to Daktarin.

Nevertheless, in the oral cavity, propolis had been found to inhibit different pathogenic microbes such as bacteria, fungi and viruses[33-35] and can be successfully applied against the different stomatological pathologic conditions: stomatitis, paradontosis, gingivitis and caries[34,36,37].

In the present study, granulation tissue was noticeably found in the early stages of wound healing in both the control group and group 2; more prominently in the control group. This is in line with **Stephens *et al.,*** [38,40] who stated that oral mucosal fibroblasts produce HGF as well as keratinocyte growth factor, platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF-2). Following injury, fibroblasts migrate into the wound, proliferate and produce the matrix proteins (fibronectin, hyaluronic acid, collagen and proteoglycans) and, in doing so, form granulation tissue [41]. They also interact with keratinocytes, releasing growth factors and cytokines that play a further role in modulating wound repair [42]. The composition of the ECM (and thus the final wound healing outcome) can be altered by the balance between the MMPs and TIMPs enzymes produced by fibroblasts.

In this study, granulation tissue decreased by day 7, but remained more in the control group compared to group 1 and group 2. No granulation tissue was found in any of the groups by day 14.

The orderly collagen formations at different stages of wound healing at different days have been recognized as histologic characteristics of healing. These include increased diameter, increased inter-fibril binding, and rearrangement of fibrils with time to become more organized in a manner that maximizes strength[43]. Based on these histological parameters it was noticed in our study that collagen fiber deposition was present in all groups starting in the early stage of wound healing (3-days). Increasing with time until it reached a maximum mature arrangement by day 14.

This is in accordance with **Cotran *et al.,*** [44] and **Barbul,** [45].

Collagen fibers orientation ranged from a mesh like organization in the control group to horizontally oriented fibrils in group 1. Mixed orientation was seen in group 2. Collagen fibrils showed organization and maturation on day 7 in all cases. Horizontal collagen orientation during wound healing had been reported by **Mustafa,** [24] and **Barbul,** [45]. Although collagen in the control group showed some delay in scar maturation compared to the other groups, collagen fibers showed maturation and horizontal organization in all cases on day 14, hence indicating complete healing.

In this current study, all cases showed complete epithelization on day 7, this is in agreement with Yilmaz et al', who when evaluated the therapeutic effectiveness of honey on oral mucosal ulcers stated that the wounds of all their studied groups were covered by new mucosa epithelium and were similar to the normal one on day 7 and 14. [46]

Conclusion:

Propolis has an enhancing effect on the healing of oral mucosal wounds. It was linked to decreased inflammatory reaction. Therapeutic value of propolis in oral mucosal wound healing is more effective compared to daktarin. Further investigations about the therapeutic effects of propolis on oral lesions might substitute or aid the conventional treatment methods. Thus, further in vivo investigations are required to support this assumption.

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Effect of Openings on the Static and Dynamic Behavior of Quadratic Folded Plate Roofing Systems

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Abstract: This paper investigates the effect of openings on the structural behaviour of quadratic folded plate Q.F.P. roofs by using different geometric configurations for the main elements of the system. The impact of such variance on the behaviour of the structure system under both static and dynamic conditions is also investigated. The selected and investigated parameters in this study are the location and size of the openings, as well as the rise of the folded plates (height). Spans of 14 m, 20 m and 26 m were selected for all of the investigated parameters. To meet the goals of this study, a 3-D Finite Element Model (FEM) was adopted to examine the suggested variables. A linear static analysis was performed to analyse the effect of the investigated parameters on the system deflections, moments, tension and compression stress. Q.F.P. slabs with rises varying from 90 cm to 180 cm were studied. The results indicated that the difference in the rise reduced the roof deflection by 72%. Moreover, the behaviour of the folded plate with openings at different locations was improved. The compression stress of the Q.F.P. roofs increased by 120% to 170% when the location of the centre openings on the models length increased from 14 m to 26 m. Folded plate openings at at the quarter length of the folded plate further reduced the compression stress by 5% to 56% when the model span changed from 14 m to 26 m. The location of the edge openings had a slight effect on the compressive stress compared to other factors. The opening location did not have a significant effect on the tensile stress of the Q.F.P. slabs. Furthermore, the results indicated that the maximum bending moment for the intermediate beams increased by 69% when the centre openings were located at the beam centre. The maximum bending moment at 0.57 L (intermediate beam length) for the quarter opening location increased by 64% compared to control model. The edge opening location had a slight effect on the diaphragm bending moment and the intermediate beam fixed-end moment. Four different opening sizes were studied, and the effect of the opening size for the 26-m model was found to increase the static and dynamic behaviour by no more than 6%. Three-dimensional dynamic modal analyses were performed, and the effect of different opening locations on the fundamental modes was investigated. The results of the modal analysis showed that the openings location did not significantly affect the fundamental frequencies or fundamental mode shapes. The results obtained from this study emphasise the importance of using an elaborate numerical analysis to address such sensitive models; furthermore, the geometric properties and openings location of each contributing element clearly affected the overall performance of the system. Finally, the model results indicated that the folded plate opening location at the centre of the beam is the most effective parameter of those investigated here, while the edge opening location in the folded plate had the lowest effect on both the static and dynamic behaviour of the investigated system.
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Key words: Folded plates; Height; Openings location; Free vibrations.

1. INTRODUCTION

The folded plate is one of the most practical structures used in civil engineering applications because it has numerous merits, including its light weight, the ease at which it is formed, its low cost and its high resistance to loads. The folded plate is used in many applications, such as roofs, sandwich plate cores and cooling towers. As the use of folded plates is advantageous compared to flat plates, their behaviour with and without openings under different load conditions must be investigated. There are several methods available for analysing this type of structure (1 to 8). While conventional analysis methods are simple and easy, they have certain limitations related to the generality of application and precision.

Early researchers solved folded plate problems in an approximate manner with the use of the beam method or a theory that neglected the relative joint displacement (2). However, these two methods could not easily address generalised folded plate problems. Computational approaches and numerical methods for the analysis of folded plates offer more precise solutions than conventional analysis methods. The methods of interest include finite strip methods; one of the earliest studies based on this method, which was introduced by **[Cheung 1969],** was that of **[Golley and Grice 1989]**, along with the study presented in the same year by **[Eterovic and Godoy 1989]**. The combined boundary element-transfer matrix method **[Ohga et al., 1991]** and finite element method (FEM) were the focus of many previous studies **[Liu and Huang, 1992; Perry et al., 1992; Duan, 2002].** The FEM is more convenient than the combined boundary element-transfer matrix method because it can be

applied to analyse large complex structures; furthermore, various types of boundary conditions and loadings can be easily implemented. For these reasons, most of the commercial software used for structural analysis uses the FEM method.

This study focused on the Q.F.P. roof because it is one of the most common types of roofs used for roofing systems. The presented parametric study on the effect of opening location on folded plate behaviour was examined using finite element analysis and linear static three-dimensional F.E. analysis. The study analysed the effects of the opening location and the rise of the folded plate on the slab deflection, intermediate beam moments, and stress distribution of the slab's maximum stress.

In some cases, Q.F.P. slabs system should include openings. These openings may be small, such as those needed to accommodate heating, plumbing, and ventilating risers, floor and roof drains, and access hatches, but in some cases, openings may be larger than the code limitation in size and position. The presence of large openings in the slab system reduces the stiffness and increases the deflection; previous studies investigated the effect of the openings on the behaviour of the flat plate structure.

Based on the output of models, design charts for this particular system will be presented to select the best opening location, and the effects of various parameters on the behaviour of the structural system will be evaluated. Recommendations will be given in accordance with the impact of the opening location on the behaviour of the structural system.

2 Finite Element Analyses

2.1 Geometry and Dimensions of the Investigated Systems

Q.F.P. panels with a width of 7.8 m were studied, as presented in Figure 1. This width was kept constant throughout the analysis, while the three different spans of 14 m, 20 m, and 26 m were changed to investigate their effect on the behaviour of the structural system. A constant opening size of 3 m×2 m was considered for each panel for openings that were located on the top surface of the folded plate slab at different positions (centre, quarter, and edge). The height of the models varied from 90 to 180 cm. A 3-D F.E. analysis was performed, and the roof was modelled using 3-D quadratic shell elements, which are presented in Figure 2. The intermediate beams, end diaphragms, and columns were modelled as 3-D frame elements, as shown in Figure 2. The length of the openings ranged from 2 m to 8 m, while a constant width of 3 m was used for the different opening locations.

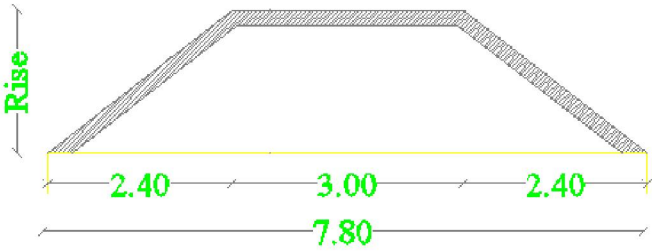


Figure 1: Cross-sectional dimensions of the investigated models

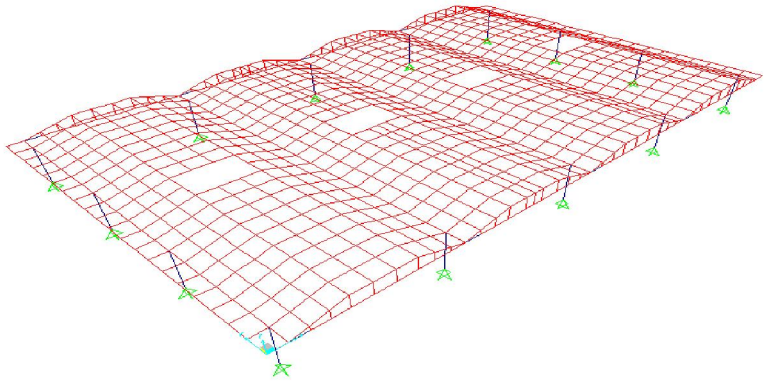


Figure 2: 3-D model of the investigated folded plate panels and centre opening location

2.2 Static Analysis of the Q.F.P. System

A 3-D static linear F.E. analysis was performed by applying each of the parameters under investigation separately. A static load of 150 kg/cm^2 was applied on all of the models to mimic flooring and service loads. Throughout the analysis, the deflections

and stresses of the folded plate were checked and the maximum bending moments at the intermediate beams and diaphragm were reported. The effect of the tested parameters will be presented in detail later.

2.2.1 Effect of the Opening Location on the Maximum Deflection of the Q.F.P. System for a Constant Rise

The first investigated parameter was the effect of the folded plate opening location on the maximum deflection at the centre of the folded plate. Spans of 14 m, 20 m, and 26 m were studied. Figure 3 shows the maximum deflection of the folded plate slabs at the central point for the different lengths used versus the opening location for the folded plate. When the openings are located at the centre of the beam, the maximum deflection for the span with a length of 26 m and rise of 0.9 m increased from 47.6 mm to 58.2 mm, with a percentage increase of 22%. When the openings were located at the quarter length of the beam, the maximum deflection of the beam centre was 51.8 mm, with a percentage increase of 9 % compared to control model. The maximum central deflection was 47.6 mm when the openings were located on the edge, which has a minor effect on the centre deflection point. The measured deflection value when the openings were located at the edge is approximately the same as in the control model.

For the 20-m Q.F.P. span, the central deflection was 15.7 mm when the rise of the folded plate was 0.9 m, as presented in Figure 3. In the most of the investigated cases, the opening location has a significant effect on the extent to which the central deflection increases. For example, when the openings were located at the centre and quarter of the beam, the deflection increased to 19.3 mm and 17 mm, respectively, corresponding to 23% and 8% increases compared to the control case.

The central deflection of the folded plate with a 14-m span was 3.73 mm; this case is considered to be the control model. The effect of the opening location on the central deflection were 4.45 mm (increasing by 19 %), 3.89 mm (increasing by 4 %), and 3.77 mm for the corresponding to centre, quarter, and edge opening locations, respectively.

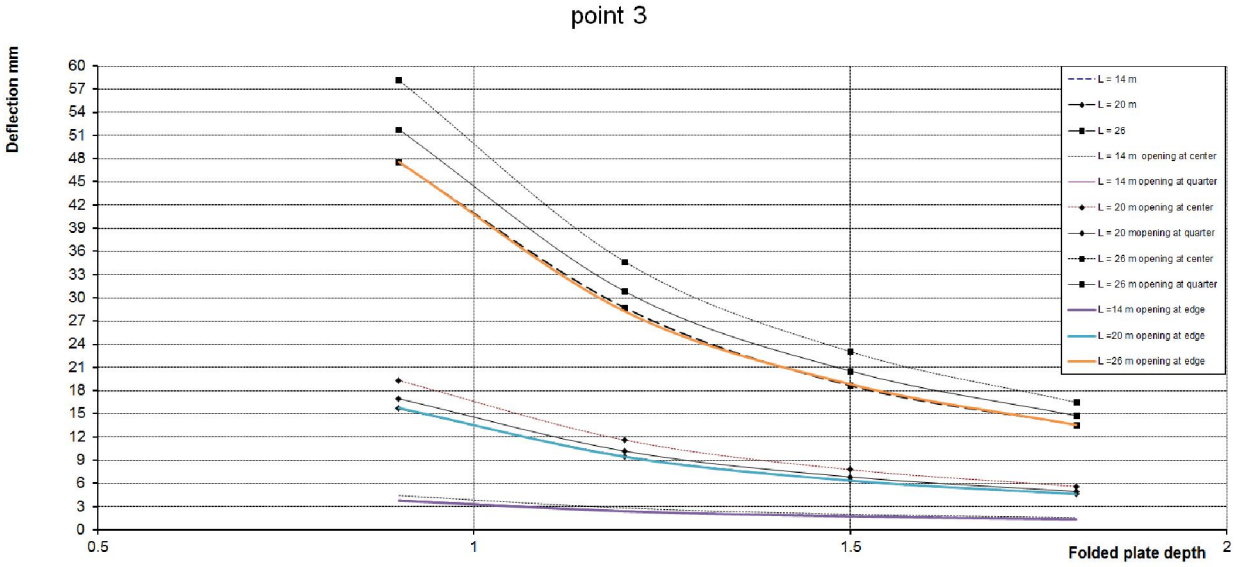


Figure 3: Maximum deflection of the Q.F.P. slabs versus different opening locations for different rises

2.2.2 Effect of the Folded Plate Rise on the Maximum Deflection

In this study, the folded plate rise was varied from 90 cm to 180 cm. The folded plate rise can significantly decrease the central deflection, as shown in Figure 3. For the longest investigated span of 26 m, the increase of the folded plate depth (rise) from 0.9 m to 1.8 m with centre opening location reduced the deflection from 58.2 mm to 16.5 mm, while the deflection induced at the quarter opening location decreased from 51.8 mm to 14.8 mm when the folded plate rise increased from 0.9 m to 1.8 m.

For the 20-m model, increasing the folded plate rise from 0.9 m to 1.8 m decreased the central deflection from 19.3 mm to 5.62 mm at the centre opening location, while the central point deflection decreased from 17 mm to 5 mm for the same increase in the folded plate rise when the openings were located at the quarter length of the beam.

For the 14-m span, the central deflection decreased by 65 % for both the centre and quarter opening locations, as presented in Figure 3. The Q.F.P. slab rise decreases the effect of the opening location on the maximum deflection.

2.2.3 Effect of the Folded Plate Opening Location on the Intermediate Beam Moment

This study considered three opening locations for the Q.F.P. slabs: at the centre, quarter and edge of the beam. These openings can significantly increase the maximum bending moment of the intermediate beam, and the value of the moment depends on the opening positions. For the 26-m span without openings, the maximum bending moment at the centre of the intermediate beam was 16.8 m.t, while the bending moment was 28.37 meter-tons (increasing by 67 %). For the 20 m and 14 m folded plate lengths, the maximum bending moments increased by 60% and 42 %, respectively. The effect of the opening location at the quarter or edge of the beam has no effect on the maximum moment at the centre of the beam, as the maximum moment is similar to that of the control model, as shown in Figure 4. The maximum moment occurred at the quarter of the intermediate beam at the opening location, and it increased by 64 %, 46% and 30% for the model lengths of 26 m, 20 m and 14 m, respectively, as shown in Figure 5. The edge opening location has a limited significant effect on the intermediate beam bending moment, as shown in Figure 6.

Figures 4-6 present the effects of the opening location and Q.F.P. rise on the bending moment of the intermediate beam. These figures show that the increase of the folded plate rise from 0.9 to 1.8 m results reduces the moment of the intermediate beam at different locations for all of the investigated models.

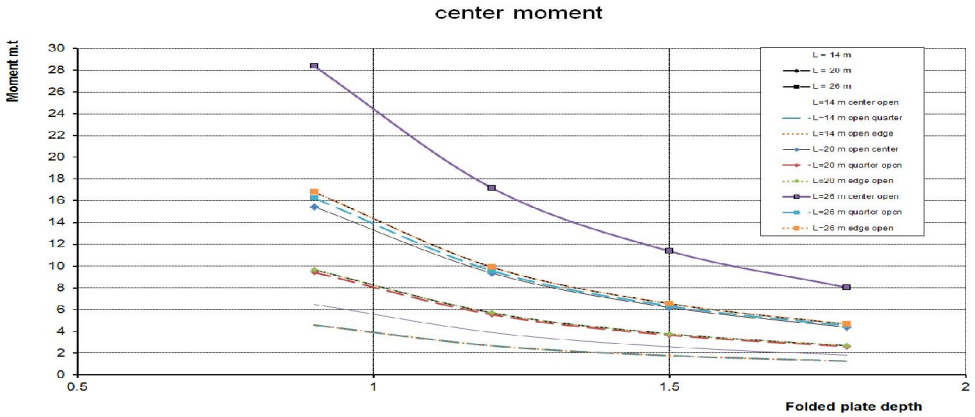


Figure 4: Maximum moment of the intermediate beam versus different opening locations

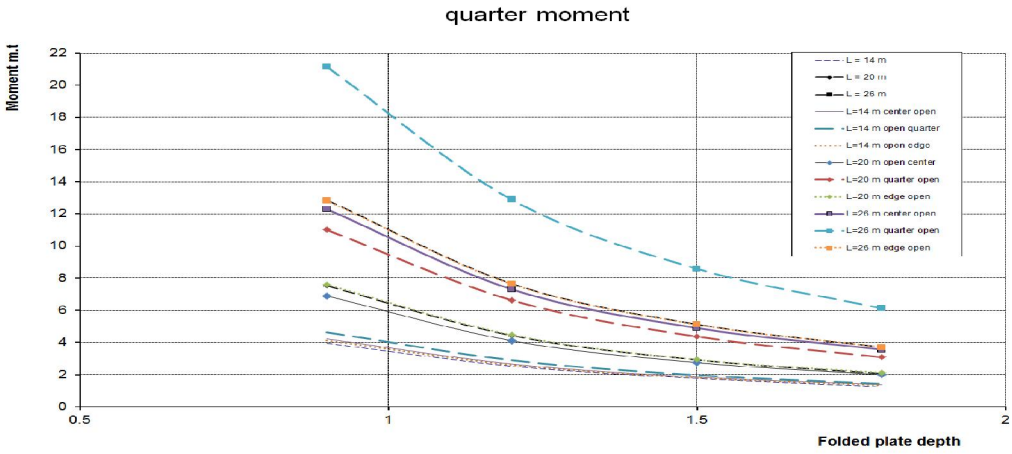


Figure 5: Intermediate beam moment at the quarter versus different opening locations

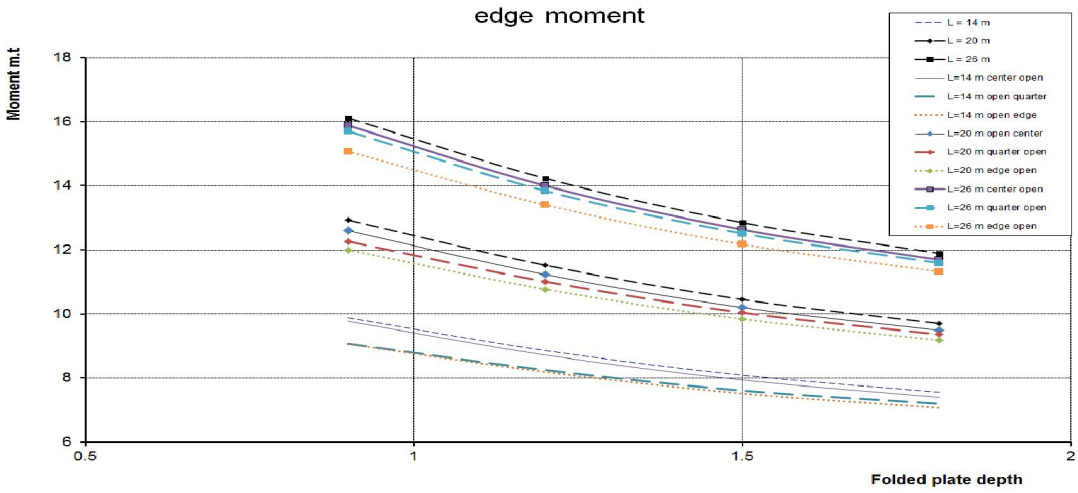


Figure 6: Fixed end moment of the intermediate beam versus different opening locations

2.2.4 Effect of the Opening Location on the Folded Plate Compression and Tension Stresses

For the minimum investigated rise of 0.9 m, the presence of different opening locations at the centre, quarter and edge of the beam results in a maximum tensile stress and maximum compression stress at the bottom and top of the beam, respectively. Changes in the opening location do not significantly affect the tensile stress, as shown in Figure 7. However, they do significantly affect the compression stresses. At the centre opening location of the 26-m, 20-m and 14-m spans, the compression stress increased by 174%, 170% and 120 %, respectively, compared to the control beam. For the quarter opening location, the compression stress increased by 56%, 30% and 5 % for the 26-m, 20-m and 14-m spans, respectively. The edge opening location did not noticeably affect the compression stress, which was maximised near the location of the openings. For example, the maximum stress occurred at 0.57 L of the folded plate length for the quarter opening, while it occurred at 0.62 L of folded plate length for the edge opening location.

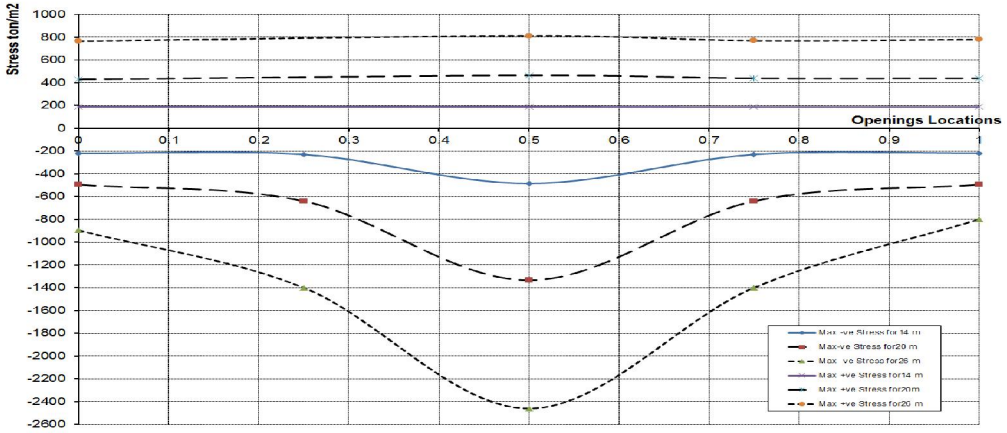


Figure 7: Maximum tension and compression stress versus different openings locations

2.2.5 Effect of the Opening Location on the Folded Plate Compression and Tension Stresses

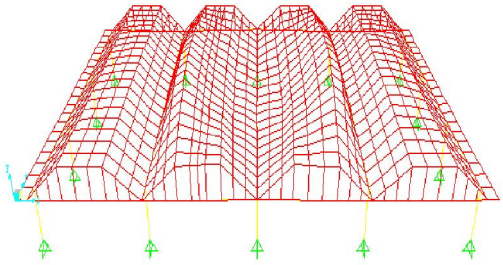
In this study, the length of the openings ranged from 2 m to 8 m, while a constant width of 3 m was used for the different opening locations. The folded plate opening sizes do not significantly increase the static or dynamic effects. For the longest investigated span of 26 m, the models indicated that the effects of these parameters are less than 6%.

2.3 FREE VIBRATION ANALYSIS

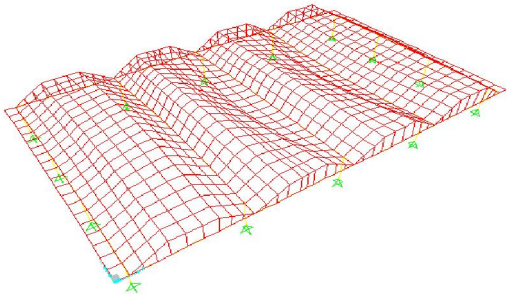
The investigated systems were subjected to a free vibration analysis. An eigenvalue analysis was adopted in the linear dynamic analysis. The arrangement of the mode shapes was consistent for the investigated spans with different parameters. Column sway dominates the first three modes, while the subsequent modes are dominated by roof deformations. Figure 8 displays the roof modes and clearly shows that the dominating mode for the Q.F.P. slab is the intermediate symmetric bending mode. The second mode was anti-symmetric bending, followed by the symmetric bending of the external spans and the alternating modes of anti-symmetric and symmetric plate bending.

In Figure 9, the fundamental frequencies of the system are provided for the Q.F.P. slabs with spans of 14, 20 and 26 meters. As shown in the figure, the first three modes were column lateral displacements. The fundamental modes for the folded plate roof began at the fourth mode. The frequency of the three fundamental modes for the 14- and 20-m roof spans were very similar, while the frequency for the 26-m span was 27% lower than these frequencies.

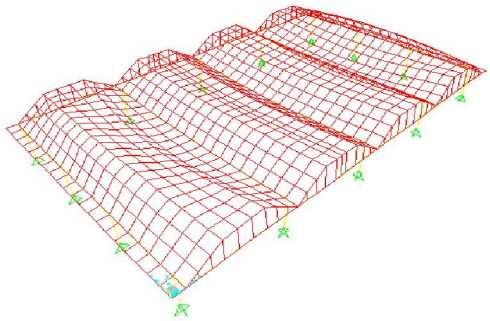
Figure 10 presents the effect of the roof rise on the fundamental frequencies of the system for the 20-m span. Rises of 90 and 180 cm were investigated, and the figure shows that the system with the higher rise has 20% higher fundamental frequencies. This result is likely due to the higher stiffness of the roof in this case. Based on the free vibration analysis, the change in the opening location has no effect on the roof modes or the fundamental frequencies.



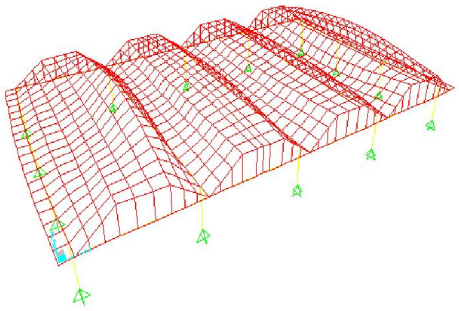
First mode shape of the Q.F.P. slab



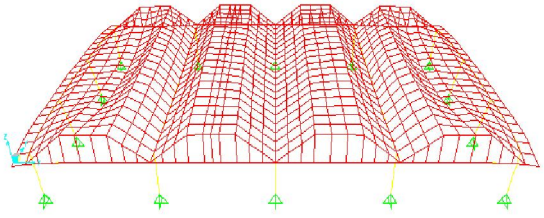
Second mode shape of the Q.F.P. slab



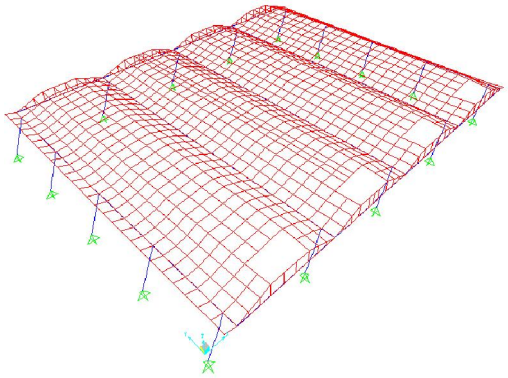
Third mode shape of the Q.F.P. slab



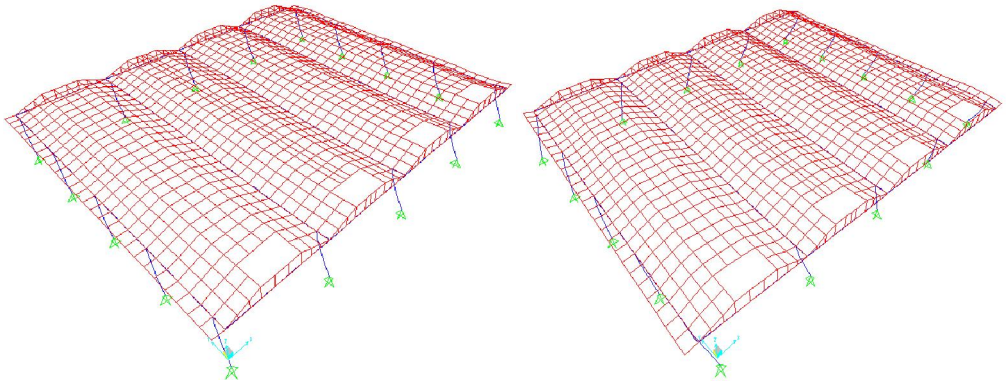
Fourth mode shape of the Q.F.P. slab



Fifth mode shape of the Q.F.P slab



Third mode shape of the Q.F.P. slab with edge openings



Fourth mode shape of the Q.F.P. slab with edge openings Fifth mode shape of the Q.F.P. slab with edge openings

Figure 8: Fundamental mode shapes of the Q.F.P. slab

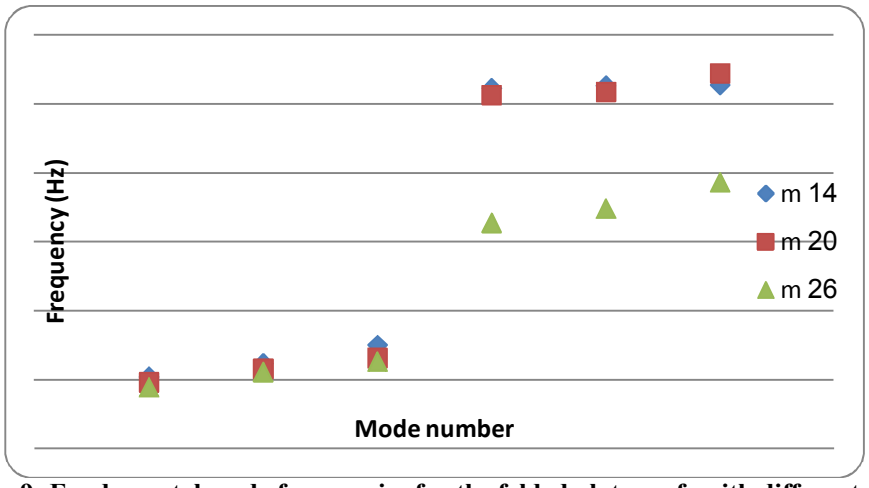


Figure 9: Fundamental mode frequencies for the folded plate roofs with different spans

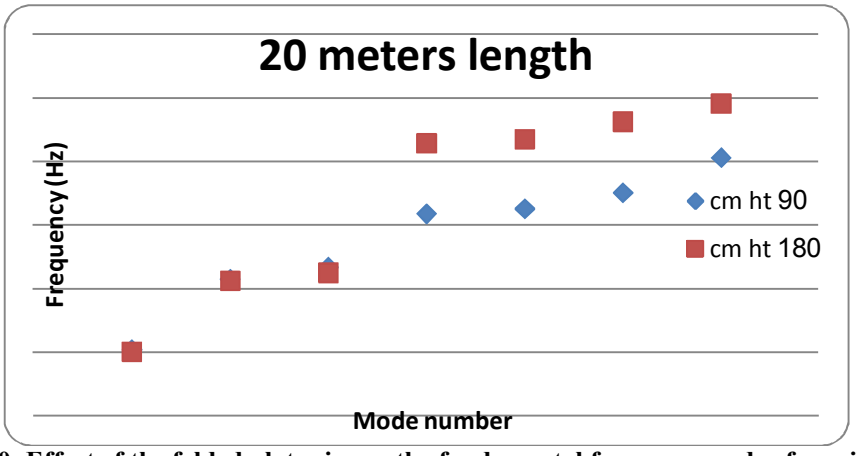


Figure 10: Effect of the folded plate rise on the fundamental frequency under free vibrations

3 Discussions of Results

For the models considered in this study, the edge opening location has a limited effect on the Q.F.P. roofs' central deflection, maximum bending moment, edge moment of the intermediate beam and diaphragm bending moment. This effect did not exceed 3%, as shown in Figure 11. The central opening location clearly affects the central deflection compared to the defections induced in both the quarter and edge opening locations. The centre opening location increased the central deflection by 14 % more than the quarter opening for the 14-m and 20-m spans and by 14% more than the quarter opening for the 26-m span.

Doubling the height of the investigated folded plate from 0.9 meters to 1.8 meters is associated with an average deflection reduction of 72%, as shown in Figure 12, which also shows that the degree to which the central deflection increases for the 20-m and 26-m spans were similar, while the effect of the opening location in the 14-m span was lower.

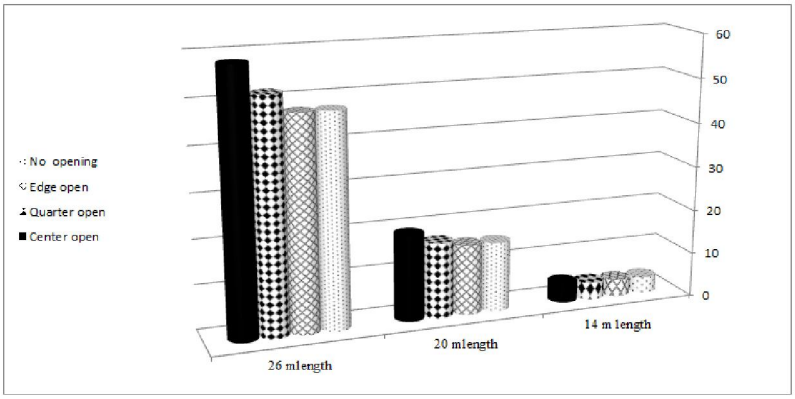


Figure 11: Maximum deflection of the Q.F.P. slabs versus different opening locations for different lengths

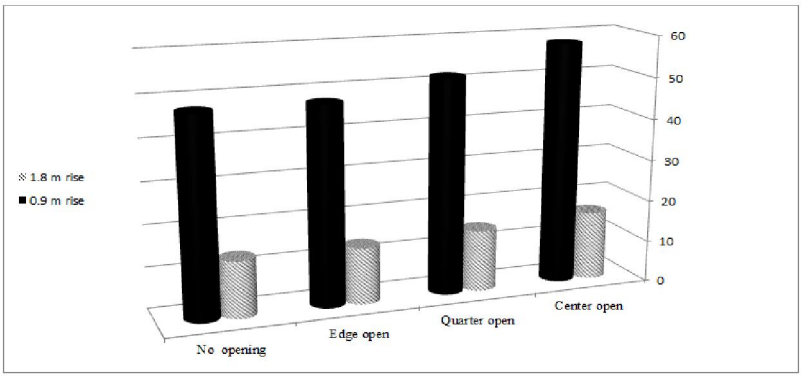


Figure 12: Maximum deflection of the Q.F.P. slabs versus different opening locations for different lengths

The maximum moment location occurred at the intermediate beam centre in the case of the control and centre opening location models. For the quarter opening location model, the maximum moment occurred at a length of 0.75 (at the opening location) for the intermediate beam length. The location of the maximum moment for the edge opening location model was similar to that of the control model, as presented in Figure 13.

Varying the opening location significantly affected the maximum moment of the intermediate beam. The centre opening location increased the maximum moment by 67%, 60% and 42% for the 26 m, 20 m and 14 m spans, respectively. The maximum moment is located at the quarter of the intermediate beam at the opening location, and it increased by 64 %, 46% and 30% in the 26-m, 20-m and 14-ms, respectively, as shown in Figure 13. The effect of the opening location on the edge moment and diaphragm bending moment did not exceed 3%. At the quarter opening location, the opening location did not appear to affect the maximum moment at the intermediate beam centre or the edge moment.

The effect of the opening location on the maximum tensile stress was minor and did not exceed 4%. However, the opening location significantly affected the compression stress, which increased by 174%, 170%, and 120% for the 26-m, 20-m and 14-m spans, respectively. The quarter opening location increased the compression stress by 56%, 30%, and 5% for the 26-m, 20-m, and 14-m spans, respectively.

The openings size does not significantly affect the static or dynamic behaviour of the beam. Increasing the opening size decreased the total load on the folded plate system (dead or live load). For the dynamic analysis, varying the opening size did not significantly affect the folded plate inertia; thus, effect of the opening size on the dynamic behaviour was not significant.

The opening locations from the centre to the edge of the beam have a limited effect on the fundamental frequency of the Q.F.P. slab. The arrangement of the mode shape patterns was not affected by variations in the opening locations from the centre to the edge of the beam. In other words, the mode shapes were of the same order for all of the investigated spans.

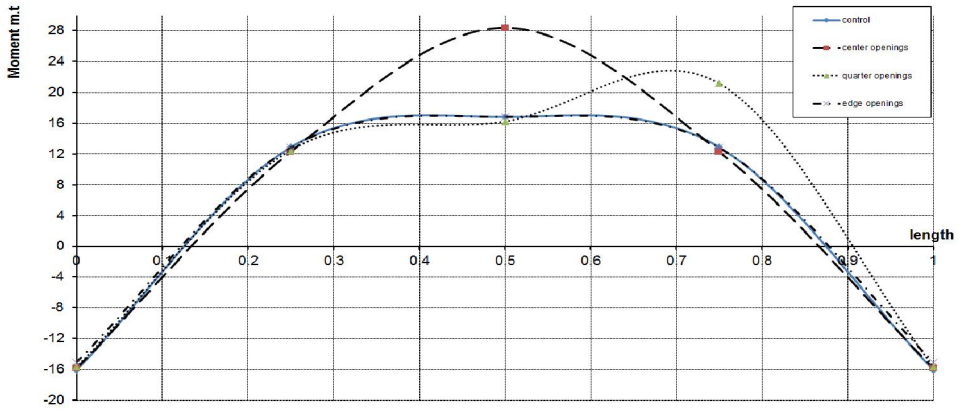


Figure 13: Intermediate beam moment versus different opening locations

4 Conclusions

- The proposed approximate static solutions for the fixed boundary conditions at the end diaphragms of the Q.F.P. slab ends (9, 10, and 11) are not reliable for the longer spans, especially for spans higher than 20 meters. Thus, the stiffness should be included in the numerical modeling.
- Increasing the folded plate rise was the most significant factor in improving the static analysis for deflection control, while increasing the roof rise increases the fundamental frequencies of the system.
- The rise effect was most effective in shorter spans.
- Increasing the folded plate rise enhanced the structural behaviour of the Q.F.P. system.
- The edge opening location has a limited effect on the Q.F.P. roofs static and dynamic behaviour.
- Centre and quarter opening locations have an effect on the change in static deflections, or straining actions of the system.
- Spans from 14 to 20 meters for the investigated Q.F.P. slabs had very close fundamental frequencies, this effect widens noticeably on analysing longer spans.
- The centre opening location significantly affected the Q.F.P. slabs deflection greater than quarter opening location by 14%.
- The variation of the opening location has a significant effect on the maximum moment of intermediate beam, while the centre opening location increased the maximum moment by 67%, 60% and 42% which corresponding spans of 26 m, 20 m, 14 m, respectively.
- The opening location has a limited effect on the edge moment and diaphragm bending moment, which did not increase by more than 5% compared to the control model.
- The quarter opening location did not affect the maximum moment at the intermediate beam centre or the edge moment. The maximum moment is located at the quarter of the intermediate beam at the opening location, and compared to the control model, it increased by 64 %, 46% and 30% for the model lengths of 26 m, 20 m and 14 m, respectively.
- The opening location has no effect on the maximum tensile stress, while it significantly affects the compression stress, which increased by 174%, 170%, 120% for the spans of 26 m, 20 m, 14 m, respectively, compared to the control model.
- The opening locations from the centre to the edge of the beam have a limited effect on the fundamental frequency of the Q.F.P. slab.
- The openings sizes do not have a significant effect on the static and dynamic behaviour of the beams.
- The arrangement of the mode shape patterns was not affected by variations in the opening locations from the centre to the edge of the beam. In other words, the mode shapes were of the same order for all of the investigated spans.

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Screening tuberculosis in the Sistan region of Iran: A Population-Based Study

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Abstract: Estimation of the prevalence of tuberculosis is one of the most important needs for the provision of health services in Iran. In this study, we investigate the prevalence of pulmonary tuberculosis in the Sistan region of Iran. A cross-sectional study was carried out during 2010 in the Sistan region of Iran. Sistan has a total population of 410,713, including 50,322 families. 21,645 of these individuals (or 5.27%) have a history of chronic cough (a cough lasting more than 2 weeks) based on our survey. Individuals who were suspected of having tuberculosis were referred to the district health center. For each case, diagnostic procedures including physical examination of the chest for evaluation of Bacillus Calmette-Guérin status, obtaining of 3 sputum smear samples, and radiography of the chest. Of 410,713 individuals, 6250 (or 1.52%) demonstrated scarring typical of BCG, and chronic cough was confirmed via clinical workup in 8140 participants. Among these participants, 4034 (0.98% of the Sistan population) presented with productive cough; sputum specimens were taken from this group. Chest radiographs showed characteristic pulmonary tuberculosis lesions in 27 (6.58%) of 410 patients in whom radiography was performed on the basis of clinical findings. Seven patients were identified as having tuberculosis through sputum smears; five other patients were found to not have tuberculosis via this method. With respect to the different types of diagnostic methods used, the differences among them for positive predictive value, specificity and sensitivity were significant (p<0.05). The likelihood ratio for chest radiography was also significantly greater than that for the sputum smear method (33.31 versus 19.1; p<0.05). Only 276 of the patients with pulmonary Tuberculosis were identified via Health Service System (HSS) screening method in Sistan, and the rest were diagnosed though recent study. Our findings suggest that the diagnostic power of chest radiograph is more than sputum smear; however, we think the HSS method for taking sputum was not a controlled one.
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Key Words: Chronic Cough, Pulmonary Tuberculosis, Sputum Smear.

Introduction:

Tuberculosis continues to be a major clinical problem in specific populations world-wide. During the last two decades, a troubling, marked increase in drug-resistant strains of *Mycobacterium tuberculosis* (MTB) has been seen, further complicating the course and treatment of this disease.

Tuberculosis is of particular concern in low and intermediate income countries such as Iran (1). In Sistan, the annual incidence rate of TB was 96 cases per 100000 population in 2005, 7 times higher than in the general population in Iran (2).The World Health Organization (WHO) recommends the DOTS strategy (for "directly observed treatment, short course") to combat TB in most high-income countries (3). For case finding, integration of active and passive discovery of cases is recommended, with a priority placed on detection of infectious cases (4, 5).

Chronic cough (>2 weeks duration) is an important symptom in the diagnosis of pulmonary tuberculosis. This symptom could be used as a screening index in the evaluation of TB prevalence in specific communities. In Zabol, however, even though the incidence rate of smear-positive pulmonary TB, miliary TB, and TB meningitis had apparently decreased the real incidence and prevalence of tuberculosis in this area is still unknown (3).

Before 1990, vaccination with BCG was not mandatorily given in Iran, and the annual risk of tuberculosis infection was estimated at 0.5-1%. These days, vaccination of ARTI is almost impossible (5, 6).
Sistan is a region in the Sistan and Baluchistan province in eastern Iran (population, 410,713). The aim of this cross-sectional study was to compare the diagnostic value of chest radiograph with sputum smear as screening methods for TB in patients with chronic (>2 weeks duration) cough(7, 8).

Materials and methods:

A total of 50,322 families were interviewed (410,713 individuals) for this study. Individuals experiencing cough of >2 weeks duration were eligible for this study during 2010. Those with suspected tuberculosis were referred to the District Health Center. For each individual enrolled in the study who was suspected of having tuberculosis, the following diagnostic procedures were performed: physical examination of the chest, determination of BCG status through inspection for typical scars, and obtaining of three sputum specimens for laboratory submission; in the lab, these samples underwent microscopy examination and Ziehl Nielsen staining. The quality of the laboratory examinations was controlled by the central laboratory in Zabol, in the center of Sistan. Whether chest radiographs were obtained was left to the discretion of the physician. For cases in which radiographs were obtained, the films were read independently by two radiologists who were aware of the subject's signs or symptoms. Disagreements on interpretation of the radiographs were settled by consensus between the two specialists who read them at first primary care physician and then radiologists read the CXR.
After performing the aforementioned steps, all diagnosed pulmonary TB cases were registered in the study and referred to public health services for standard treatment. The diagnosis of tuberculosis was based on a combination of clinical findings, laboratory reports and response to treatment. Data analyses were performed using SPSS z-tests (SPSS/IBM, Armonk, NY, United States) to compare the sputum smear tests with radiography with respect to positive predictive value, negative predictive value, specificity, and sensitivity.

Results:
Of the 410,713 individuals initially included, 21,645 (8213 female and 13,432 male) had a history of more than 2 weeks' cough. Of these 21,645 individuals, 6250 had scarring that was typical of BCG. Chronic cough was confirmed in 8140 participants through clinical workup. Additionally, seven new pulmonary TB cases were diagnoses through direct microscopy examination of sputum smear samples. (Table and Figure 1)
Chest radiography suggested the presence of pulmonary TB in 27 cases; seven of these had positive sputum smears, 10 had negative sputum smears, and 10 had old TB lesions. Five of these 27 patients had no history of pulmonary TB. (Table 2)
Sensitivity, specificity, PPV, NPV, and the likelihood ratio for sputum smear samples and chest radiography are shown in. (Table 3)

Discussion:
The reported incidence of pulmonary TB is lower in comparison with the actual incidence of disease in Sistan shown during this the study. 5270 individuals presented with chronic cough; 4034 of these presented with positive cough, and 0.173% of these were smear positive (7 of 4034). A similar study in Iran indicated that 1.5% of these types of cases had sputum smear samples that were positive for TB. Another study showed that 20% of cases with chronic cough had smear samples that showed the presence of TB (10). In Sistan in 2010, the prevalence of pulmonary TB and smear-positive pulmonary TB was 96 and 67 per 100,000 population, respectively.
During the present study, all of those with positive sputum smear samples presented clinically with anorexia and weight loss. Fever, cough, and weight loss have been reported as the most common symptoms of pulmonary TB in different studies (9, 11, 12). However, fever and chronic cough were not sensitive enough for adequate TB diagnosis in some studies (13). Overall, chest radiography has greater diagnostic ability than does sputum smear samples (9, 14); this was confirmed again in the present study, which found that chest radiography is significantly better than sputum testing for the diagnosis of TB.
The use of radiography for diagnosis of pulmonary TB has some limitations. Considering active pulmonary TB, 46.4 of suspected patients had positive results on chest radiography (active disease or old TB lesions). Of total tuberculosis cases and suspected cases, active disease was identified in 44.4% of them by means of chest radiography; 25.9% had positive results via sputum microscopy/laboratory examination. Annual incidence of pulmonary TB and the incidence of TB as shown on smear samples were 100 and 19.3 per 100,000 populations, respectively. The Iranian Health Service System identified 67 smear-positive cases of TB in 2006, while the recent study identified 12 additional cases with pulmonary tuberculosis at the same time. Therefore, the Health Service System only identified 90.54% of patients with tuberculosis. In our opinion, the only key for diminishing this difference is revising the application of diagnostic tools.

Conclusion Our findings suggest that the diagnostic power of chest radiograph is more than sputum smear; however, we think the HSS method for taking sputum was not a controlled one.

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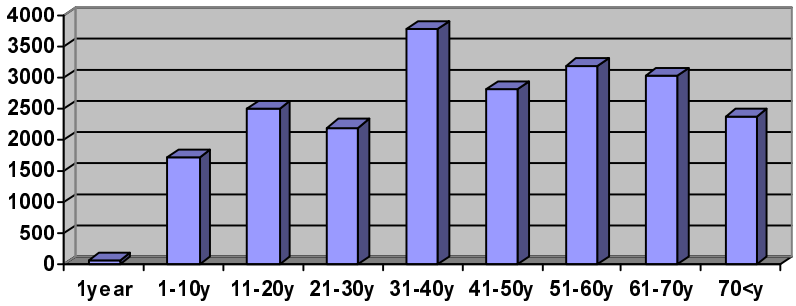

Figure 1. Frequency distribution of chronic cough in different age groups, Zabol, in 2010.

Table1. Results of sputum – smear direct microscopy

Productive cough	**Sputum- smear**		
4034	Total	positive	**negative**
	3670 (90.9%)	**7(0.19%)**	**3663(99.81%)**

Table2.Radiologic finding of patients with chronic cough

Lesion	**Number of patients**
Compatible with pulmonary TB	27(6.6%)
Non-Tuberculosis	190(46.4%)
Heart and alveolar lesion	14 (3.4%)
Non specific	20(4.9%)
Normal graph	159 (38.7%)
Total	410(100%)

Table3. Statistical comparison between sputum smear and chest X-ray

	Sputum smear	**Chest X-ray**	**P value**
sensitivity	57.7	96	0.00
specificity	100	85	0.023
PPV	100	54	0.00
NPV	9.1	100	0.11
Likelihood ratio	19.1	33.31	0.00

Natural Radioactivity and Heavy Metals in Milk Consumed in Saudi Arabia and Population Dose Rate Estimates

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Abstract

This paper represents an important part of the Saudi Food and Drug Authority plane to reach its aims regarding the safety and effectiveness of food for humans.The results of radioactivity analysis carried out for ^{40}K,^{232}Th and^{226}Ra in powdered infant's milk used in Saudi Arabia (Jeddah city).The main detected activity corresponding to^{40}K was within the range reported in different parts of the world with average activity of 234.18±1.9BqKg^{-1}, while the average activities of^{226}Ra,^{232}Th were 0.46 Bqkg^{-1}, and 0.35 Bqkg^{-1}, respectively, although the activity of some samples were below the detection limit. The total average effective dose due to annual intake of ^{226}Ra, ^{232}Th and ^{40}K from the ingestion of the powdered milk for infants were estimated to be 410μSv for infant ≤1Y and157 μSv for infants (1-2Y) ,which are lower than allowed value (1mSv). The heavy metals analyses were done by atomic absorption spectrophotometer. The geometric mean of Fe, Zn, Mn,Cu and Pb in the samples of powdered milk was found to be 3.033 ,2.91, 0.031 ,0.182 and 0.034 mg/ kg respectively, where as the daily intake was computed to be 0.186, 0.179, 0.002 ,0.001 and 0.002 mg / day, respectively. The results showed that the intake of heavy metals through the ingestion of milk did not exceed the limit of one as proposed by US-IPA. This study could be useful as a baseline data for radiation and heavy metals exposure to infant's milk and their impact on infant's health**[**J. H. Al-Zahrani. **Natural Radioactivity and Heavy Metal s in Milk Consumed in Saudi Arabia and Population Dose Rate Estimates.** Life Sci J 2012;9(2):651-656]. (ISSN:1097-8135). http://www.lifesciencesite.com. 98

Keywords: Natural Radioactivity, Milk, Heavy Metals, Ingestion dose, Hazard quotient

1-Introduction:

Milk is an important vector of radio nuclides and heavy metals to man that may get into the environment from the mining activities .The radio nuclides and heavy metals enter the human body mainly by two routes namely : inhalation and ingestion, Licata *et al.,(* 2004).Also milk is one of the important food for human nutrition and contains all the macronutrients namely protein, carbohydrates, fat, vitamins (A,D and B groups) and trace elements particularly calcium, phosphate, magnesium, zinc and selenium, Abollino *et al.,(*1998); Buldini *et al.,(* 2002). Milk is the main basic foodstuffs for the infants especially for infants less than one year because they generally consume more milk on apery body weight basis than adults. So, the assessment of radioactivity and heavy metals levels in the powdered infant's milk and the associated doses are of crucial importance for controlling the radiation levels and necessary in establishing rules and regulations relating to radiation protection ,Quindos *et al.*,(1994) . It is also important to understand the behaviour of natural radio nuclides and heavy metals in the environment because such informations can be used as the associated parameter values for radiological assessment (Vera et al. (2003). In addition Potassium is an essential constituent of cellular tissue,^{40} K is one of the most important natural radio nuclides . Also, the heavy metals such as Fe ,Zn,Cu , Mn and Pb are essential at very low concentrations for the survival of all forms of life .

There is increasing world wide concern about quality of powdered milk , studies in recent years have been indicated,Shukla(1994) Melquiades *et al (*2001) ; Melquiades and Appoloni (2002) ; Al-Masri *et al (*2004) ; Navarrte *et al* (2007) ; Desmani *et al (*2009) ; Zaid et al (2010) ;Shanti *et al* (2010); Marko and Borut (2011) ; Soma *et al (*2011) .

In Saudi Arabia no surveys of radioactivity in powdered infant's milk have been carried out and no baselines of concentration of natural and anthropogenic radioisotopes have been reported. Therefore, the establishment of radio-isotope concentrations will prove meaningful information that can contribute to knowledge of population exposure and to the setting up of original baseline, IAEA (1989).

The aim of this study was to investigate the concentration of some long-lived radio nuclides (^{226}Ra, ^{232}Th and ^{40}K) and the concentration of some heavy metals such as Fe , Zn , Cu , Mn and Pb in the powdered infant's milk . In addition estimation of the annual internal dose from the intake of natural isotopes and heavy metals . These measurements can be useful as baseline values for the estimation of the internal radiation and heavy metals doses .

2 . Materials and Methods:

Twenty five samples of different types of powdered infant's milk were collected from the local markets in Saudi Arabia (Jeddah City) between 2010-2012 . The

Type of samples are listed in Table (1). The powdered samples were stored in tight plastic containers for four weeks to allow radioactive equilibrium to be reached between parents and their daughter radio nuclides , Ibrahim and Pimpl (1994).Detection and measurements of the radio nuclides in the powdered infant's samples were carried out by gamma ray spectrometer using a NaI (TI) detector 3x3 inch with a 1024-chanel computer analyzer.The detector has a peak efficiency of $1.2x10^{-5}$at1332.5KevCo-60and an energy resolution (FWHM)of7.5%for662kev.samples were accounted 10 hours, the activity concentration of Pb^{214} (352Kev) and Bi^{214} (609 Kev,1120Kev) were chosen to provide an estimate of ^{226}Ra ,while that of the daughter radionuclidesTi^{208} (2651Kev)Pb^{212} (239Kev) Ac^{228} (911Kev) were chosen as indicator of ^{232}Th,^{40} K was directly measured using its single photo peak at 1460 KeV emitter ,jibiri *et al*.,(2007). The activity *concentra*tion (AE,i) in Bq kg^{-1}, for a radionuclide i with a detected photo peak at energy E,was obtained from the following equation, Noorddin (1999) :

$AE;i\ (Bq\ kg^{-1}) = NE;i / e_E\ t\ cd\ M$ ....(1)

Where:

***NE;i** = is the net peak-area of the radionuclide i at energy E , e_E is the detector energy-dependent efficiency at energy E , t is the counting live time in sec, cd is the gamma-ray yield per disintegration of the nuclide i for its transition at energy E , M is the mass of the sample.*

Heavy metals were analysed using an atomic absorption spectrophotometer (A Analyst 700) reagents blank determinations were used to correct the instrument readings. Also, after every 4 samples readings standards were run to make sure that the obtained results were within ranges.

3. Results and Discussions

3.1. Radioactivity analysis of infant's milk

The measured activity concentration of ^{226}R, ^{232}Th and ^{40}K detected in the samples of powdered infant's milk under study including their uncertainty are summarized in Table (1).It can be noticed that ^{40}K was detected in most of samples and varied between 210.21±.31 $Bqkg^{-1}$ to 257.51±3.33 $Bqkg^{-1}$ with an average value of 234.18 ±1.9 $Bqkg^{-1}$.The measured concentration ranged from 0.25±0.03 $Bqkg^{-1}$ to 0.85±.12 $Bqkg^{-1}$ and from 0.09±.02 Bqkg to 0.76±.12 $Bqkg^{-1}$ for^{226}Ra and ^{232}Th respectively. On the other hand, the highest values of ^{40}K were detected with activities 250.8±2.55 , 257.51±3.33 and 252.3±275 $Bqkg^{-1}$in samples No. (12, 14 and 20) respectively. The lowest concentration of ^{40}K was found 210.20±.31$Bqkg^{-1}$ in sample No.(24) . While the lowest concentrations of ^{226}Ra and ^{232}Th were 0.25±.03 $Bqkg^{-1}$ and 0.09±0.02 $Bqkg^{-1}$ in samples No.(1) and No.(15), respectively.The ^{226} Ra,^{232} Th,^{40} K activities measured in the present work were comparable with completion of others activated values of milk samples around the world were presented in Table (2). It is important to remark that the ^{226}Ra ^{232}Th and ^{40}K activities levels determined in the present study are similar to those of powdered milk consumed in other countries, Melquiades *et al.*, (2002); Al-Marsi *etal.*,(2004); Hosseni *et al.,(*2006) ;Ibrahim *et al.* ,(2007);Zaid *et al*.,(2010)

Table(1) :activity concentrations of ^{226}Ra,^{232}Th And ^{40}K in powered infant's milk (Bqkg

Table (2): Comparison of the average concentrations of

No.	Samples	226Ra	232Th	40K
1	Semilac Gain	0.25±0.03	-----	-----
2	Eptamil	0.55±0.10	0.29+0.02	210.7±2.26
3	Bi0mil	0.32±.02	0.76±.12	222.3±0.47
4	Iasomil	0.68±.08	--------	-------
5	Babelac	0.61±.07	---------	-------
6	Hiap	0.30±.03	---------	--------
7	Expret	0.44±.0.03	0.64±.06	216.9±0.9
8	Novalac	0.49±.04	-------	-------
9	Maial mam	0.28±.02	0.45±.05	-------
10	Meloppa	0.33±.02	0.23±.07	-------
11	Fabimilk	0.85±.12	0.62±.04	------
12	Saha	……	……	250.8±2.55
13	Smilac total	--------	0.30±.09	-------
14	Gain kids	---------	0.65±.14	257.51±3.33
15	France	---------	0.09±0.02	229.9±2.12
16	Blemil plus	--------	0.45±0.04	226.1±2.37
17	Eptajenuer	---------	--------	248.7±3.31
18	Gain plus	-------	0.48±0.06	247.3±2.73
19	Brame care	--------	0.39±0.02	219.1±3.05
20	Nan	-------	0.41±0.07	252.3±2.57
21	Novalac	-------	--------	243.2±0.41
22	Ronagrow	-------	-------	219.9±0.88
23	Larilac	--------	0.73±0.03	227.2±2.76
24	Soupermail	--------	0.56±.03	210.21±0.31
25	Promil	--------	0.22±0.07	231.5±0. 61
	Average	0.46	0.35	234±1.9

Table (2): Comparison of the average concentrations of ^{226}Ra, ^{232}Th and ^{40}Kin powered infant's milk with those published data in powder milk(Bqkg^{-1})

Region	^{226}Ra	^{232}Th	^{40}K	Reference
Present Work	0.25 – 0.85	0.09 - 0.76	210 – 257	present Work
Iran/France	0.05	0.142	434	Hosseni *et al.,* (2006)
Jordan	0.5 --2.14	0.78--1.28	349 —392	Zaid *et al.,*(2010)
Newzealand	0.149--0.186	0.147 –.166	594-605	Hosseni *et al.,* (2006)
France	0.05±.011	0.142±.026	434.1	Hosseni *et al, ,*(2006)
Brazil	----	1.7 – 3.7	489	Melquiades *et al.,* (2002)
Egypt	------	-----	222.11	Ibrahim *et al.* (2007)
Syria	-----	----	129- -435	Al-Marsi *et al.,*(2004)

3.2. Internal dose of the radio nuclides from ingested milk

Radiation doses to population from intake of radio nuclides in foods can be calculated from the Formula reported in Reference (UNSCEAR 2000) :

D = C A R --------- (2)

Where :

D *is the effective dose by ingestion of the radionuclide (Sv Y^{-1}),* ***A****is the activity concentration of the radionuclides in the sample (Bqkg^{-1}) ,* ***C*** *is the internal dose conversion factor by ingestion of the radionuclides (Sv Bq^{-1}) ,* ***R*** *is the annual intake of milk (Kg Y^{-1}) which depends on a given age* (ICRP 1996).

Annual effective ingestion dose due to milk consumption strongly depends on the milk consumption. In our study the average mass of the milk consumed by the infant ≤ 1Y and infant 1-2Y were 22.4KgY^{-1} and 15KgY^{-1} ,UNSCEEAR(1993).

Table 3:The dose conversion factor of ^{40}K, ^{226}Ra, ^{232}Th for the age infants (≤1Y)and (1-2Y

Dose conversion factors (Sv Bq-1)			
	^{40}K	^{226}Ra	^{232}Th
Infants ≤1Y	6.2×10^{-8}	4.7×10^{-6}	4.6×10^{-6}
Infants (1-2Y)	4.2×10^{-8}	9.6×10^{-7}	4.5×10^{-7}

For the calculation, the recommended conversion factors ICRP (1996) in Table (3) with Table (1) were used . The results listed in Table (4) showed that the average annual doses received from the intake of ^{40}K, ^{232}Th, ^{226}Ra due to the ingestion of the powdered infant's milk were 410 µSv Y^{-1} and 157 µSv Y^{-1} for the ages ≤ 1Y and 1-2 year respectively .Also ^{40}K gives the largest contribution to the total average annual effective dose due to the high consumption rate of milk in the first year, after the age of one year the baby starts to have more solid foodstuff than milk .These results for all ages of infants are within the typical world wide range of annual dose (200–800 mSv) due to the ingestion of all natural radiation sources UNCEAR(2000) .

Table 4: Annual radionuclide intake and effective ingestion dose due to the intake of ^{226} Ra , ^{232}Th and^{40}K in powdered infant's milk

Radionuclide		In*take* *(BqY^{-1})*		Ingestion dose (µSv Y^{-1})	
		Infants		*Infants*	
		≤1Y	1-2 Y	≤ 1Y	1-2Y
^{40}K	Minimum	4709	3153	291.94	132.43
	Maximum	5768	3882	357.63	163.07
	Average	5246	3512	325	148
^{226}Ra	Minimum	5.6	4.0	26.32	3.6
	Maximum	19	13	89.49	12.24
	Average	10	7.0	48	7.0
^{232}Th	Minimum	2.0	1.35	9.27	0.61
	Maximum	17	11	78.31	5.13
	Average	8.0	5.0	36	2.0
Total Average				410	157

3.3. Heavy metals analysis of infant's milk

The range of Fe, Zn, Mn ,Cu and Pb in the milk samples were 2.125 - 3.971 mg/kg , 2.04 – 3.943mg/kg ,0.005 - 0.063 mg/kg , 0.019 – 0.336 mg/kg and 0.012 – 0.098 mg/kg respectively, Table (5). The geometric mean concentration of Fe , Zn , Mn , Cu and Pb was found to be 3.033 ,2.91 ,0.031 ,0.182 and 0.034 mg/kg respectively .The mean of each heavy metal was compared with acceptable limits recorded by International Dairy Federation ,IDF(1979) in which the limits were given as 0.37 ,3.28 , 0.025 , 0.1 and 0.049 mg/kg for Fe , Zn , Mn , Cu and Pb respectively. It was appeared that all heavy metals values reported in this study were within the accepted limits, except the value of iron , which was found exceeded the permissible limit, but it is similar to average value 4.02 – 3.94 mg/kg in milk reported by Jelena *et al.,(*2007) and the value 4.91 mg /l represented by Soma *et al.,(*2011).The infants need iron unusually o rapid growth. In addition, the institute of medicine has estimated that growing infant needs to gain iron of 0.7 mg per day, IMDR (2001).

3.4. Daily intake of heavy metals and hazard quotient

Risk from heavy metals intake through ingestion may be characterized using a hazard quotient (HQ).This is the ratio of the average daily dose (ADD ;milligrams per kilogram of body weight per day) of a chemical to a reference dose (Rf Do; milligrams per kilograms per day)defined as the maximum tolerable daily intake of specific metal that dose not result in any deleterious health effects:

HQ = ADD/RfDo --------(3)

The HQ was estimated for the heavy metals by the intake of the milk , if HQ > 1.0 ,then the ADD of a particular metal exceeds the RfDo ,indicating that there is a potential risk associated with that metal .

The average daily dose(ADD) was calculated by dividing the intake by the average body weight of infants 8.5 Kg and 14.6 Kg for infants ≤ 1Y and infants (1-2Y) respectively ,Dang et al .,(1996).The daily intake was estimated taking into account

Table 5 . Concentration of heavy metals in infant's milk

Samples	Concentration of heavy metals (mg/kg)					Sampels	Concentration of heavy metals (mg/kg)				
	Fe	*Mn*	*Zn*	*Cu*	*Pb*		*Fe*	*Mn*	*Zn*	*Cu*	*Pb*
Semilac Gain	3.271	0.022	2.142	0.132	0.025	**Gain kids**	3.124	0.037	3.153	0.300	0.018
Eptamil	3.367	0.026	3.549	0.213	0.098	**France**	3.057	0.026	2.593	0.146	0.026
Bi0mil	3.614	0.015	2.348	0.202	0.045	**Blemil plus**	3.478	0.042	3.046	0.215	0.038
Iasomil	3.271	0.037	3.362	0.243	0.027	**Eptajenuer**	3.001	0.009	3.182	0.135	0.021
Babelac	3.1.22	0.031	2.611	0.122	0.021	**Gain plus**	3.345	0.005	3.943	0.221	0.097
Hiap	2.535	0.008	3.139	0.162	0.023	**Brame care**	3.971	0.032	3.087	0.143	0.016
Expret	3.208	0.063	4.343	0.120	0.015	**Nan**	3.036	0.026	4.085	0.205	0.042
Novalac	3.114	0.052	3.849	0.232	0.019	**Novalac**	2.858	0.025	2.195	0.335	0.059
Maial mam	3.421	0.012	2.457	0.202	0.024	**Ronagrow**	3.593	0.054	2.041	0.146	0.043
Meloppa	3.225	0.046	3.449	0.143	0.032	**Larilac**	2.125	0.018	3.278	0.019	0.012
Fabimilk	2.223	0.036	2.510	0.119	0.015	**Soupermail**	2.146	0.058	2.127	0.152	0.061
Ssaha	3.118	0.041	2.321	0.336	0.016	**Promil**	2.374	0.021	2.846	0.141	0.024
Smilactotal	2.231	0.035	3.081	0.155	0.041						
Geomean	Fe 3.033	Mn 0.031	Zn 2.91	Cu 0.182	Pb 0.034						

the geometric mean concentration of the metals in milk and the average milk consumption per day for infants≤1y and infants (1-2Y) respectively UNSCEEAR(1993).The RfDo of all the heavy metals except Pb were considered from US-EPA(2003). The RfDo of Pb was taken from WHO(1993).

Estimated exposure and hazard quotient due to intake of infant's milk are given in Table (6). Results showed that the daily intake of Fe ,Mn , Zn, Cu and Pb was 0.186,0.002,0.011,0.021,0.0002 mg/day respectively. These results are normally significantly lower than the recommended desirable levels of , 3.0 – 5.0 mg/kg and 0.5 – 1.0 mg/kg for Zn and Cu respectively, FAO/WHO (1992).The hazarded quotients(HQ) of the heavy metals suggest that the heavy metals in the infant's milk does not pose any apparent threat to the infants, where the HQs of all the considered heavy metals were below the value of (1) as suggested by US-EPA .The HQ ranges from 0.0017(Mn) to 0.07 (Zn).

Table 6 Intake and hazard quotient of heavy metals milk due to ingestion of infant's

Heavy metals	[a]RfDo (mg/kg body weight/day	Geomean (mg/kg)	[b]Intake (mg /day)	[b]ADD (mg/kg body weight/day)	HQ (hazard Quotient)
Fe	7.0×10^{-1}	3.033	0.186	0.022	0.031
Mn	1.4×10^{-1}	0.031	0.002	0.0002	.0017
Cu	4.0×10^{-2}	0.182	0.011	0.0013	0.033
Zn	3.0×10^{-1}	2.91	0.179	0.021	0.07
Pb	3.5×10^{-3}	0.034	0.002	0.0003	0.007

[a]RfDo (Reference oral dose) , [b]Intake and [b]ADD (for infants age ≤1 Y)

Conclusions

Natural radioactivity such as ^{226}Ra , ^{232}Th ,and ^{40}K radio nuclides were determined for most available powdered infant's milk consumed in Saudi Arabia. The main gamma activity arises from ^{40} K which was a detected value to be within the world wide ranges as reported in other regions around the Natural radioactivity such as ^{226}Ra , ^{232}Th ,and ^{40}K radio nuclides were determined for most available powdered infant's milk consumed in Saudi Arabia. The main gamma activity arises from ^{40} K which was a detected value to be within the world wide ranges as reported in other regions around the world. ^{226}Ra and ^{232}Th activities were below the detection limits. In addition the annual effective internal dose due to the intake of powdered infant's milk was calculated, the main contributor was ^{40}K at the first year due to high consumption rate of milk concentration of heavy metals in infant's milk will provide baseline data and there is a require for intensive of the sampling for quantification of the result. The contribution to the dietary intake of heavy metals was lower than Provisional Tolerable Daily Intake reported by the Joint, FAO/WHO (1992).The largest contributors to the dose received from ingestion of milk in general was due to natural radio nuclides, particularly ^{40}K which was an essential constituent of cellular tissue.^{40} K is one of the most important natural radio nuclides. Regular monitoring of these radio nuclides and the metals in the milk and in other food to prevent excessive build up of the metals in the food chain.

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Effect of Acute Apelin Injection on Cardiac Muscle Performance in both Normal and Diabetic Rats

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Abstract: Background: Apelin is an adipokine originally identified as the endogenous ligand of the G protein coupled receptor APJ. Several studies have demonstrated that apelin and its receptor are involved in the regulation of cardiovascular function. Apelin was also found to have a positive inotropic effect in both rat and human hearts. However, this effect in case of cardiovascular diseases is controversial. Diabetes mellitus is one of the major risk factors for cardiovascular disease which is the leading cause of death in those patients**. Aim**: This study was designed to detect possible acute effects of *in vivo* apelin-13 injection on cardiac performance in both normal and diabetic state, with a trial to clarify possible involved mechanisms .**Material & methods**: This study was conducted on 72 healthy, adult, male albino rats. The animals were divided equally into three main groups: **Group I:** Control group. **Group II:** Streptozotocin -induced diabetic non treated rats. **Group III**: Insulin treated diabetic rats. **Experimental design:** In the three groups we examined the effect of acute injection of apelin-13 (10 nmol /kg b.wt) alone or in the presence of propranolol (0.2mg/kg b.wt), verapamil (4.8mg/kg), benzamil HCL (Na/ Ca^{+2} exchange (NCX) blocker) (10 nmol/kg), on cardiac muscle performance. **Results**: The present results demonstrated that apelin-13 administration significantly increased cardiac muscle performance ($p<0.001$) without any significant changes in heart rate, in all groups, as evidenced by the significant increase in ($+dT_{max}/t_{max}$) and ($-dT_{max}/t_r$). In addition, this increase was more significant in diabetic rats in comparison with that of both control and diabetic treated rats. Moreover, the observed effects are independent of the voltage- gated calcium channels or B- adrenergic receptors but appear to involve activation of the sacrolemmal Na^{++} /Ca^{++} exchanger (NCX). **Conclusion:** apelin-13 exerted both positive inotropic and lusiotropic effects without affection of the heart rate in vivo, which was more significant in diabetic rats in comparison with that of both normal and insulin treated rats. Our results also suggested that this response to apelin involved activation of Na^{+}- Ca^{+2} exchange channels (NCX). Therefore, the use of apelin may be investigated as a potential therapeutic target for diabetic cardiomyopathy. However, the impact of chronic administration requires further attention.
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Key words: Apelin, diabetes, heart pressure, rats

1. Introduction

Apelin is a bioactive peptide originally identified from bovine stomach extracts as the endogenous ligand of the G protein coupled receptor

APJ **(Boucher *et al.*, 2005)**. Apelin is considered as an adipokine, essentially as the result of its increased expression during adipocyte differentiation and its release by differentiated adipose cells into the medium culture (**Wei *et al.*, 2005**).

The next studies have demonstrated that apelin and its receptor are widely expressed in the central nervous system (CNS) and peripheral tissues, and involved in the regulation of cardiovascular function **(Hosoya *et al.*, 2000, Lee *et al.*, 2000, Kawamata *et al.*, 2001 , Macaluso *et al.*, 2011).**

Most importantly, apelin has been shown to act as an endogenous inotrope regulating cardiac contractility (**Ashley *et al.*, 2005, Jia *et al.*, 2006, Zeng *et al.*, 2007)** and playing an important role in paracrine signaling in the heart (**Chen *et al.*, 2003 and Földes *et al.*, 2003**).

Diabetes mellitus is one of the major risk factors for cardiovascular disease which is the leading cause of death in those patients**.** Aside from large vessel disease and accelerated atherosclerosis, which is very common in diabetes, diabetic cardiomyopathy is a clinical condition diagnosed when ventricular dysfunction develops in patients with diabetes in the absence of coronary atherosclerosis and hypertension **(Avogaro *et al.*, 2004).**

Apelin expression in adipose tissue is regulated by nutritional status, such as fasting and refeeding **(Boucher *et al.*, 2005)**, insulin **(Wei *et al.*, 2005)** and tumor necrosis factor- alpha **(Daviaud *et al.*, 2006)**. Mice with streptozotocin-induced diabetes mellitus had decreased apelin expression **(Boucher *et al.*, 2005)**, whereas apelin levels were increased in obese, hyperinsulinemic humans compared to normal weight subjects **(Heinonen *et al.*, 2005 and Boucher *et al.*, 2005)**.

In addition, accumulating evidence supports apelin involvement in cardiovascular function, but its

causative relationship with ischemic heart disease is controversial **(Ronkainen *et al.,* 2007, Chandrasekaran *et al.,* 2008 and Rastaldo *et al.,* 2011).** Limited evidence has emerged, indicating the association of reduced apelin with coronary atherosclerosis **(Weir *et al.,* 2009).** Consistent with previous studies of **Li *et al.* (2008), Kadoglou *et al.* (2010)** found lower apelin levels in patients with coronary artery diseases (CAD) than in the healthy controls. Besides this finding, they confirmed the correlation of low apelin concentrations with a CAD presence and severity. Moreover, lower plasma apelin was associated with left ventricular systolic and diastolic function impairment **(Przewlocka-Kosmala *et al.,* 2011)**

Importantly, the latter relationship was independent of other traditional cardiovascular risk factors. Taken together, apelin emerged as a novel biomarker of coronary atherosclerosis development and severity, but this result remains to be proved prospectively **(Kadoglou *et al.,* 2010)**.

Up to our knowledge, there is no information on the functional in vivo effects of apelin in case of diabetic cardiomyopathy. Moreover, the possible mechanisms of action of apelin on cardiac performance have not yet been sufficiently cleared.

This study was designed to detect possible acute in vivo effects of apelin on cardiac performance in both normal and diabetic state, with a trial to clarify possible involved mechanisms.

2. Animals and methods

Animals:

This study was conducted on 72 healthy, adult, male albino rats weighing 180- 200 gm. The animals had free access to water and chow and were kept at room temperature.

Ethical committee approval for the study was obtained from Zagazig University

The animals were divided equally into 3main groups:

Group I: To study the acute effect of apelin-13 injection (10 nmol /kg) **(Cheng et al., 2003)** on cardiac muscle performance of normal rats.

Group II: To study the acute effect of apelin-13 injection (10 nmol /kg) **(Cheng et al., 2003)** on cardiac muscle performance of strptozotocin -induced type 1 diabetic non treated rats.

Diabetes was induced by a single intra-peritoneal injection of freshly prepared solution of streptozotocin 65 mg/kg of body weight dissolved in 0.2 mmol/L sodium citrate, at pH 4.5 **(Lutz and Pardridge, 1993)** and the rats maintained for 6 weeks **(Srinivasan et al., 1997, Shenoy and Goyal 2002)**.

Three days later, diabetes induction was confirmed through measurement of blood glucose level in each animal (blood was sampled from the tail vein) with the One Touch Ultra Glucometer **(Yves and Theo, 2007)** and rats with blood glucose levels more than 250 mg/dl were selected for experiments **(Coskun et al., 2004)**. The rats were provided with oral 10% glucose solution after 6 hours of streptozotocin administration for the next 48 hours.

Group III: To study the acute effect of apelin-13 injection (10 nmol /kg) on cardiac muscle performance of strptozotocin -induced type 1 diabetic insulin treated rats. These animals were treated with regular (R) and NPH (N) insulin (2UR at diagnosis of diabetes and then 1R/3N at 6 P.M and 1R/1N at 9 A.M daily subcutaneously for 6 weeks after induction of diabetes **(Sivitz et al., 1998).**

Methods:

Recording of cardiac muscle performance parameters via D1 isometric transducer (Bioscience, London) attached to **a** 4-channel oscillograph "MD4" (Bioscience, London)

The rats were anaesthetized by intraperitoneal injection of ethyl carbamate (urethane) in a dose 1.75-2 gm /kg body weight injected intraperitonealy as 25 % freshly prepared aqueous solution **(Gosh, 1971).** Tracheotomy was performed on the neck to open a direct airway through an incision in the trachea and connected to the artificial ventilator. The rats were ventilated with room air at 60-70 breaths/ min. The right jugular vein was cannulated to infuse saline or drugs throughout the experiment. Upon completion of the surgical procedures, the animals were allowed to stabilize, generally for 30 min.

A 6-0 prolene suture was fixed to the ventricle and passed via thoracotomy to be attached to the hock of D1 isometric transducer (the baseline tension of the rat heart is adjusted at 2.00 grams). The FC 117 direct input coupler is fixed to one channel of the oscillograph and connected to D1 isometric transducer. Calibration of the isometric transducer using increasing weights, and recording the corresponding pen deflection was done before starting anesthesia.

Experimental design

Experiment I: to study the acute effect of apelin-13 injection (10 nmol/kg) **(Cheng et al., 2003)** on cardiac muscle performance in the three main groups (n=18)

Experiment II: to study the acute effect of apelin-13 injection (10nmol/kg) on cardiac muscle performance 10 minutes after the propranolol injection (0.2mg/kg) **(Vongpatanasin et al., 1999)** in the three main groups (n=18)

Experiment III: to study the acute effect of apelin-13 (10 nmol/kg) on cardiac muscle performance

10minutes after the verapamil (ca $^{+2}$ channel blocker) injection (4.8mg/kg) **(Persson et al., 2007)** in the three main groups (n=18).

Experiment IV: to study the acute effect of apelin-13 injection (10nmol/kg) **(Cheng et al., 2003)** on cardiac muscle performance 10 minutes after benzamil HCL (Na/ Ca^{+2} exchange (NCX) blocker) injection (10 nmol/kg) **(Nishimura et al., 1998)** in the three main groups (n=18).

NB: The maximal effect of apelin injection on cardiac muscle performance was calculated and statstatically investigated in all experiments (this effect was about 5-10 minutes after its injection).

Calculation of the studied parameters

1-Maximum tension developed ($+dT_{max}$): It was obtained from the calibration of tension on the graph in grams.

2-Time to reach maximum tension (t_{max}): From the point of maximum tension a vertical was drawn to meet the baseline of the recorded tension on the graph. The distance on the baseline from the onset of tension rise till vertical line was measured. As the speed of the oscillograph equal to 50 mm/ sec, so every 1mm measured on the baseline equal to 0.02sec. According to the latter equation the time to reach maximum tension **(t_{max})** was calculated in seconds.

3- Rate of developing tension ($+dT_{max}/ t_{max}$): By dividing Maximum tension developed **($+dT_{max}$)** by time to reach maximum tension **(t_{max})** was calculated as gm/ sec.

4-Time of cardiac relaxation (t_r): It was calculated by measuring the distance on the baseline from the point of maximum tension till return to basal tension. The time of cardiac relaxation was assessed as every 1mm equals 0.02 sec.

5-Rate of cardiac relaxation ($-dT_{max}/t_r$): By dividing the maximum tension developed by the time of cardiac relaxation **(t_r)**; the rate of cardiac relaxation **($-dT_{max}/t_r$)** was calculated and expressed as gm/sec.

6- Calculation of the heart rate/ minute was carried out by counting the number of the heart cycles (n) per fixed distance of chart paper **(Gay, 1965)**.

Statistical analysis:

Data were presented as mean ± SD. Statistical significance was determined by one way analysis of variance (ANOVA) between the three main groups, and student's t test (paired and unpaired) in the same group. P values less than 0.05 were considered to be significant. In statistical analysis, SPSS program version 10.0 for Windows (SPSS Inc. Chicago, IL, USA) was used**.**

3. Results

Table 1: Shows blood glucose levels (mg/dl) at the end of the study period in all groups. Serum glucose levels in group II (mean ± SD) (413.5 ± 85.49mg/dl) was significantly increased ($P< 0.001$) when compared with that of group I (78.1 ± **6**.32mg/dl). Moreover, in group III serum glucose levels were significantly decreased and return to the normal levels when compared with that of group II (81.77 ± 5.88mg/dl& $P < 0.001$).

Table 2 and record 1: Show cardiac contractility parameter; the rate of development of tension (+dTmax/tmax) [gram/second] and cardiac relaxation parameter; rate of relaxation (-dTmax/ tr) [gram/second] and heart rate in the three main groups: There was a significant decrease in (+dTmax/tmax)[gram/second] (mean± SD) (91.7± 5.2 gram/second) in diabetic group in comparison with that of both Control (110.3± 12.8 gram/second, $P<0.01$) and insulin treated (104.8± 10.7 gram/second, $P<0.05$) groups.

In addition, there was a significant decrease in (-dTmax/ tr) [gram/second]: (mean± SD) (31.2± 3.6 gram/second) in diabetic group in comparison with that of both control (36.1± 2.3 gram/second, $P<0.01$) and insulin treated groups (36.8± 0.8 gram/second, $P<0.01$).

In addition, there was a significant decrease in heart rate (mean± SD) (320± 15.5 beat\ min) in diabetic group in comparison with that of both control (355± 22.6 beat\ min, $P<0.05$) and insulin treated (350± 31 beat\ min, $P<0.05$) groups.

Table 3 and record 2: Show the effect of I.V. bolus injection of apelin-13 (10 nmol/kg) on cardiac contractility parameter; the rate of development of tension (+dTmax/tmax) [gram/second] in the three main groups.

In group I: there was a significant ($P< 0.001$) increase in (+dTmax/tmax) from (mean± SD) (110.3± 12.8 gram/second) to (133.3± 12.9 gram/second) after apelin injection.

In group II: there was a significant ($P< 0.001$) increase in (+dTmax/tmax) from (mean± SD) (91.7± 5.2 gram/second) to (120.5± 5.8 gram/second) after apelin injection.

In group III: there was a significant ($P< 0.001$) increase in (+dTmax/tmax) from (mean± SD) (104.7± 10.7 gram/second) to (127,5± 10 gram/second) after apelin injection.

Moreover, the percentage of increase was more significant in diabetic group (group II), (mean± SD) was (31.5±2.6) compared to that of both group I (21.2±3.1, $P < 0.001$) and group III (20.2±3, $P < 0.001$).

Table 4 and record 2: Show the effect of I.V. bolus injection of apelin-13 (10 nmol/kg) on cardiac

relaxation parameter; rate of relaxation (-dTmax/tr) [gram/second] in the three main groups.

In group I: there was a significant (P< 0.001) increase in (-dTmax/tr) from (mean± SD) (36.1± 2.3 gram/second) to (44.8± 3.1 gram/second) after apelin injection.

In group II: there was a significant (*P*< 0.001) increase in (-dTmax/tr) from (mean± SD) (30.8±2.8 gram/second) to (40.5± 2.9 gram/second) after apelin injection.

In group III: there was a significant (*P*<0.001) increase in (-dTmax/tr) from (mean±SD) (37±1 gram/second) to (46.1±1.2 gram/second) after apelin injection.

Moreover, the percentage of increase was more significant in diabetic group (group II), (mean±SD) was (29.8±3.2) compared to that of both group I (23.1±4.1, P<0.01) and group III (25.5±3, P<0.05).

Table 3 and record 3: Show the effect of I.V. bolus injection of apelin-13 (10 nmol/kg) on cardiac contractility parameter; the rate of development of tension (+dTmax/tmax) [gram/second] in the presence of verapamil (4.8 mg/kg) in the three main groups.

In group I: there was a significant (*P*<0.001) increase in the rate of development of tension (+dTmax/tmax) after apelin-13 injection in the presence of verapamil, (mean± SD) was (94.3± 9.3 gram/second) compared to (79± 7.7 gram/second) before apelin-13 injection.

In group II: there was a significant (P<0.001) increase in the rate of development of tension (+dTmax/tmax) after apelin-13 injection in the presence of verapamil, (mean± SD) was (83.8± 13 gram/second) compared to (64.2± 9.3 gram/second) before its injection.

In group III: there was a significant (P<0.01) increase in the rate of development of tension (+dTmax/tmax) after apelin-13 injection in the presence of verapamil, (mean± SD) was (85.3± 9.6 gram/second) compared to (72.6± 7.1 gram/second) before its injection.

Furthermore, no significant difference was detected in the percentage of increase in (+dTmax/tmax) in the presence of verapamil in comparison to that produced by apelin alone in all groups.

Table 4 and record 3: Show the effect of I.V. bolus injection of apelin-13 (10 nmol/kg) on cardiac relaxation parameter; rate of relaxation ($-dT_{max}/t_r$) [gram/second] in the presence of verapamil injection (4.8mg/kg) in the three main groups.

In group I: there was a significant (P<0.001) increase in the rate of relaxation ($-dT_{max}/t_r$) after apelin-13 injection in the presence of verapamil, (mean± SD) was (37.2± 2.5 gram/second) compared to (29.8± 1.4 gram/second) before apelin-13 injection.

In group II: there was a significant (P<0.001) increase in the rate of relaxation ($-dT_{max}/t_r$) after apelin-13 injection in the presence of verapamil, (mean± SD) was (35.5± 2.9 gram/second) compared to (27.4± 2.2 gram/second) before its injection.

In group III: there was a significant (P<0.001) increase in the rate of relaxation ($-dT_{max}/t_r$) after apelin-13 injection in the presence of verapamil, (mean± SD) was (38± 2.5 gram/second) compared to (30.7± 1.9 gram/second) before its injection.

Furthermore, no significant difference was detected in the percentage of increase in ($-dT_{max}/t_r$) in the presence of verapamil in comparison to that produced by apelin alone in all groups**.**

Table 3 and record 4: Show the effect of I.V. bolus injection of apelin-13 (10 nmol/kg) on cardiac contractility parameter; the rate of development of tension ($+dT_{max}/t_{max}$) [gram/second] in the presence of propranolol injection (0.2mg/kg) in the three main groups.

In group I: there was a significant (P<0.001) increase in the rate of development of tension ($+dT_{max}/t_{max}$) after apelin-13 injection in the presence of propranolol, (mean± SD) was (91.5± 6.5 gram/second) compared to (76.3± 6.5 gram/second) before apelin-13 injection.

In group II: there was a significant (P<0.001) increase in the rate of development of tension ($+dT_{max}/t_{max}$) after apelin-13 injection in the presence of propranolol, (mean± SD) was (66.6± 4.5 gram/second) compared to (52± 3.7 gram/second) before its injection.

In group III: there was a significant (P<0.01) increase in the rate of development of tension ($+dT_{max}/t_{max}$) after apelin-13 injection in the presence of propranolol, (mean± SD) was (87.7± 7.2 gram/second) compared to (72.3± 6.4 gram/second) before its injection.

Furthermore, no significant difference was detected in the percentage of increase in ($+dT_{max}/t_{max}$) in the presence of propranolol in comparison to that produced by apelin alone in all groups.

Table 4 and record 4: Show the effect of I.V. bolus injection of apelin-13 (10 nmol/kg) on cardiac relaxation parameter; rate of relaxation ($-dT_{max}/t_r$) [gram/second] in the presence of propranolol injection (0.2mg/kg) in the three main groups.

In group I: there was a significant (P<0.001) increase in the rate of relaxation ($-dT_{max}/t_r$) after apelin-13 injection in the presence of propranolol, (mean± SD) was (38.5± 2.4 gram/second) compared to (31.7± 1.8 gram/second) before apelin-13 injection.

In group II: there was a significant (P<0.001) increase in the rate of relaxation ($-dT_{max}/t_r$) after apelin-13 injection injection in the presence of propranolol, mean± SD) was (36.7± 1.3 gram/second)

compared to (28.2± 0.7 gram/second) before its injection.

In group III: there was a significant (P<0.001) increase in the rate of relaxation ($-dT_{max}/t_r$) after apelin-13 injection in the presence of propranolol, (mean± SD) was (37.8± 2.2 gram/second) compared to (30.2± 1.9 gram/second) before its injection.

Furthermore, no significant difference was detected in the percentage of increase in ($-dT_{max}/t_r$) in the presence of propranolol in comparison to that produced by apelin alone in all groups.

Table 3 and record 5: Show the effect of I.V. bolus injection of apelin-13 (10 nmol/kg) on cardiac contractility parameter; the rate of development of tension ($+dT_{max}/t_{max}$) [gram/second]in the presence of benzamil hydrochloride injection (10 nmol/kg) in the three main groups.

In group I: there was a significant (P<0.01) increase in the rate of development of tension ($+dT_{max}/t_{max}$) after apelin-13 injection in the presence of benzamil hydrochloride, (mean± SD) was (120.3± 13.1 gram/second) compared to (111.8± 12.8 gram/second) before apelin-13 injection.

In group II: there was a significance (P<0.001) increase the rate of development of tension ($+dT_{max}/t_{max}$) after apelin-13 injection injection in the presence of benzamil hydrochloride, (mean± SD) was (106.5± 5.3 gram/second) compared to (90.6± 5 gram/second) before its injection.

In group III: there was a significance (P<0.001) increase the rate of development of tension ($+dT_{max}/t_{max}$) after apelin-13 injection in the presence of benzamil hydrochloride, (mean± SD) was (118.6± 9.5 gram/second) compared to (109.6± 8.4 gram/second) before its injection.

Furthermore, benzamil hydrochloride injection partially blocked the action of apelin as evidenced by the significant decrease in the percentage of increase in ($+dT_{max}/t_{max}$) in comparison with that produced by apelin alone in all groups (p<0.001).

Table 4 and record 5: Show the effect of I.V. bolus injection of apelin-13 (10 nmol/kg) on cardiac relaxation parameter; rate of relaxation ($-dT_{max}/t_r$) [gram/second] in the presence of benzamil hydrochloride injection (10 nmol/kg) in the three main groups.

In group I: there was a significant (P<0.01) increase in the rate of relaxation (-dTmax/tr) after apelin-13 injection in the presence of benzamil hydrochloride, (mean± SD) was (41± 1.4 gram/second) compared to (37.3± 0.9 gram/second) before apelin-13 injection.

In group II: there was a significant (P<0.001) increase in the rate of relaxation (-dTmax/tr) after apelin-13 injection in the presence of benzamil hydrochloride, (mean± SD) was (35.8± 2.9 gram/second) compared to (30.1±1.9 gram/second) before its injection.

In group III: there was a significant (P<0.001) increase in the rate of relaxation (-dTmax/tr) after apelin-13 injection in the presence of benzamil hydrochloride, (mean± SD) was (41.2± 4.2 gram/second) compared to (37.4± 3.7 gram/second) before its injection. Furthermore, benzamil hydrochloride injection partially blocked the action of apelin as evidenced by thesignificant decrease in the percentage of increase in (-dTmax/tr) in comparison with that produced by apelin alone in all groups (P<0.001).

Table 5: Shows the effect of I.V. bolus injection of apelin-13 at a dose of 10 nmol/kg on HR (beat\ min) in the three main groups.

In group I: there was a non-significant (P>0.05) change in HR after apelin-13 injection, (mean± SD) was (360± 19 beat\ mim) compared to (355± 22.6 beat\ mim) before its injection.

In group II: there was a non-significant (P>0.05) change in HR after apelin-13 injection, (mean± SD) was (325±12.2 beat\ mim) compared to (320± 15.5 beat\ mim) before its injection.

In group III: there was a non-significant (P>0.05) change in HR after apelin-13 injection, (mean± SD) was (355± 35.1 beat\ mim) compared to (350± 31 beat\ min) before its injection.

Table 1: Shows blood glucose levels (mg/dl) at the end of the studied period in all groups.

	Control	Diabetic	Diabetic treated
$\bar{X}$	78.1	413.5	81.5
SD	6.32	85.49	5.88
P value of LSD vs control	P<0.001		NS
P value of LSD vs diabetic		P<0.001	

NS: non-significant

Table (2): Shows the rate of development of tension ($+dT_{max}/t_{max}$)[gram/second]and rate of relaxation ($-dT_{max}/t_r$)[gram/second], and heart rate (beat/min) in all groups:

	$(+dT_{max}/t_{max})$ [gram/second]			**$(-dT_{max}/t_r)$** [gram/second]			**HR (beat/min.)**		
	Control	Diabetic	Diabetic treated	Control	Diabetic	Diabetic treated	Control	diabetic	Diabetic treated
$\bar{X}$	110.3	91.7	104.7	36.1	31.2	36.8	355	320	350
SD	12.8	5.2	10.7	2.3	3.6	0.8	22.6	15.5	31
P value of LSD vs control	P< 0.01		NS	<0.05		NS	<0.05		NS
Vs diabetic			P< 0.05			P<0.01			<0.05

NS: non-significant

Table (3): Shows the effect of I.V. bolus injection of apelin-13 (10 nmol/kg) alone or in the presence of verapamil (4.8mg/kg), propranolol (0.2mg/kg) or benzamil Hcl (10 nmol/kg) on the rate of development of tension ($+dT_{max}/t_{max}$) [gram/second] in the three main groups.

		Control	**Diabetic**	**Diabetic Treated**
Apelin	*Before*	110.3±12.8	91.7±5.2	104.7±10.7
	After	133.3±12.9***	120.5±5.8***	127.5±10***
	% of increase	21.2±3.1	31.5±2.6***$	20.2±3***¥
Verapamil	*verapamil*	79±7.7	64.2±9.3	72.6±7.1
	verapamil + Apelin	94.3±9.3***	83.8±13***	85.3±9.6**
	% of increase	19.4±1.7€	27.5±2.6€	21.1±3.4€
Propranolol	*Propranolol.*	76.3±6.5	52±3.7	72.3±6.4
	Propranolol + Apelin	91.5±6.5***	66.6±4.5***	87.7±7.2**
	% of increase	19.9±2.5€	28±2.4€	21.4±2.4€
Benzamil Hcl	*Benzamil*	111.8±12.8	90.6±5	109.6±8.4
	Benzamil +Apelin	120.3±13.1**	106.5±5.3***	118.6±9.5***
	% of increase	7.5±2.4***€	17.7±2.2***€	8.2±1.9***€

** **Significant VS. pre-injection values of apelin P< 0.01**
*** **Significant VS. pre-injection values of apelin P< 0.001**
$ **VS control**
¥ **VS diabetic.**
€ **VS % of increase with Apelin alone**

Table (4): The effect of I.V. bolus injection of apelin-13 (10 nmol/kg) alone or in the presence of verapamil (4.8mg/kg) ,propranolol (0.2mg/kg) or benzamil hydrochloride (10 nmol/kg) on the rate of relaxation ($-dT_{max}/ t_r$) [gram/second] in the three main groups

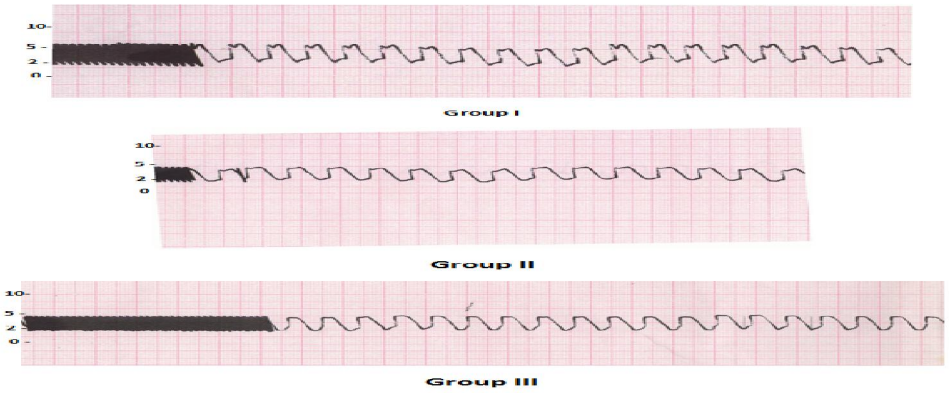
		Control	Diabetic	Diabetic Treated
Apelin	*Before*	36.1±2.3	30.8±2.8	37±1
	After	44.8±3.1***	40.5±2.9***	46.1±1.2***
	% of increase	23.1±4.1	29.8±3.2**$	25.5±3*¥
Verapamil	*Verapamil*	29.8±1.4	27.4±2.2	30.7±1.9
	verapamil + Apelin	37.2±2.5***	35.5±2.9***	38±2.5***
	% of increase	23.3±1.4€	29.8±1.5€	23.2±2.8€
Propranolol	*Propranolol.*	31.7±1.8	28.2±0.7	30.2±1.9
	Propranolol + Apelin	38.5±2.4***	36.7±1.3***	37.8±2.2***
	% of increase	22.8±2.8€	30.8±2.5€	25±3.1€
Benzamil Hcl	*Benzamil*	37.3±0.9	30.1±1.9	37.4±3.7
	Benzamil +Apelin	41±1.4**	35.8±2.9***	41.2±4.2***
	% of increase	9.7±2.6***€	19±4.3***€	9.7±2.6***€

** **Significant VS. pre-injection values of apelin P< 0.01**
*** **Significant VS. pre-injection values of apelin P< 0.001**
$ **VS control**
¥ **VS diabetic .**
€ **VS % of increase with Apelin alone**

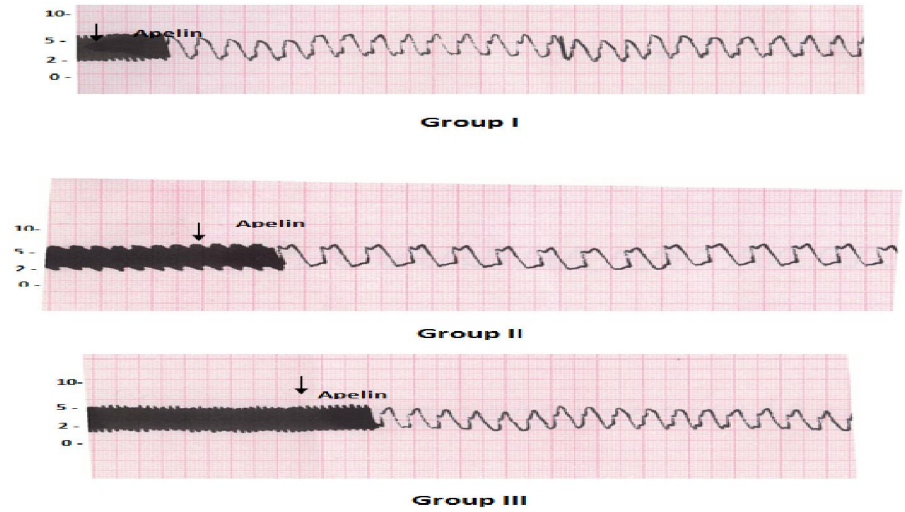
Table (5): The effect of I.V. bolus injection of apelin-13 at a dose of 10 nmol/kg on HR (beat\ min) in the three main groups.

		Control	Diabetic	Diabetic Treated
Apelin	Before	355±22.6	320±15.5	350±31
	After	360±19	325±12.2	355±35.1
P value of paired t test		NS	NS	NS

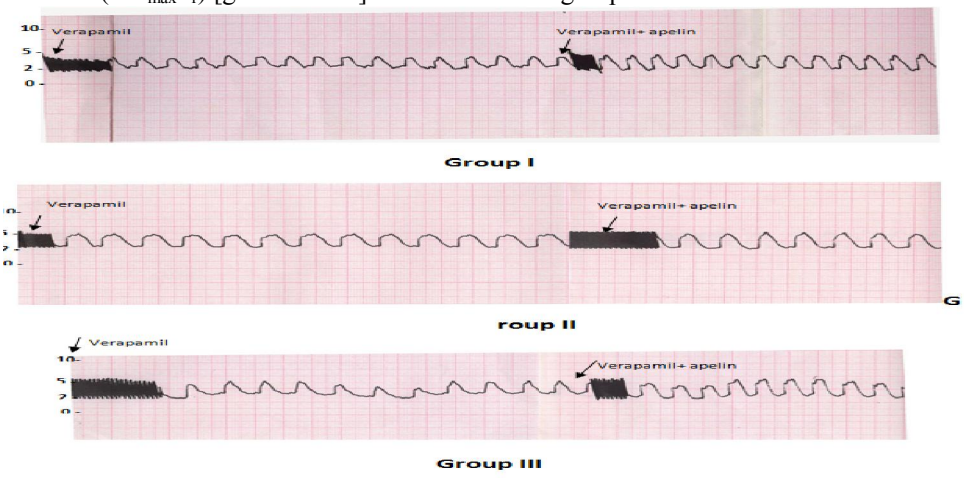
NS:non-significant



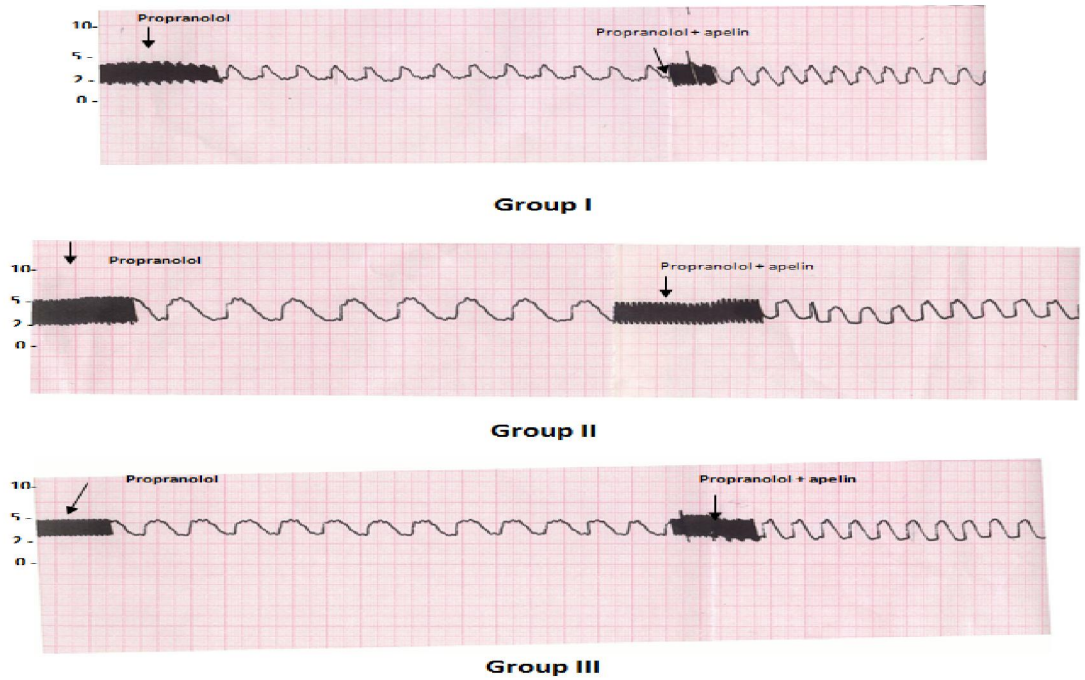
Record 1: Shows (+ dT_{max}/t_{max}) [gram/second] and (-dT_{max}/t_r) [gram/second] in the three main groups.



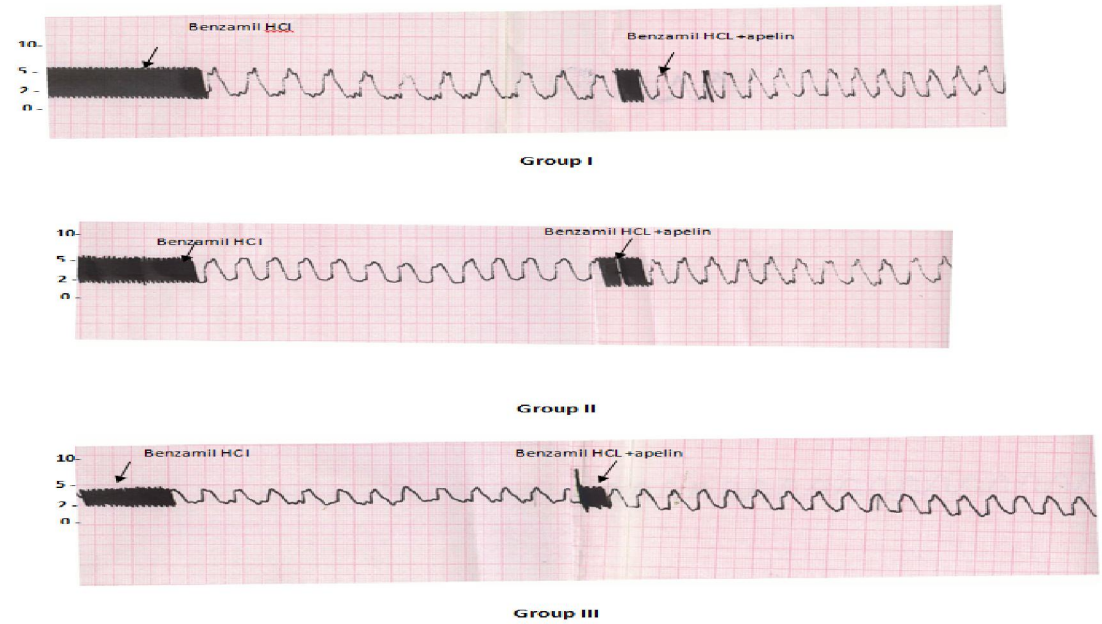
Record 2: Show the effect of I.V. bolus injection of apelin-13 (10 nmol/kg) on cardiac contractility parameter; the rate of development of tension (+dT_{max}/t_{max}) [gram/second] and cardiac relaxation parameter; rate of relaxation (-dT_{max}/t_r) [gram/second] in the three main groups.



Record 3: Show the effect of I.V. bolus injection of apelin-13 (10 nmol/kg) on cardiac contractility parameter; the rate of development of tension (+dTmax/tmax) [gram/second] and cardiac relaxation parameter; rate of relaxation (-dTmax/tr) [gram/second] in the presence of verapamil injection (4.8 mg/kg) in the three main groups.



Record 3: Shows the effect of I.V. bolus injection of apelin-13 (10 nmol/kg) on cardiac contractility parameter; the rate of development of tension ($+dT_{max}/t_{max}$) [gram/second] and on cardiac relaxation parameter; rate of relaxation ($-dT_{max}/t_r$) [gram/second] in the presence of propranolol injection (0.2mg/kg) in the three main groups.



Record 4: Shows the effect of I.V. bolus injection of apelin-13 (10 nmol/kg) the rate of development of tension ($+dT_{max}/t_{max}$) [gram/second] and the rate of relaxation ($-dT_{max}/t_r$) [gram/second] in the presence of benzamil hydrochloride injection (10 nmol/kg) in the three main groups.

4. Discussion

Apelin is the endogenous ligand for the previously orphaned G-protein–coupled receptor, APJ. This novel pathway is widely expressed in the cardiovascular system and is emerging as an important mediator of cardiovascular homeostasis **(Japp *et al.*, 2010).**

In our study, streptozotocin induced diabetic rats had a significant weight loss (about 25%) and displayed typical manifestations of diabetes mellitus such as polydypsia, polyurea, and hyperglycemia. The results of this study showed that both contraction and relaxation of cardiac muscle were significantly reduced in case of diabetic rats as indicated by the significant decrease in ($+dT_{max}/t_{max}$) and ($-dT_{max}/t_r$) in comparison with that of both control and insulin treated groups. In addition, there was a significant decrease in heart rate in the diabetic rats as compared with that of the two other groups of rats.

Our results are in agreement with those of who concluded that STZ-diabetic rats exhibited a significant decrease in indices of both contractility and relaxation as compared to control rats and STZ-diabetic rats treated with insulin **(Borges *et al.*, 2006).**

This can be explained as follows; in STZ diabetic rats the ability of the sarcoplasmic reticulum to take up and release calcium is depressed. Similarly reports for decreases in Na^+/K^+ATPase and adenylyl cyclase accompanied by decreases in sodium/calcium exchanges and calcium pump activity have been documented in diabetes **(Nordin and Gilat, 1990)**. In addition to cardiomyopathy, alteration in the lipid metabolism seems to be another factor involved in cardiac depression **(Shenoy and Goyal, 2002).**

Furthermore, myocardial dysfunction is an important feature that might be associated with a number of intrinsic alterations of cardiac myocytes **(Ren and Bode, 2000)**. There are several studies *in vivo* (anesthetized animals) and *in vitro* (Langhendorff and isolated myocytes) showing an impairment of Ca^{++} homeostasis and Ca^{++}signaling in diabetes **(Ren *et al.*, 2000, and Choi *et al.*, 2002)**.The most significant abnormalities involved delay of the relaxation process, slow relaxation ratio and delay in peak ratio of isometric and isotonic relaxation **(Choi *et al.*, 2002)**.

Finally, an impairment of sympathetic innervations of the heart, frequently observed in diabetes **(Maeda *et al.*, 1995 and Fazan *et al.*, 1999)**, should be also taken into consideration in the impairment of myocardial contractility found in STZ-diabetic rats.

Moreover, the development of STZ-induced bradycardia has been attributed to a down regulation of myocardial beta adrenoceptors **(Baba and Ishikawa, 1992)**, and depression of myocardial calcium metabolism **(Nordin and Gilat, 1990)**.

In addition, in the present study, treatment with insulin prevented the occurrence of alterations caused by diabetes, i.e. bradycardia and low ($+dT_{max}/t_{max}$) and ($-dT_{max}/t_r$).

Although several studies demonstrated that insulin can prevent, or even reverse, the derangements caused by chronic diabetes (**Fein *et al.*, 1981, Schaan *et al.*, 1997**). Nevertheless, the mechanism responsible for this protective effect is still unknown, because diabetes is a long-standing metabolic disorder with several outcomes. It has been demonstrated in normal cardiac myocytes that insulin speeds the glucose transport into the cell **(Bayliss *et al.*, 1928)**. However, it has been demonstrated also that insulin promotes a positive inotropic effect independent of glucose uptake **(Oye and Sinclair, 1966)**.

Our results are in line with those of **Stroedter *et al.* (1995)** who reported the improvement of cardiac performance in diabetes following the subcutaneous administration of insulin. They also suggested that the dysfunction of the heart observed in diabetes may be caused by conspicuous alterations of myocardial metabolism caused by insulin deficiency, which can be reversed by means of exogenous replacement of the hormone.

Moreover, the results of this work demonstrated that apelin-13 administration significantly increased cardiac muscle performance without any significant changes in heart rate , in all groups, as evidenced by the significant increase in ($+dT_{max}/t_{max}$) and ($-dT_{max}/t_r$). In addition, this increase was more significant in diabetic rats in comparison with that of both control and diabetic treated rats

Our results are supported by those of other investigators who concluded that acute apelin infusion increases cardiac contractility and cardiac output **(Berry *et al.*, 2004, Jia *et al.*, 2006, Atluri *et al.*, 2007)**, furthermore, other studies reported significant increase in the diastolic function of the heart after apelin injection **(Berry *et al.*, 2004, , Pan *et al.*, 2010).**

The mechanisms by which apelin exerts its inotropic effects have been only partially elucidated and remain the subject of debate. However, in our study the observed effects are independent of ATP calcium channels or B- adrenergic receptors but appear to involve activation of the sacrolemmal Na^{++}/Ca^{++} exchanger (NCX), as verapamil failed to attenuate the inotropic response to apelin. Moreover, the effect of apelin remained unchanged in the presence of propranolol .On the other hand, administration of benzamil HCL (Na/Ca^{+2} exchange (NCX) blocker) partially blocked the effect of apelin-13 injection on the cardiac performance.

Our results are in line with **Dai *et al.* (2006)** who reported that in intact rat hearts, inhibition of NCX suppresses the apelin-induced inotropic response indicating that this mechanism may contribute to apelin-mediated inotropic activity, they

also concluded that apelin increased the amplitude of the intracellular $Ca2^{+}$ transient **(Dai *et al.*, 2006).** Moreover, **Kentish, 1999** concluded that, apelin does not alter voltage-gated Ca^{++} channels in cardiomyocytes.

In addition, the positive inotropic effect of apelin is independent of angiotensin II, endothelin-1, catecholamines and nitric oxide release **(Szokodi *et al.*, 2002)** but appear to involve activation of the sacrolemmal Na^{+}/H+ exchanger (NHE), probably through phospholipase C and protein kinase C-dependent pathways **(Szokodi *et al.*, 2002, Farkasfalvi *et al.*, 2007)**. In single cardiomyocytes, NHE activity increases following exposure to apelin while, in intact rat hearts, the inotropic response to apelin is markedly attenuated by a specific inhibitor of NHE. Stimulation of NHE can lead to intracellular alkalinization and sensitization of cardiac myofilaments to intracellular Ca^{++} **(Karmazyn *et al.*, 1999)**. In keeping with this, the increased NHE activity is accompanied by an increase in intracellular pH **(Farkasfalvi *et al.*, 2007)**. Moreover, activation of NHE can also indirectly increase intracellular Ca^{++} as the resulting accumulation of Na^{+} within cells stimulates the reverse mode Na^{+}/Ca^{++} exchanger (NCX) **(Karmazyn *et al.*, 1999, Kentish *et al.*, 1999).**

Thus the inotropic effects of apelin may involve increased intracellular Ca^{++} availability in addition to enhanced myofilament responsiveness to Ca^{++} ions **(Japp and Newby, 2008).**

The results of the above studies suggest that activation of NHE and NCX contributes to the inotropic effect of apelin, whereas voltage-activated Ca2are not involved, whatever, the finding that 40% of the apelin-induced positive inotropic effect remained unaffected even after combined inhibition of NHE and NCX indicates the existence of additional signaling mechanisms **(Berry *et al.*, 2004).**

Furthermore, the effect of apelin on myocardial efficiency could be mediated also via PKC **(Ashley *et al.*, 2005)** this is because cardiac apelin–APJ signaling is abrogated by PKC inhibitors and PKC phosphorylation of the cardiac fibers has been shown to reduce the requirements of the contractile apparatus for both calcium and ATP (promoting efficient ATP utilization) **(Pi *et al.*, 2003)**. Furthermore, apelin injection increased coronary blood flow to the cardiac muscle **(Japp *et al.*, 2010).**

In addition, our results are supported by the following studies who reported that apelin has positive inotropic effects in vivo in both normal rat hearts and rat hearts in failure after myocardial infarction (**Szokodi *et al.*, 2002, Berry *et al.*, 2004, Dai *et al.*, 2006),** and so apelin may have used as an acute inotropic agent in patients with ischemic heart failure **(Berry *et al.*, 2004)**. Interestingly, an apelin-knockout mice showed severely impaired heart contractility **(Kuba *et al.*, 2007)**, which suggests that decrease in endogenous apelin plays a pivotal role in heart failure **(Berry *et al.*, 2004, Atluri *et al.*, 2007; Sheikh *et al.*, 2008).**

Lastly, the findings of the more significant effects in diabetic rats in comparison with that of both control and insulin treated rats, might be explained, at least partially, by means of an up-regulation of APJ receptors exhibited by STZ-diabetic rats, which may be due to decrease in apelin synthesis and secretion in the injured endothelium and myocardium **(Jia *et al.*, 2006)**, even though this hypothesis deserves better investigation.

In addition to the above explanation, **Dray *et al.* (2008)** demonstrated that acute injection of apelin was able to improve glucose tolerance and to increase glucose utilization in heart; this noticeable effect needs to be further depicted.

However, in addition to confirming the *in vivo* positive inotropic effect, **Ladeiras-Lopes *et al.* (2008)** demonstrated that apelin has a negative inotropic effect in isolated cardiac muscle, suggesting other cells may be required in addition to myocardial cells so that positive inotropic effect is revealed.

As regards the effect of apelin-13 on the heart rate, our results are in agreement with those of **Lee *et al.* (2000)**, who reported insignificant changes in heart rate after apelin injection. While those results are in disagreement with the results of other investigators who concluded that apelin injection decreased heart rate in rodents **(Tatemoto *et al.*, 1998).**

Moreover, our finding also in controversy to those of other investigators who reported that IV apelin injection increased heart rate in conscious sheep and both anaesthetized and conscious rats **(Cheng *et al.*, 2003, Charles *et al.*, 2006)**.

The reason for these discrepancies among findings is unclear; however, possible explanations are as follows: in case of anaesthetized rats, anesthetics are well known to affect the sympathetic nervous system **(Kagiyama *et al.*, 2005)**. Moreover, the diversity of the previous results may be due to differences in methodology and the different doses of apelin administered **(Chamdrasekaran *et al.*, 2008).** In addition, cardiovascular response to apelin may exhibit interspecies differences **(Japp and Newby *et al.*, 2008)**

Conclusion,

Apelin-13 exerted both positive inotropic and lusiotropic effects without affection of the heart rate in vivo, which was more significant in diabetic rats in comparison with that of both normal and insulin

treated rats. Our results also suggested that this response to apelin involved activation of Na^{+}- Ca^{+2} exchange channels (NCX).

Since different mechanisms are responsible for the diabetic cardiomyopathy and response rate to treatments is far from homogenous and ideal, the search for additional therapeutic agents continues. Therefore, the use of apelin may be investigated as a potential therapeutic target for this pathology. Furthermore, studies examining the effects of chronic apelin administration on long-term cardiac function will also be useful in assessing apelin treatment of chronic heart failure

Finally, more studies are recommended to investigate not yet discovered mechanism/s of apelin actions on the cardiovascular system on the cardiac performance.

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Phenolic and biological activities of *Vitex trifolia* aerials parts

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Abstract: *Vitex trifolia* (Family: Verbenaceae) grows as an herbaceous plant in Egypt. Most of the Verbenaceae plants contain phenolic compounds which have important pharmacologically properties. *V. trifolia* aerial parts methanol extract was fractionated by repeated column chromatographic separation to obtain a phenyl ethanoid which isolated for the first time from the genus *Vitex* along with five phenolic metabolites. The identification and structure elucidation of the isolated compounds were based on chemical and spectral data (UV, ESI-MS, ^{1}HNMR, ^{13}CNMR, HMQC and HMBC) and also by direct comparison with respectively published data. Cytotoxic activities of the plant aerial parts extracts (methanol, ethyl acetate and chloroform) have been studied herein for the first time using, brine shrimp bioassay method (LC_{50} values 140 mg ml^{-1}, 165 mg ml^{-1} and 180 mg ml^{-1}, respectively). Total phenolic content of different aerial parts extracts and the antioxidant activity of the major isolates has been studied as well. The radical scavenging activities of compounds 1 - 3 were measured and compound 1 have been identified as the most promising compound.

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1. Introduction

The *Vitex* genus family Verbenaceae is comprised of about 250 species of shrubs and trees; it's widely cultivated in warm temperate and subtropical regions. *Vitex trifolia* L. is a deciduous shrub which commonly known as common chaste tree (1) , the plant has been used as an anti-inflammatory (2), antibacterial (3,4), antipyretic (5), hepatoprotective (6), trypanosidal, and sedative for headache, rheumatism, and the common cold in Asian countries (7), It's also used for the treatment of cough, febrifuge, fever and amenorrhea (8). The plant is known to possess various active constituents viz., essential oil (9), halimane-type diterpenes, vitetrifolins (10,11), flavonoids (12,13), chalcones (14), triterpens (15,16), lignans (17,18), iridoides (19, 20), and ecdysteroids (21). In continuation of our studies on biologically active substances from medicinal plants, we reported herein the isolation and characterization of six phenolic compounds **1-6** from *V. trifolia* aerial parts methanol extract. In addition the cytotoxicity towards brine shrimps larva was determined for the three aerial parts extracts (methanol, chloroform and ethyl acetate). Total phenolic content of different aerial parts extracts and the antioxidant capacity of compounds **1 - 3** have been studied as well.

2. Material and Methods

Plant material

Aerial parts of *Vitex trifolia* were collected from shibin El-kanater Garden (El- kaluobia city, the North - East of Egypt) in January 2008, the plant was authenticated by Mohamed El-Kassas (Professors of Taxonomy, Department of Botany, Faculty of Science, Cairo University, Giza, Egypt), voucher specimens (Reg. No.: V-1) are kept in the herbarium of City for Scientific Research and Technological Application.

Experimental

The NMR spectrum was recorded at 300 (^{1}H) and 75 (^{13}C) MH_Z. on a Varian Mercury 300 NMR spectrometers and δ values were reported as ppm relative to TMS in DMSO-d_6. ESI-MS analyses were measured on a Finnegan LCQ deco LC/MS and double focusing sector field MAT 90 MS spectrometers (Finnegan, Bremen, Germany). For column chromatography (CC), Sephadex LH-20 (Pharmacia, Uppsala, Sweden), microcrystalline cellulose (Merck, Darmstadt, Germany) and polyamide 6S (Riedel de Haën AG, Seelze, Germany) were used. For paper chromatography Whatman No. 1 sheets (England) were used. The spots were detected by spraying anisaldehyde-H_2SO_4 reagent followed by heating. UV spectra of pure samples were recorded, separately, as MeOH solutions and with different diagnostic UV shift reagents on a Shimadzu UV 240 spectrophotometer.

Extraction and isolation

The air-dried powdered aerial parts of *V. trifolia* (800 g) were exhaustively extracted under reflux with

hot 80% MeOH (3 x 5 L). After evaporation of the solvent, the dry residue obtained, was defatted with petroleum ether (60-80 °C) (3 x 1 L) to give residue which was extracted by suspended in water and extracted with $CHCl_3$ (3 x 500 mL) and ethyle acetate separately (3 X 500). The water soluble portions from $CHCl_3$ and EtOAc were collected and desalted by precipitation with excess MeOH. After evaporation of the MeOH, the extract (100 g) was suspended in H_2O and fractionated on a polyamide column (110 × 6 cm, 300 g) using a stepwise gradient from H_2O, H_2O/MeOH mixtures up to pure MeOH for elution. Based on chromatographic properties (Co-PC) with the use of UV light, 1% $FeCl_3$ and Naturstoff spray reagents for detection, the individual 65 fractions (150 ml) each were collected in 6 fractions (A – F). Fractions A and B (H_2O, 15 g) were exhibited free sugar characters. 2D – PC of fraction C (10% methanol, 20 g) showed a major blue spot, it was purified by a Sephadex LH – 20 column using 20% aqueous MeOH to afforded a pure sample of compound **1**. Five dark purple spots were detected on 2D – PC of fraction D (20-30%- MeOH, 20 g), which gave yellowish green fluorescence under UV after spraying with Na / PE. Separation of the individual major compounds was carried out on a cellulose column using H_2O / MeOH (7:3), this fractionation led to the isolation of chromatographically pure samples of compounds **2** and **3**. Fraction E (40-70% MeOH, 15 g) showed three dark purple minor spots on the 2D – Pc of this fraction, they were changed to orange and greenish yellow upon spraying with Naturstoff reagent. Application of fraction E on cellulose column using *n*-butanol saturated with water resulted in three subfractions. Final purification of this subfractions were carried out by successive fractionation on Sephadex LH-20 CC using H_2O / MeOH (60%), resulted in chromatographically minor samples of compounds. Two dark purple spots and a blue one were detected on 2D-PC of fraction F (80-100% MeOH, 20 g). It was rechromatographed on a Sephadex LH-20 column using 95% EtOH as an eluent to afford pure samples of compounds **4**, **5** and **6**. All separation processes were followed up by Comp. PC using Whatman No. 1 paper with (S_1) *n*-BuOH – HOAc – H_2O (4:1:5, upper layer) and (S_2) 15% aqueous HOAc as a solvent systems.

Determination of total phenolic content

Total phenolic content determined with the Folin-Ciocaltea (FC) (22). 100 µl of the aerial parts extract dissolved in methanol (equivalents to 1mg of extract) were mixed with 750 µl of (FC) reagent (previously diluted 10-fold with distilled water) and allowed to stand at 22°C for 5 min; 750 µl of Na_2CO_3 (60 g /l) solution were added to the mixture. After 90 min, the absorbance was measured at 750 nm. All determinations were carried out in triplicate. The standard curve of gallic acid was carried out and the equation Y = 2.4412 X was obtained. This equation was used to obtain the gallic acid equivalents (mg of gallic acid per mg dry weight extract). The results were represented in table 3

Antioxidant activity

The DPPH is a purple stable organic radical with an absorption band in the range of 515-528 nm; when the radical accept an electron or a free radical species, the result is a visually noticeable discoloration from purple to yellow. Because the DPPH radical can accommodate many samples in a short period of time and is sensitive enough to detect active molecules at low concentrations.

a- **DPPH radical scavenging activity**

The ability of *V. trifolia* pure compounds **1-3** to scavenge DPPH radicals was evaluated according to the procedure described by **Molyneux and Songklanakarin** (23). To 1 ml of each sample at a concentration of 100µg/ml was mixed with 1ml of 0.1m M DPPH in methanol. The mixture was then shaken and left for 20 min. at room temperature in the dark. The absorbance was measured at 517 nm using a spectrophotometer. Ascorbic acid was used as a reference standard. All experimental were carried out in triplicate. The activity of each sample was expressed as percentage DPPH radical scavenging relative to the control using the following equation: DPPH radical scavenging % = [(control absorbance - sample absorbance)/control absorbance] × 100. The scavenging effect (antioxidant activity) of each sample was expressed as SC_{50} which is the concentration of the extract required for 50% scavenging of DPPH radicals compared with that of the standard ascorbic acid (Table 4).

B-Evaluation of total antioxidant capacity.

An aliquot of 0.1 ml of sample solution was combined in an Eppendorf tube with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent used for the sample, and it was incubated under the same conditions as the rest of the sample (24).

All chemicals and solvents used in the two previous antioxidant methods were of analytical grade. DPPH (2, 2-diphenyl 1-picrylhydrazyl) were purchased from Sigma Co. (USA), while the other reagents sodium phosphate, ammonium molebdate,

ascorbic acid and sulfuric acid were purchased from Merk Chemical Co. (Germany).

Brine shrimp lethality bioassay

The cytotoxicity was measured by brine shrimp lethality bioassay (25), where eggs of brine shrimp *Artemia salina* (Atremia Inc., California) were allowed to hatch into their larvae under convenient conditions. Assays were performed in test tubes with ten larvae in each, and methanol, ethyl acetate, and chloroform extracts were separately dissolved in distilled water to give six assay concentrations (1000, 800, 600, 400, 200, 100 and, 10 mg /ml) solubility was aided by DMSO; the final volumes were adjusted to 5 ml sea salt solution immediately after adding the shrimp. After 24 h, the number of surviving shrimp at each dose was recorded. Each dose was examined in triplicate. The same steps carry out for potassium dichromate as positive control and DMSO as negative control. The values and the statistical analysis of the results were calculated and carried according to **Reed-Muench** method (26, 27)

The statistical analysis

Values were expressed as mean ± SEM. Statistical difference between groups were computed by one-way analysis of variance (ANOVA). Tukey-Kramer multiple comparison tests were used to compare between treated and control groups. The level of significance was accepted at $p < 0.05$. Statistical analysis was performed by the aid of Instat version 2 computer program (Graph pad software, Inc., San Diego, USA).

3. Result and Discussion

The defatted total aqueous methanol extract of *V. trifolia* aerial parts was fractionated by repeated column chromatographic separation to obtain compounds **1-6**. Based on chemical and physicochemical analyses, they were identified as 2-(3,4-dihydroxyphenyl)ethyl-2-*O*-[6-deoxy- *α*-L-mannopyranosyl-4′-(3,4-dihydroxyphenyl)- 2-propenoate]-β-D-glucopyranoside **1**, vitexin **2**, isovitexin **3**, luteolin-7-*O*-β- glucuronopyranoside **4**, quercitrin **5** and methyl caffeiate **6**.

Compound **1:** was expected to be a phenylethanoid on the basis of its chromatographic properties, UV spectral data and acid hydrolysis products. Its negative ESI-MS spectrum gave a dimeric adduct and molecular ion peaks at *m/z* = 1247.0 and 623.1 assignable to $[2M-H]^-$ and $[M-H]^-$, respectively, together with three fragment ions at 461.1, 179.1 and 161.1 for $[M-H\text{-caffeate}]^-$, $[\text{caffeate}]^-$ and $[\text{caffeate-}H_2O]^-$, respectively to prove its identity as 3,4- dihydroxyphenethyl-alcohol-caffeoyl-rhamnosylglucoside. In the aromatic region of its ^{1}H NMR spectrum (Table 1), an A^2X^2-spin coupling system of H-7''' and H-8''' at 7.59 and 6.29 ppm, respectively (each *d*, *J* = 15.9 Hz) together with an ABM one of H-2''', H-6''' and H-5''' at 7.17, 7.05 and 6.87 ppm, respectively were indicative an *E*-caffeoyl moiety. The characteristic signals of the aglycone moiety (3, 4-dihydroxyphenethyl alcohol) were assigned in the form of an ABM spin coupling system at 6.77, 6.73 and 6.57 ppm for H-2, H-5 and H-6, respectively in the aromatic region and its characteristic AX-system of two triplets for H-7 and H-8 at 2.78 and 3.84, respectively in the aliphatic region. The ^{1}H-NMR also showed two anomeric proton signals at 4.43 ppm (H-1', *d*, *J* = 7.8 Hz) and at 5.29 (H-1", brs) together with the signal at 1.11 (3H, *d*, *J* = 6 Hz- CH_3-6"). The glucoside moiety adopts an *O*-β-$^4C_1$-pyranose, while rhamnosyl adopts an *O*-α-$^1C_4$-pyranose depending on all of their *δ*- and *J*-values in ^{1}H and ^{13}C-spectra (Table 1). The connectivity of the caffeoyl moiety on OH-4 of glucose was followed from the downfield location of H-4' as t-like at 4.90, whereas, the connectivity of *O*-rhamnosyl on C-2-glucoside was concluded from the characteristic downfield shift of its ^{13}C NMR signal to 79.66 ppm. Depending on the assignment of all other signals of ^{1}H and ^{13}C NMR with the aid of the cross peaks in 2D-NMR spectra (^{1}H^{1}H-COSY, HMQC and HMBC) and comparison with previously reported data. (28, 29), compound **1** was identified as 2-(3,4-dihydroxyphenyl)ethyl-2-*O*-[6-deoxy-α-L-mannopyranosyl-4-(3,4-dihydroxyphenyl)- 2-propenoate]-β-D-glucopyranoside (28, 29).

Compounds **2, 3** both occur as yellow amorphous powder they gave dark purple spots under UV light (365 nm), and this color changes with NH_3, $FeCl_3$ and Naturstoff reagent, (yellow, green and greenish yellow, respectively). In compound **2** R_f: 0.59 (S_1), 0.55 (S_2). -UV spectral data λ_{max} (nm), (MeOH) =: 344.5, 269.5, 229.5, 288.5 and 248 where in compound 3 R_f: 0.51 (S_1), 0.54 (S_2). -UV spectral data λ_{max} (nm), (MeOH) =: 345, 270.5, 228, 289.5 and 247. On complete acid hydrolyses they remained without any change and this indicated that compounds **2, 3** are C-glycosides. -Negative ESI-MS spectrum of both compounds showed a molecular ion peak at *m/z* = 431 $[M-H]^-$, corresponding to a molecular weight of 432, which was in complete accordance with apigenin C-hexoside. The fragment ion peak at *m/z* = 269 $[M\text{ -H- hexose}]^-$ in both compounds corresponding to apigenin. $^{-1}$H-NMR spectrum of compound **2** and **3** (Table 2) showed two doublets at δ (7.95 – 7.84), and δ (6.92-6.9) both integrating for two protons and are assigned to (H-2' / 6') and (H-3' / 5') of the 1', 4' disubstituted B-ring, respectively. Signals at δ 6.55 and 6.50 ppm are due to protons attached at C-3 in the apigenin skeleton of the compounds **2** and 3, respectively. Absence of the

characteristic signal of H-8 led us to identify compound **2** as 8-subistetuted apigenin, while the absence of characteristic signal of H-6 in compound **3** led us to identify it as 6-subistetuted apigenin. Presence of C-β- glycoside moiety in the structure of **2** and **3** were concluded depending on the anomeric protons coupling at δ 4.7 and 4.98 ppm, respectively with characteristic high *J*-values ($9H_Z$) in both compounds. In the ^{13}C NMR spectrum of compound **2** and **3** (Table 2), the signals at (δ128.9 and 128.36 ppm) and (δ 115.9 and 116.11 ppm) revealing to C (2' / 6') and (3' / 5'), respectively. The methane carbon signal at δ 102.59 and 102.6 ppm were characteristic for C-3 apigenin aglycone in **2** and **3**, respectively. The characteristic downfield shifts of C-8 to δ = 104.6 ppm (Δ ≈ 10 ppm) and upfield of both C-7 and C-9 to δ 161.2 and 156 ppm, respectively was diagnostic for 8-*C*-glycosedation in compound **2**, while the characteristic downfield shifts of C-6 to δ 109.1 (Δ ≈ 10 ppm) and upfield of both C-7 and C-5 to δ 161.43 and 160.7 ppm, respectively was diagnostic for 6-*C*-glycosedation in compound **3**. C-β- glycoside moiety in the two structures can be obtained from the signal at around 74 ppm, indicating that the anomeric carbon is attached to C- atom and not to the usual oxygen in the both aglycone. Six C-resonances of the β-C- glucopyranoside moiety were assigned in the range of 82-60 ppm in the both compounds. The remaining carbon resonances of both compounds were completely assigned by comparison with previously corresponding data (30, 31). Therefore, **2** and **3** were finally identified as vitexin and isovitexin, respectively.

Compound **4**: occurs as yellow amorphous powder, R_f: 0.7 (S1), 0.3 (S2). -UV/V λ_{max} (MeOH) =: 345; (NaOMe): 266 sh, 329sh, 401; (NaOAc): 268, 326sh, 386; (H_3BO_3): 260, 300 sh, 370,428 sh; ($AlCl_3$): 272, 300 sh, 325, 426; (HCl): 272, 290, 355, 383. It was found to be substituted at 7-OH by absence of bathochromic shift upon addition of NaOAc. On complete acid hydrolyses of **4**, letulin was detected in the organic phase while, glucuronic acid was detected in the aqueous phase (Co-PC with authentic samples). ESI–MS of **4** gave a molecular ion peak at *m/z* = 461 corresponding to M.Wt = 462. The fragment ion peak at *m/z* 285 = [aglycone-H] $^-$ which consistence with letulin and was attributed to the loss of glucuronic acid moiety. ^1H NMR (300 MHz DMSO-d_6) δ: = 7.39 (1H, *d*, *J* = 2.1 Hz, H-2'), 7.35 (1H, *d*, *J* = 2.1 Hz, H-6'), 6.89 (1H, *d*, *J* = 8.4 Hz, H-5'), 6.75 (1H, *d*, *J* = 1.8 Hz H-8), 6.66 (1H, *s*, H-3), 6.38 (1H, *d*, *J* = 2.1 Hz, H-6), 5.16 (1H, *d*, *J* = 6.9 Hz, H-1") 3.90 - 3.30 (remaining sugar protons). ^{13}C NMR (75 MHz DMSO-d_6) δ: = 182.53 (C-4), 171.41 (C-6"). 165.24 (C-2), 163.33 (C-7), 161.81 (C-5), 157.62 (C-9), 150.73 (C-4'), 146.53 (C-3'), 121.93 (C-1'), 119.75 (C-6'), 116.89 (C-5'), 114.39 (C-2'),106.1 (C-10), 103.79 (C- 3), 100.16 (C- 1"), 100.05 (C-6), 95.32 (C-8), 76.59 (C-5"), 75.68 (C-3"), 73.54 (C-2"), 72.14 (C-4"). $^{-1}$HNMR spectrum exhibited signals at δ- values 7.39 ppm (*d*, *J* = 2.1 H_Z, H-2'), 7.35 ppm (*dd*, *J* = 8.4 and 2.1 H_Z, H-6'), and 6.89 ppm (*d*, *J* = 8.4 H_Z, H-5'), characteristic for ABX spin coupling system for a 3', 4'- disubstituted B– ring. The glucuronosedation at 7- OH was concluded from downfield shift of both H-6 and H-8 (≈ + Δ 0.2 ppm) and the β – anomeric proton signal at 5.16 (*J* = $7H_Z$) (30). $^{-13}$CNMR spectrum of **4** showed 21 carbon signals fifteen of them were attributed to the aglycone moiety and assigned by comparison with corresponding data (31), while the sex remaining signals for the glucuronic acid moiety. Slight upfield shift of C-7 and downfield shift of both C-6 and C-8 were further confirmation for the glucuronosedation at 7-OH (32). So the structure of **4** was deduced as letulin 7-*O*- glucuronopyranoside. (30, 31)

Compound **5**: occurs as yellow amorphous powder R_f: 0.6 (S1), 0.4 (S2). -UV λ_{max} (MeOH) =: 259, 355, 300 sh.; (NaOMe): 270, 325sh, 399; (NaOAc): 270, 325sh, 460; (NaOAc /H_3O_3): 269, 325,390; ($AlCl_3$): 239, 275, 343, 425. It appeared as dark purple fluorescence under UV light turned to orange with Naturstoff spray reagent. On complete acid hydrolyses quercetin was detected in the organic phase, while rhamnose was detected in the aqueous phase (Co-PC with authentic samples).$^{-1}$H NMR (300 MHz DMSO-d_6) δ: = 7.3 (1H, *d*, *J* = 1.8 Hz, H-2'), 7.26 (1H, *dd*, *J* = 8.4, 1.2 Hz, H-6'), 6.87 (1H, *d*, *J* = 8.4 Hz, H-5'), 6.39 (1H, *d*, *J* = 2.1 Hz, H-8), 6.21(1H, *d*, *J* = 2.1 Hz, H-6), 5.26 (brs, H-1"), 3.98 (brs, H-2"), 3.6 (brd, H-3") 3.80 - 3.30 (remaining sugar protons), 1.2 (1H, *d*, *J* = 5.7 Hz, H-6"). $^{-13}$C NMR (75 MHz DMSO-d_6) *δ*: = 177.33 (C-4), 164.1 (C-7), 161.28 (C-5), 156.31 (C-9), 156.17 (C-2), 148.42 (C-4'), 144.9 (C-3'),132.91 (C-3), 121.73 (C-1'), 121.24 (C-6'), 116.03 (C-5'), 115.18 (C-2'),104.06 (C-10), 102.2 (C-1"), 98.43 (C-6), 93.59 (C-8), 71.94 (C-4"), 70.66 (C-2"), 70.31 (C-3"), 68.34 (C- 5"),17.26 (C-6").

Negative ESI-Ms spectrum of compound **5:** showed a molecular ion peak at *m/z* = 447.1 $[M-H]^-$, together with a fragment ion peak at 301.2 [M-H - 146]$^-$, corresponding to the loss of rhamnose moiety which confirmed the presence of quercetin as aglycone**.** The UV methanol spectrum showed characteristic absorption bands at λ_{max} 259 and 355 nm for band II and I, respectively for quercetin moiety. The bathochromic shift observed upon addition of NaOMe (+42 nm in band I) with increase in the intensity proved the presence of free 4-OH. Compound **5:** showed in the aromatic region of its ^{1}HNMR spectrum two characteristic spin coupling systems, the first one occur as an ABX of three types

of protons at δ = 7.3 (1H, *d*, *J* = 1.8 Hz), 7.26 (1H, *dd*, *J* = 8.4, 1.2 Hz) and 6.87 (1H, *d*, *J* = 8.4 Hz) assignable to H-2', 6' and 5', respectively of 3', 4'-dihydroxy B-ring. The second coupling system occurs as an AM of two meta coupled protons at δ = 6.39 (1H, *d*, *J* = 2.1 Hz), 6.21 (1H, *d*, *J* = 2.1Hz) assignable for H-8 and H-6, respectively of 5, 7-dihydroxy A-ring. The presence of 3-*O*-rhamnopyranosyl in **5** was concluded from its aliphatic proton signal (brs) at δ = 5.26 ppm. Stereo structure of sugar moiety was established as α-$^{1}C_{4}$-pyranose based on the typical *J*- and δ-values in both of its $^{-1}$H- and $^{-13}$C-NMR signals. The remaining carbon resonances of **5** were completely assigned by comparison with previously corresponding data (31). Therefore, compound **5**: was finally identified as quercetin 3-*O*- α -L-$^{1}C_{4}$-rhamnopyranoside (querecterin) (32).

Compound **6**: occurs as pale yellow amorphous powder. -UV-spectral data λ_{max} (nm), (MeOH): 235 sh., 325, 340; (+NaOMe): 250, 300, and 350. It gave a blue fluorescent spot under -UV- light which turned into greenish blue with NA/PE and gave blue color with $FeCl_3$ spray reagents. $^{-1}$H-NMR spectral data revealed the presence of caffeoyl moiety from the ABM spin coupling system at *δ* = 6.90 ppm (1H, *d*, *J* = 1.8 H_Z), *δ* = 6.82 ppm (1H, *dd*, *J* = 1.8, 8.5 H_Z) and *δ* = 6.68 ppm (1H, *d*, *J* = 8.5 H_Z) for the three aromatic protons H-2, H-6 and H-5, respectively. In addition the AX spin coupling system at *δ* = 7.46 ppm and 6.16 ppm (each 1H, *d*, *J* = 16 H_Z) for the *E*-olefinic protons of H-7 and H-8, respectively. Furthermore the singlet signal at 3.51(3H, *s*, OCH_3) in the aliphatic region was assigned to methoxy group. Compound **6** was completely assigned by comparison with authentic samples and previously corresponding data (33). From the previous data compound **6** was identified as *E*- methyl caffeate. The results in (Table 3) showed that methanol extract had the highest amount of total phenolic content (3.5 ± 0.054 mg gallic equivalent per mg dry extract) followed by ethyl acetate extract (1.38 ± 0.038 mg gallic equivalent per mg dry extract) and the lowest one was chloroform extract (0.87 ± 0.015 mg gallic equivalent per mg dry extract).

The brine shrimp lethality assay is considered a useful tool for preliminary assessment of toxicity. The brine shrimp bioassay can be employed for this purpose as it appears to be a convenient, rapid and inexpensive. All extracts used showed significant lethality against brine shrimp. The methanol extract was found to be the most active extract which has LC_{50} value about 140 mg /mg followed by ethyl acetate 165 mg /mg and chloroform 180 mg /mg.

Antioxidative and radical scavenging properties of compounds (**1-3**) were evaluated using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical and phosphomolybdenum method (Table 4). The antioxidant results showed that compound **1** is a highest scavenger of the artificial radical. Compound **1** is phenylethanoid (*E*-isomer) glycoside and identified as strong antioxidant (34). It is suggested that the 3, 4-dihydroxyphenethyl alcohol group might be more responsible for their activities than the caffeoyl group (35).

In conclusion, we have clearly shown that different extracts *of Vitex trifolia* displays cytotoxic activity on the brine shrimp lethality bioassay. Also the methanol extract has the highest value of total phenolic content due to the presence of flavonoids followed by chloroform and ethyl acetate extracts, and the antioxidant results showed that compound **1** is the strongest antioxidant.

Table 1: ^{1}H, ^{13}CNMR spectral data of 1 (300/75 MHz, DMSO-d_6)

No.	δ_{H1}	δ_{C1}
1	…….	131.25
2	6.77 *d* (1.6)	116.87
3	……..	144.25
4	……..	145.7
5	6.73 *d* (8.1)	115.95
6	6.57 *dd* (8.1, 18)	121.05
7	2.78 *t*-like (7.5)	36.24
8	3.84 *t*-like (7.5)	72.17
1'	4.43 *d* (7.8)	103.78
2'	4.01*	79.65
3'	……	73.94
4'	4.903 *t-like* (10)	72.17
5'	……	76.27
6'	4.02 *brd* (12)	62.36
6'	3.37 *	62.36
1"	5.29 *d* (1.5)	101.85
2"	3.89 *brs*	71.53
3"	……	70.28
4"	……..	71.98
5"	3.71 *m*	69.47
6"	1.11 *d* (6)	18.54
1"'	……	127.62
2"'	7.17 *d* (2.1)	115.26
3"'	…….	148.89
4"'	……	146.25
5"'	6.87 *d* (8.1)	116.36
6"'	7.05 *dd* (8.1, 2.1)	122.77
7"'	7.59 *d* (15.9)	147.76
8"'	6.29 *d* (15.9)	115.15
9"'	…….	169

δ in ppm and *J* values in H_Z, are given in parentheses, *Unresolved protons

Table 2 ^{1}H, ^{13}CNMR spectral data of 2, 3 (300/75 MHz, DMSO-d_6)

No.	δ_{H2}	δc_2	δ_{H3}	δc_3
2	…….	163.9	……..	163.23
3	6.55 *s*	103.4	6.50 *s*	102.6
4	……	182.0	…….	181.83
5	……..	160.4	……...	160.7
6	6.45 *s*	98.4		109.1
7		161.2		161.43
8		104.6	6.44 *s*	94.02
9		156		156.46
10		104.5		102.6
1'		121.6		121.09
2'	7.95 *d* (8.3)	128.9	7.84 *d* (8.1)	128.36
3'	6.9 *d* (8.5)	115.9	6.92 *d* (8.1)	116.11
4'		161.01		161.43
5'	6.9 *d* (8.5)	115.9	6.92 *d* (8.1)	116.11
6'	7.95 *d* (8.3)	128.9	7.84 *d* (8.1)	128.36
1''	4.7 *d* (9)	73.5	4.98 *d* (8.1)	73.32
2''		71		70.54
3''		78.7		79.1
4''	4.11 *d* (7.8)	70.6	4.11 *d* (7.8)	70.27
5''	3.79- 3.34		3.79- 3.34	
6''	3.79- 3.34		3.79- 3.34	

δ in ppm and *J* values in H_Z, are given in parentheses.

Table 3 Total phenolic content TPC (mg Gallic equivalent/ mg dry extract) and Cytotoxicity expressed as % mortality and LC_{50} of *Vitex trifolia* aerial parts different extracts. Values of mortality and total phenolic content given are mean ± SE. (N=6). (Mean±SD).

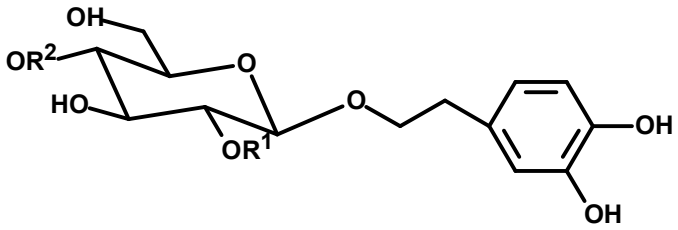
Extracts	Concentration mg ml^{-1} (Mean±SD)							TPC
	10	100	200	400	600	800	1000	
MeOH	9.33± 0.21	34.67±0.23	74.33±0.22	85.00±0.37	93.67±0.22	98.67±0.42	99.33±0.21	3.5 ± 0.054
$CHCl_3$	10.0± 0.36	34.00±0.37	58.66±0.56	79.66±0.20	91.33±0.21	96.67±0.21	99.67±0.22	1.38 ± 0.038
EtOAc	8.33± 0.23	26.00±0.43	57.67±0.22	67.66±0.21	79.32±0.20	94.00±0.37	99.32±0.42	0.87± 0.015

TPC = Total phenolic content

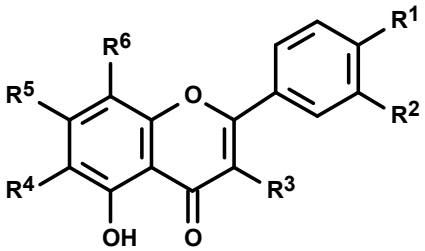
Table 4 DPPH as indicated by the low SC50 value and total antioxidant capacity (mg of ascorbic acid equivalent / g of extract). (Mean±SD)

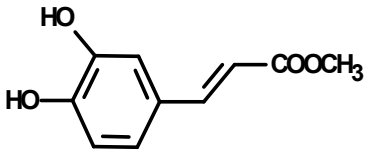
sample	$DPPHSC_{50}$ (µg/ ml)	Total antioxidant capacity (mg AAE/ g extract)
Compound 1	4.70 ± 0.12	900.21 ± 0.36
Compound 2	15.36 ± 0.07	315.35 ± 13.55
Compound 3	30.48 ±0.47	355.71 ± 1.36
Ascorbic acid	7.90 ± 0.20	----------

Results are (means ± S.D. (standard deviation) (n = 3); AAE (ascorbic equivalent).



1- R^1 = α-L- rhamnopyranoside, R^2 = *E*- caffeoyl





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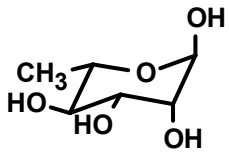
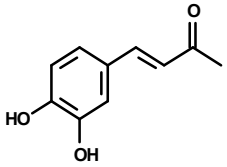
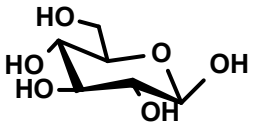
2- $R^2 = R^3 = R^4$ = H, $R^1 = R^5$ = OH, R^6 = C- glucopyranoside
3- $R^2 = R^3 = R^6$ = H, $R^1 = R^5$ = OH, R^4 = C- glucopyranoside
4- $R^3 = R^4 = R^6$ = H, $R^1 = R^2$ = OH, R^5 = O- glucuronopyranoside
5- $R^4 = R^6$ = H, $R^1 = R^2 = R^5$ = OH, R^3 = O- α-L- rhamnopyranoside



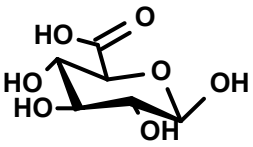
α-L- rhamnopyranoside



E- Caffeoyl



glucopyranoside



Glucuronopyranoside

Figure 1. Chemical structures of compounds isolated from *Vitex trifolia* aerial parts

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Determinants of medical care service personnel knowledge sharing intention: an empirical study

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Abstract: Historically, knowledge management plays a predominant role in enhancing organizational performance. Organizations adopt what methods to capture useful knowledge from knowledge repositories for accumulation, is the largest challenge in the organization. The organization will be encourage and instruct inter-organization members vigorous to promote the exchange and sharing of knowledge attitude and intention to facilitate organizational competitiveness and operating performance. In the health care domain, reliance on staff expertise to perform the operation is very professional practice. Medical service personnel in the implementation of daily operation often need to communicate with colleagues and work together to exclude the health care problem. This research review the theory of reasoned action, and individual's intrinsic motivations with extrinsic factors to observe related impact on the antecedents of knowledge sharing intention. Further, to this end, the authors integrate knowledge sharing literature to inference and develop a conceptual framework to empirical factors interrelationships in medical care service area.

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Keywords: medical care service personnel, extrinsic rewards, resource availability, anticipated reciprocal relationships, theory of reasoned action

1. Introduction

In the twenty-first century, we are face on the knowledge economy era. The medical care staff is more focused on learning new professional skill and services for accumulated their professional knowledge to increase organization performance. This situation will continued influence to various health care related groups and organizations. Within the health care industry, practitioner will face on increasingly challenges and competition in the operating environment [1]. Health care provider is full of all kinds of specialization and knowledge related to the operation the patient lives, the slightest negligence will be cause inconvenience to patients, or even killed, the newspapers of such rumors frequently seen, therefore, how the medical organization to strengthen and training members enhancing professional knowledge and impetus the knowledge exchanges, is an important issue. The medical industry is a knowledge-intensive industry, remain competitive advantage and accumulate knowledge is the organizational main subject. As a result, the knowledge management efficiency of the organization has become the most important and critical test in facing turbulence environment [1]. According to prior literatures finding, a number of dilemmas associated with knowledge sharing (exchange) in a organization. For instance, how to encourage self-interest organization employees to participate in the organization and to openly share valuable knowledge with other organization employees are critical roles in the medical care service (MCS) industrial. [3]. In essence, the personal behavior intention is filled with nature of elusive. Among them, concern about both individual and supervisor manager motivation are key of successful management activity. Consequently, exploring MCS personnel (MCSP) knowledge sharing intention will assist industrial to establish knowledge management policy. With this purpose in mind, the aim of this study is to deepen our comprehension of the antecedents that increase or decrease employees' tendencies to engage in knowledge-sharing intentions. For this reason, knowledge sharing intentions are likely to be influenced not only by individual motivations but also by contextual forces [4].

Based on previous literatures, the study propose a theoretical model which include extrinsic factor and individual beliefs combine with the theory of reasoned action (TRA) [5]. At the same time, by delivering questionnaires to the relevant MCSP fill out and collect their arguments for analysis.

Our essay is organized as follows, first, review previous literature arguments to develop a conceptual model for knowledge sharing intentions and depicted in the figure 1. The following section proposes and describes the elicited external beliefs and demonstrated within the relationship to TRA. The third section presents the research model and develops the research hypothesis to empirical relationships of the factor. Finally, we present the six positive

hypothesis between six constructs, introduce methodology, discuss the study finding and its contributions following.

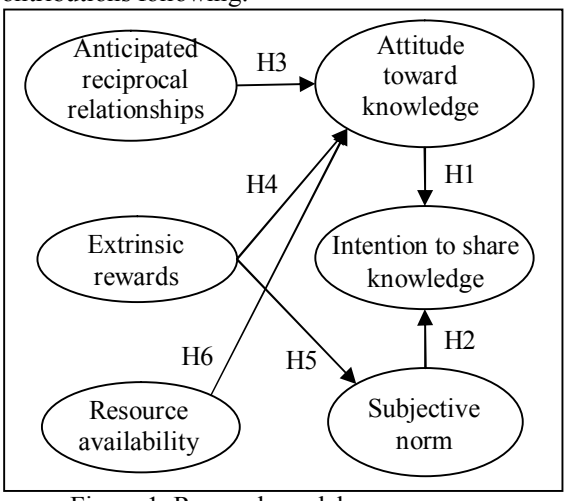

Figure 1. Research model

2. Methodology

2.1 Sample collection

Based on previous TRA and beliefs of individual literatures argument that regarding MCSP execution tasks and similar goals of this study. The suitable sample size and questionnaire of this study was determined and collected by reference literature related to this study. The contained 21 items questionnaire of this research is also conducted by using the Likert five point classification scale also ranging from "disagree strongly" (1) to "agree strongly" (5). According to pilot test, the formal questionnaire was delivered in medical care organizations such as hospital, physician clinic, and pharmacy in all regions of Taiwan. The sample resource and diversity by MCSP gender, age, degree, department, position and experience. During the March, 2011 to May, 2011, a total of 700 sets hard and soft copies of the survey questionnaires are distributed via email, and public places. Due to reminder mail and go through reminder telephone call for MCSP participator, the total 355 completed sets of the questionnaires were returned (given an overall response rate of 50.7%), however, 36 respondents with incomplete filled out questionnaires which are exclusive for further statistical analysis. Finally, 319 respondents was used in the data analysis. The respondents' characteristics and demographics are shown in Table 1.

2.2 Statistical analysis

In this study, all of the collected data was tested by SPSS 16.0 and LISREL 8.52 version of the statistical package software for used to analysis the whole received respondents' data and complete the results.

2.3 Measure

This study apply explanatory factor analysis (EFA), confirmatory factor analysis (CFA), reliability analysis (RA) and path analysis to extraction the collected data into certain factors and supplemented by structure equation modeling (SEM) analysis to estimate constructs interrelated [6-7].

Table 1. Respondents' characteristics and demographics (n=319)

Measure	Items	Frequency	Percentage (%)
Gender	Male	136	42.6%
	Female	183	57.4%
Age	18~28	95	29.8%
	29~39	82	25.7%
	40~44	64	20.0%
	45~49	43	13.5%
	50~	35	11.0%
Degree	Senior school	124	38.9%
	Bachelor	105	33.0%
	Master	85	26.6%
	PhD.	5	0.2%
Department	Hospital	187	58.6%
	Physician clinic	99	31.0%
	Pharmacy	33	10.3%
Position	Department employee	276	86.5%
	Department supervisor	27	8.5%
	Senior manager	16	5.0%
Experience	~5 years	57	17.9%
	6~12 years	97	30.4%
	13~20 years	109	34.2%
	21~ years	56	17.6%

3. Results

3.1 Research finding

The results from EFA, RA, and CFA which were show that all of above standard coefficient. In addition, within the six hypothesis by SEM analysis in research model, H2 is non-significant (coefficient=0.07) revealed that MCSP are not limited the self's norm to process knowledge sharing. Otherwise, the H3 present negative significant also expose interpersonal reciprocal relationships may not help to MCSP adopt knowledge sharing intention. The analysis data from the empirical evidence are stated in Table 2 and Figure 2.

Table 2. Results of statistics analysis

Construct	EFA		RA	CFA
Intention to knowledge sharing	ITT1	0.854	0.923	P value=0.000 GFI=0.938 AGFI=0.916 RMSEA=0.041
	ITT2	0.854		
	ITT3	0.768		
	ITT4	0.760		
Attitude toward knowledge	ATT1	0.831	0.931	
	ATT2	0.816		
	ATT3	0.786		
	ATT4	0.785		
Subjective norm	SN1	0.861	0.912	
	SN2	0.850		
	SN3	0.844		
	SN4	0.833		
Anticipated reciprocal relationships	PR2	0.901	0.935	
	PR3	0.891		
	PR4	0.889		
Extrinsic rewards	ER1	0.814	0.922	
	ER2	0.809		
	ER4	0.781		
Resource availability	RA1	0.822	0.894	
	RA2	0.814		
	RA3	0.807		

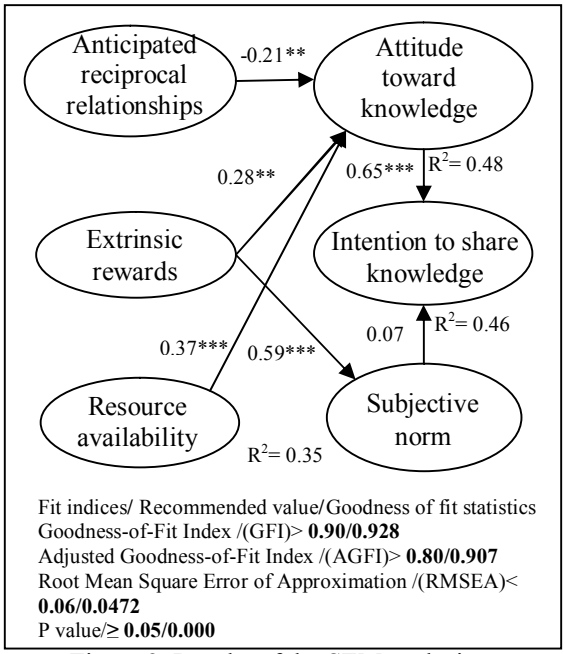

Figure 2. Results of the SEM analysis
Note: *: p<0..05; **:p<0.01; ***:p<0.001

4. Discussion

In this study, TRA as the main core structure, and integrate anticipated reciprocal relationships, extrinsic rewards and resources availability as the antecedents to explore relationship the MCS personnel's knowledge sharing intention. In light with the prior literatures, only a few scholars to discuss the availability of resources influence the individual intent to knowledge sharing. This research analyzing the relevant literatures and empirical evidence that supported or non-supported six hypothesis. In addition, this study also filled out the gap of academic theory and MCPS practices. Future study could be expand this research findings to investigate other industrial and organization.

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Sputum Cytology – An Underutilized Diagnostic Tool: A Single Institute Experience

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Abstract: Objective: Sputum cytology is a valuable diagnostic tool which under underutilized in our clinical practice. The aim of this study is to report our experience utilizing this tool to diagnose various respiratory disorders and to report the utility and accuracy of this procedure in our institute. **Design:** A retrospective analysis of all sputum smears from Jan 1995 to December 2010. **Settings:** The Department of Pathology at King Abdulaziz University hospital Jeddah, Western region of Saudi Arabia. **Subjects and Methods:** All sputum cytology samples received at the Department of Pathology were reviewed. **Interventions:** Cytology smears, clinical history and surgical follow-ups were reviewed. **Main Outcome measures:** The data was analyzed to calculate sensitivity, specificity, and predictive values. **Results:** A total of 191 cases of sputum cytology were examined during this period and only 38 (20%) patients had a subsequent follow up biopsies. Cytology diagnosis was categorized (reporting system of our laboratory) as atypical in 4 cases, malignant in 5 cases, inflammatory in 17 cases, insufficient in 21 cases, and negative in 144 patients. The subsequent histological follow-up in 38 cases was categorized as malignant in 21cases, inflammatory /benign in 12 cases and negative in 5 cases. Cross-tabulating the cytology with surgical follow-up revealed 3 true-positive, one false-positive, 16 true-negative, and 15 false-negative cases. **Conclusion:** Sputum cytology showed high specificity (94%) and positive-predictive value (75%) and low sensitivity (16.7%) and a negative-predictive value (52%). The low sensitivity limited the sputum-cytology as a screening tool. But in patients suspected of having malignant lesions the high specificity of this tool can be utilized to get diagnosis before proceeding to invasive procedures.
[Awatif Jamal and Ibrahim Mansoor. **Sputum Cytology – An Underutilized Diagnostic Tool: A Single Institute Experience.** Life Sci J 2012; 9(2):681-685]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 102

Key Words: Sputum, Mucus, Cytology.

1. Introduction

Lung cancer outcome has improved a lot by early screening and detection by multiple modern diagnostic techniques. The current the methodologies available that can be utilized in reaching a diagnosis of lung cancer are: sputum cytology; flexible bronchoscopy [FB]; and transthoracic needle aspiration (TTNA). Recently positron emission tomography (PET) scanning has emerged to help in achieving the diagnosis and staging of lung cancer as well [1].

In order to decide which specific diagnostic modality to be used effectively, it should fulfill two main criterions: the first criterion is that it should have maximum yield with regards to diagnosis and staging of the disease. Secondly it should be minimally invasive. The diagnostic modality should also take into account the patient clinical condition and the treatment plan which will be offered to them after the specific diagnosis [1].

Sputum cytology examination was widely used at the end of the 20th century for discovering lung cancer in high-risk patient at an early stage and it was viewed by clinicians as a simple, non-invasive, cheap investigation. Early reports on sputum cytology suggested that positive identification of lung cancer could be achieved in 57% to 66% of patients with a clinically obvious tumor [2].

Literature of Sputum cytology established that this non-invasive method is an acceptable method of establishing the diagnosis of cancer in suspected patients. Several studies identified that sensitivity of sputum cytology in diagnosing suspected lung lesion ranged from 0.42 to 0.97, while the specificity ranged from 0.68 to 1.0. These studies also showed that the pooled sensitivity of sputum cytology was 0.66, and the pooled specificity was 0.99 [1,3]. **Sing *et al.*** and **Rivera *et al.*** declared in their studies that sputum specimens are most valuable in the detection of early and peripheral carcinomas and that the diagnostic yield in lung carcinoma depends on the location of the tumor, the histological type and the stage [4,1].

Studies from National Cancer Institute (NCI) has reviewed the role of sputum cytology as a screening tool and has shown 85 to 90% 5-year survival rate in small number of patients with negative radiological examination for lung cancer and positive by sputum cytology alone. The most common histological type of carcinoma encountered in these studies was squamous cell carcinoma [5].

The diagnostic accuracy of sputum cytology also depends on the number of samples obtained, the preservation technique of the sample, the location of the tumor (central vs peripheral) and the size of the tumor. Patients yielding positive sputum cytology often

have: bloody sputum; low FEV1 values; large tumor volume (> 2.4 cm); centrally located tumors; and squamous cell histology [1].

2. Material and Methods

All consecutive sputum samples received at King Abdulaziz University Hospital, Department of Histopathology were reviewed over the period of fifteen years (from January 1st, 1995 to December 31st, 2010).

All the sputum cytology specimens in this study where collected and prepared in adherence (as much as possible) to the international guide lines for sputum cytology specimen collection and preparation.

A series of three (3) sputum specimens, one each day for three days, were collected as recommended by the university laboratory policy and procedure. Deep cough specimens were required if malignancy was suspected. Specimens are immediately processed to avoid cell degeneration and growth of contaminating micro-organisms. The gross/ physical specimen characteristics (volume in mL, gross appearance) were described and recorded in the final sputum cytology report (macroscopic section).

Pick-and-smear preparations were made from 'suspicious' areas of the specimen, which may appear as gray/white or blood-tinged mucus strands. Different areas of the specimen were sampled. Specimen was placed onto appropriately labeled frosted-end glass slides for direct smearing; the material was spread and manipulated between two opposing slides to produce a thin, mirror image, even layer of specimen on each slide

Four smears are prepared. Three smears are fixed immediately in 95% alcohol (or equivalent) and one smear is air-dried; three smears are stained with the Papanicolaou stain and one is stained with Diff-Quik. Mucoid specimens are treated with Mucolex solution (Shandon), equal volume of Mucolex solution is added to the specimen, mix then centrifuge.

Sputum specimens are regarding as being unsatisfactory when no pulmonary macrophages are identified. All stained slides are screened for abnormal findings. No radiological or demographic data was collected since it was beyond the scope of this study. A summary of the findings is prepared using current acceptable terminology based on established cytomorphologic criteria and adopted as protocol reporting system in our laboratory, and released on a standard report format.

A total of 191 sputum cytology cases were collected and categorized as: Atypical, Malignant, Inflammatory, Insufficient and Negative (Table 1). The records and the histopathological slides of 38 patients that had subsequent surgical follow up biopsies were retrieved and examined. The surgical histopathological categories were as follow: Malignant, Inflammatory and Negative (Table 2). Correlation of the sputum cytology and surgical biopsy was performed (Table 3), sensitivity, specificity, positive and negative predictive values were calculated.

3. Results

A total of 191 Sputum smear /cytology diagnosis were collected and categorized as atypical, malignant, inflammatory, insufficient and negative (Table 1). The detailed cytological diagnosis were: 4 cases atypical cells, 5 malignant cases of which 3 were adenocarcinoma, 1case non-small cell carcinoma and 1 case squamous cell carcinoma.. Inflammatory cases were 17 of which 8 cases were fungal infection (5 Candida, 2 aspergillus, 1 fungal hyphae unclassified), 4 cases of tuberculosis infection and 5 cases were only abundant inflammation. Insufficient or inadequate for diagnosis were 21 cases and negative for malignancy 144 cases.

The subsequent histological follow-up in 38 cases was categorized as malignant in 21 cases, inflammatory /benign in 12 cases and negative in 5 cases (Table 2). Of the 21 malignant cases in surgical follow-up, we identified 3cases of adenocarcinoma that were diagnosed on both surgical follow-up and cytology as adenocarcinom (true-positive), 3 cases were diagnosed as inflammatory/ benign on cytology while on surgical follow-up were malignant (false negative). 3 cases were insufficient for diagnosis, and 12 cases were diagnosed as negative for malignancy on cytology while were malignant on surgical follow-up biopsies (false negative) (Table 3). 16 cases were diagnosed as negative on both cytology and surgical follow-up (true negative). One case was diagnosed as positive on cytology but was negative on surgical follow-up (false positive). Excluding the 3 non-diagnostic cases(insufficient for diagnosis), there were 3 true-positive cases, 16 true-negative cases, one false-positive case and 15 false-negative cases. The sensitivity was 16.7%, specificity of 94%, positive predictive value of 75% and negative predictive value of 52%.

Table 1: Summary of sputum cytology diagnosis categories

Cytology Dx Categories	**Count**
Atypical	4
Malignant	5
Inflammatory	17
Insufficient	21
Negative	144
Grand Total	**191**

Table 2: Summary of surgical diagnosis categories

Surgical Dx Categories	Count
Malignant	21
Inflammatory	12
Negative	5
Grand Total	**38**

Table 3: Cytohistological correlation of sputum cytology and surgical biopsy follow-up.

Cytology Dx Category	Surgical Dx Categories			
	Inflammatory	Malignant	Negative	Total
Atypical		1		1
Malignant		2	1	3
Inflammatory		3	1	4
Insufficient		3		3
Negative	12	12	3	27
Grand Total	**12**	**21**	**5**	**38**

4. Discussion

An adequate sputum sample is composed of mucus and various types of respiratory cells that are cleared by the mucociliary apparatus and includes bronchial epithelial cells, few squamous cells and abundant alveolar macrophages. Before the development of fibreoptic bronchoscopy (FB), sputum cytology was the only alternative to thoracotomy for tissue diagnosis of many pulmonary neoplasms. At that time sputum was the most common respiratory tract specimen examined because it is relatively easy to collect and causes minimal discomfort to the patient. Unfortunately, utilization of sputum cytology as the mainstay in respiratory cytology has declined significantly probably due to the advent of bronchoscopy, tans thoracic imaging guided fine needle aspiration (FNA), trans-bronchial needle aspiration, EUS-guided FNA and due to the low-sensitivity of sputum cytology. **Kennedy *et al.*** underlined the valuable role of sputum cytology in early detection of lung cancer in selected patients and suggested reevaluation of the role of sputum cytology [5]. Furthermore, many studies have reported that sputum cytology has resulted in detection of lung cancer at an early stage and improved 5-years survival rate [5].

Regrettably, screening asymptomatic smokers with sputum cytology does not decrease mortality from lung cancer due to its low-sensitivity. However; sputum cytology has a high specificity and positive predictive value and can be clinically very useful for symptomatic individual [4, 6]. It has been shown that sputum cytology has an important role especially in patients with relative or absolute, contraindication to bronchoscopy examination. Moreover, it is reported that sputum cytology can be helpful in diagnosing peripheral lesions, inaccessible to bronchoscopy, or if bronchoscopy has failed in providing diagnostic material [2]. Furthermore, sputum cytology has a role if a tissue diagnosis is needed to direct the patient treatment, and surgery is unlikely to be performed, then sputum cytology represent the appropriate non-invasive procedure [2]. **Rivera and Mehta** recommends sputum cytology for patients who present with centrally located tumors (*i.e.*, SCLC or squamous call carcinoma) and in those who present with hemoptysis, with a central lesion with or without radiographic evidence of metastatic disease, and in whom a semi-invasive procedure such as bronchoscopy or TTNA might pose a higher risk [1].

Multiple studies have stated that the accuracy of sputum cytology and sensitivity of diagnosing lung cancer is difficult to summarize because of a range of methodological problems which are strongly related to the number of sputum samples and the specimen adequacy [1]. **Schreiber *et al.*** and **Rivera *et al.*** emphasized the importance of adequate sampling and claimed that many institutions have no established Programs for sputum collection and processing and that explain the lower sensitivity reported in many studies [3, 1]. To have a yielding sputum cytology results more attention has to be given to the optimal collection and submission of the sputum and to the method of sputum preparation [7, 11, 12] .Deep cough specimen of the lower respiratory tract containing numerous pulmonary macrophages is mandatory to obtain [9]. In fact sputum induction increases the detection of lung cancer [10]. Furthermore, it was emphasized that positive samples with higher sputum cytology yield are often submitted by specialist physicians such as chest physicians [2].

Collecting multiple sputum samples over several days optimizes sensitivity of sputum cytology. **Bocking *et al.*** have shown that the sensitivity of sputum cytology in detecting lung cancer is highly dependent on the number of sputum specimens

collected per patient, ranging from approximately 0.68 for a single specimen, to 0.78 for two specimens, to 0.85– 0.86 for three or more specimens [7]. In another study the sensitivity of sputum cytology for the diagnosis of malignancy increases with the number of specimens examined, from 42% with a single specimen to 91% with five specimens [8].The type of patients from whom the sputum was collected was another concern. Elderly individual with productive cough and exacerbation of chronic obstructive air way disease or congestive heart failure and with low clinical suspicion of malignance have low yield of positive sputum cytology [2].

The specificity of sputum examination is high, ranging from 96% to 99%, and the positive and negative predictive values are 100% and 15%, respectively reported by **Fraire *et al.*** [13]. The present study is comparable to other studies in the literature and it reports high specificity (94%) and positive predictive value (75%) and showed a lower sensitivity (16.7%) and negative predictive value (52%). The diagnostic yield in lung carcinoma as was stated by **Sing *et al.*** [4] and **Rivera *et al.*** [1] is dependent on the location of the tumor, the histological type and the stage of the tumor and that sputum specimen are most valuable in the detection of early and peripheral carcinomas [4]. Accuracy in tumor classification is 75% to 80% [14] and is tumor type dependent [15]. It is evident from the literature that sensitivity of sputum cytology depends on the location of the malignant tumor: 46% to 77% of central lung cancers but only 31% to 47% of peripheral cancers [4, 6], In another study by **Schreiber *et al.*** the sputum cytology sensitivity was higher for central lesions than for peripheral lesions (0.71 vs 0.49, respectively) [3].

The lower sensitivity in our study can be explained by the uncontrolled protocol for sputum specimen collection, the time of collection, variation between physician and their confidence in the sputum cytology, the frequency and the number of specimen obtained by patient as well as some variations in the laboratory preparation techniques. This retrospective study establishes the high reliability and specifity of the sputum cytology examination and discloses the underutilization of this test at our institution. Furthermore, our study encourages the use of this simple non-invasive procedure in high risk patient with high clinical suspicion of lung cancer, especially in patient complaining of hemoptasis, or have central lesion and or when performing bronchoscopy is contraindicated or inappropriate. The study highlighted careful evaluation and the use of other investigational modalities in case of negative sputum cytology; since many studies have underlined that a single negative sputum result does not guarantee the absence of a malignancy, especially in a patient suspected of having lung cancer.

5. Conclusion

Sputum cytology is highly specific diagnostic tool in expert hands and to improve the yield of this simple non invasive examination a basic recommendation is to be followed: collection of multiple sputum specimens from the suspected patients involving specialist physicians is highly encouraged. Furthermore, attention has to be made during collection, submission and preparation of the sputum to improve the quality of the specimen and cytology results. Unified protocol or an institutional guide for collection, submission and preparation of the specimen should be generated and followed if increased sensitivity and specificity of sputum cytology is aimed.

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Prognostic Value of Cyclin E and Cyclin Dependent Kinase Inhibitor (P27) Gene Expression in Non – Hodgkin's Lymphoma

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Abstract: The eukaryotic cell cycle is controlled by protein kinase complexes composed of cyclins and cyclin dependent Kinases (Cdks). The activity of Cdks is regulated by binding of positive effectors, the cyclins, and by association-dissociation of inhibitory subunits, designated cyclin dependent kinase inhibitors (CKIs). Cyclins, Cdks, and CKIs are frequently altered in human cancer. P27 is a CKI that regulates progression from G1 into S phase and appears to play a role in both cell growth and differentiation. Cyclin E, in conjunction with its kinase partner Cdk2, regulates many aspects of cell division. Many human cancers express high levels of cyclin E, and this is thought to directly contribute to cell transformation and tumor aggressiveness. The aim of this study was to study the prognostic value of p27 and cyclin E protein expression levels in relation to the staging of NHL, laboratory data, clinical manifestations and to predict patient's survival. The patients were subjected to full work-up for diagnosis of NHL and Western blot analysis for detection of p27 and cyclin E protein expression.40 newly diagnosed patients suffering from non-Hodgkin's lymphoma (NHL) in different stages and twenty healthy subjects were the subject matter of this study. Our results showed over expression of cyclin E and down regulation of p27, which was significantly associated with advanced staging of the disease. There was a positive correlation between age and over expression of cyclin E and inverse correlation with p27 expression. Over expression of cyclin E and down regulation of p27 were significantly associated with laboratory and clinical findings, delayed remission, increased relapse and increased death rate. Conclusion: p27 and cyclin E expression are significant, independent prognostic factors and reliable molecular markers in predicting recurrence and selection of patients for adjuvant therapy in malignant lymphoma.

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Keyword: Cylin E, p27, non Hodgkin's lymphoma, gene expression

1. Introduction:

Non-Hodgkin's lymphoma (NHL) is a group of closely related B and T-cell cancers of the lymphatic system. The incidence of NHL is rising, particularly in countries of the industrialized world. The incidence of NHL in the United States has increased by 5% over the past 15 years. The cause of this increased incidence is not fully understood, but several risk factors have been blamed including: exposure to chemicals, viral infections, organ transplantation and blood transfusion, family history, and lifestyle factors [(1)].

In general, the incidence of NHL is 50% higher in men than in women [(2)]. The cancer can develop in people at all ages, including children, although it is most common in those aging from 45 – 60 years.

The eukaryotic cell cycle is controlled by protein kinase complexes composed of cyclins and cyclin dependent Kinases (Cdks). The activity of Cdks is regulated by binding of positive effectors, the cyclins, and by association-dissociation of inhibitory subunits, designated cyclin dependent kinase inhibitors (CKIs) [(3)].

Two families of CKIs have been identified; INK4 and Cip/Kip families. The INK4 Family members including $P15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, and $p19^{INK4D}$ bind to and inhibit Cyclin-D-dependant kinases (cdk4 and cdk6).The Cip/Kip family members, including $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$ preferentially inhibit cdk2 [(4)].

Cyclins, Cdks, and CKIs are frequently altered in human cancer. $P27^{KIP1}$ is a CKI that regulates progression from G1 into S phase by inhibiting a variety of Cyclin-Cdk complexes. P27 appears to play a role in both cell growth and differentiation [(5)].

Cyclin E, in conjunction with its kinase partner Cdk2, regulates many aspects of cell division [(6)]. Cyclin E-Cdk2 exerts its cell cycle regulatory activities by phosphorylating substrates involved in G1 progression, S-phase entry and centrosome duplication, as well as a kinase- independent function involved in exiting quiescence [(7)]. Many human cancers express high levels of cyclin E, and this is

thought to directly contribute to cell transformation and tumor aggressiveness [8].

P27Kip1 protein level changes during cell cycle progression, accumulating when cells progress through G1 and sharply decreasing just before cells enter S phase. Additionally, p27Kip1 protein levels rise when cells exit the cell cycle to G0, and decreases when cells enter the cell cycle again [9]. These alternations in p27Kip1 levels are caused by regulation at the protein degradation level [10].

Deregulation of cell cycle control is a critical step in the development of human cancers and, therefore, knowledge of the expression of cell cycle regulatory proteins in tumor cells is essential for understanding tumor cell behavior and may be important for predicting prognosis of cancer patients [11].

Aim of work:

The aim of the present work is to study the expression level of the cell cycle regulators cyclin E and p27 proteins by Western blot analysis on 40 newly diagnosed patients with NHL in different disease stages and detect their correlation with the staging of NHL, laboratory data, clinical manifestation and patient's survival.

2. Subjects and Methods:

40 newly diagnosed patients suffering from NHL, were selected from the inpatients of the Oncology Unit of Tanta University Hospital and the National Cancer Institute (NCI), Cairo University. The study was designed to continue for 18 months of follow up. Twenty healthy subjects matched in age and sex, were also included, as a control group. The subjects included in this study were divided into two main groups:

Group 1: Patients suffering from NHL in different stages of the disease at presentation and after treatment. They were 25 males and 15 females, their ages ranged from 25-75 years. They were 6 patients with Mucosa Associated Lymphoma Tissue (MALT), 6 patients with Follicular lymphoma, 7 patients with Diffuse large B-cell lymphoma, 5 patients with Anaplastic large T-cell lymphoma, 5 patients with Peripheral T-cell lymphoma, 4 patients with B-cell Burkitt like lymphoma, 4 patients with Mantle cell lymphoma and 3 patients with Marginal zone lymphoma (nodal).

They were divided into four subgroups according to Ann Arbor staging system:

Group Ia: Included 7 patients in stage I suffering from one cervical or axillary lymph node enlargement and discovered accidentally during routine examination of other diseases.

Group Ib: included 8 patients in stage II suffering from both cervical and axillary lymph nodes enlargement with or without splenomegaly or B symptoms (night sweating, unexplained fever and unexplained loss of more than 10% of the body weight in the last 6 months).

Group Ic: included 15 patients in stage III suffering from both cervical and axillary or inguinal or abdominal lymph nodes enlargement, ascitis, pleural effusions or B symptoms.

Group Id: included 10 patients in stage IV suffering from both, cervical and axillary or inguinal or abdominal lymph nodes enlargement, spenomegaly, B symptoms and bone marrow infiltration with or without hepatomegaly or ascitis or pleural effusion.

Group II: normal control group; twenty healthy subjects selected from hospital staff. They were 12 males and 8 females, their ages ranged from 25-60 years.

*Patients with exclusion criteria were out of our study; including patients with inflammatory disease, malignant disease other than NHL, and previous exposure to chemotherapy.

*The Standard CHOP treatment protocol was adopted to our patients: C-Cyclophosphmide 750 mg/m^2 I.V for 1 day. H-Doxorubicin (Adriamycin) 50 mg/m^2 I.V for 1 day. O-Vincristine (Oncovin) 1.4 mg/m^2 I.V for 1 day; and -Prednisone 100 mg daily for 5 days.

This protocol was given one time every 3 weeks for 6 doses.

According to response to treatment, patients of group Ic and Id were further subdivided into Icc, Idc (cured) and Icn, Idn (non-cured), respectively.

*Patients were in complete remission when complete absence of symptoms and signs occurred and laboratory investigation returned to normal levels, while its persistence indicated non-cure.

Patients' group was subjected to the following:

A) Full work–up for diagnosis of NHL:(1)Detailed history and clinical investigation searching for important signs of prognostic significance as night sweating, unexplained fever, unexplained loss of more than 10% of the body weight in the last 6 months, lymphadenopathy, organomegaly, ascitis and pleural effusion.(2)Radiological examination including chest x-ray, CT scan chest, pelvi-abdominal CT scan and ultra-sonography.(3)Laboratory investigation: four ml venous blood was collected under complete aseptic technique, delivered into two tubes; one containing EDTA for CBC and the other was plain for LDH and uric acid assessment. BM

aspiration for cytochemistry and immunophenotyping was done.

B)10 ml venous blood was collected into heparinized tubes for mononuclear cell separation, and protein extraction for detection of Cyclin E and p27 by size separation of the proteins in the mixture on polyacrylamide gel electrophoresis (PAGE). The separated proteins were then transferred to poly vinylidenediflouride (PVDF) membrane (Bio-Rad Laboratory). Detection of the protein under investigation by its specific antibody and determination of its size relative to standard protein of known size was performed.

Informed consent was taken from every patient and control before enrollment in the study and the research was approved by the Ethical committee of Tanta University.

Statistical methods:

Data was analyzed using SPSSwin statistical package version 17 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation. Qualitative data were expressed as frequency and percentage. Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using Mann-Whitney test (non-parametric t-test). Comparison between 3 groups was done using Kruskal-Wallis test (nonparametric ANOVA). A *p*-value < 0.05 was considered significant and of < 0.001 was considered highly significant.

3. Results:

NHL Patients were divided according to Ann Arbor staging system, as shown in Tables 1 and 2.

Stage I (Group Ia): Seven patients, 4 males (57.1%) and 3 were females (49.9%). Their age ranged from 32 to 59 with mean of 51.14 ± 10.22 years. Stage II (Group Ib): Eight patients, 6 males (75%) and 2 females (25%). Their age ranged from 25 to 62 with mean of 49.87 ± 12.94 years. Stage III (Group Ic): Fifteen patients, 9 males (60%) and 6 females (40%). Their ages ranged from 38 - 75 with mean of 57.6 ± 8.77 years. (Group Id): Ten patients, 6 males (60%) and 4 females (40%).Their ages ranged from 48 - 75 with mean of 60.4 ± 7.5 years. As regards the control group, their ages ranged from 25 to 60 years with a mean of 49.95+ 6.81 years. There was a high statistically significant difference between the expression of p27 and cyclin E in patients and controls (p =0.0001 for both). Figure (1) demonstrates Western blot analysis of cyclin E and p27 in patients compared to controls.

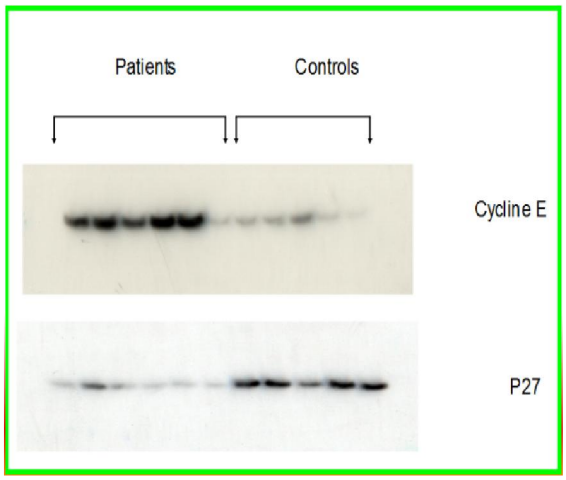

Fig. (1): Cyclin E and P27 protein expression in NHL cases by Western Blot analysis in comparison to controls.

Table (1): Distribution of patients according to age

	Age (years)			
	G Ia	**G Ib**	**G Ic**	**G Id**
Range	32 – 59	25 – 62	38 – 75	48 – 75
Mean	51.14	49.87	57.60	60.40
± SD	10.22	12.94	8.77	7.50
	p-value = 0.079			

Table (2): Relation between sex and the stage of NHL

		Sex		
		Male	**Female**	**Total**
G Ia	**N**	4	3	7
	%	57.1	42.9	100
G Ib	**N**	6	2	8
	%	75	25	100
G Ic	**N**	9	6	15
	%	60	40	100
G Id	**N**	6	4	10
	%	60	40	100
Total	**N**	25	15	40
	%	62.5	37.5	100
	p-value = 0.876			

Down regulation of p27 and over expression of Cyclin E was significantly correlated with advancement of the stage of NHL with *p*-value of 0.001 as shown in tables 3 and 4.

Table (3): Relation between p27 expression and the stage of NHL

Age (years)	P27	
	Normal expression	Down regulation
Mean	47.15	59.70
±SD	11.18	6.76
P. value = 0.000*		

Table (4): Relation between cyclin E expression and stage of NHL

		P27		
		Normal expression	Down regulation	Total
G Ia	N	6	1	7
	%	85.7	14.3	100
G Ib	N	6	2	8
	%	75	25	100
G Ic	N	1	14	15
	%	6.7	93.3	100
G Id	N	0	10	10
	%	0	100	100
Total	N	13	27	40
	%	32.5	67.5	100
	p-value = 0.001*			

Table (5): P27 expression in relation to age

Age (years)	P27	
	Normal expression	Down regulation
Mean	47.15	59.70
±SD	11.18	6.76
P. value = 0.000*		

Table (6): Cyclin E expression in relation to age

Age (years)	Cyclin E	
	Over expression	Normal expression
Mean	62.77	49.77
±SD	5.41	9.52
P. value = 0.000*		

As regards the relation of expression of p27 and cyclin E with sex, no statistical significance could be detected between p27 down regulation or cylin E over expression and sex (p value = 0.138 and 0.251 respectively). However, there was a highly significant association between down regulation of p27 and over expression of cyclin E and increasing age with a p value of 0.0001 for each, as shown in tables 5 and 6. Elevated LDH and reduction in Hb were highly significant with down regulation of p27 with a p value of 0.0001. Thrombocytopenia was associated with down regulation of p27 (P=0.002). There was no significant relation between p27 down regulation and WDCs count with a p value of 0.097, nor with hyperuricemia with a p value of 0.065 (Table 7).

Table (7): P27 expression in relation to Laboratory results

		P27				*P.* value
		Normal expression		Down regulation		
		N	%	N	%	
LDH	N	12	92.3	6	22.2	0.000*
	↑	1	7.7	21	77.8	
HB	N	12	92.3	9	33.3	0.000*
	↓	1	7.7	18	66.7	
Platelet	N	13	100	14	51.9	0.002*
	↓	0	0	13	48.1	
WBCs	N	13	100	22	81.5	0.097
	↓	0	0	5	18.5	
Uric acid	N	13	100	21	77.8	0.065
	↑	0	0	6	22.2	

Elevated LDH and reduced Hb were highly significant with over expression of cyclin E with a p value of 0.0001. Cyclin E over expression was associated with leucopenia (P=0.008), thrombocytopenia (P=0.0001) and hyperuricemia (P = 0.003) (Table 8).

Table (8): Cyclin E expression in relation to laboratory results

		Cyclin E				*p.* value
		Over expression		Normal expression		
		N	%	N	%	
LDH	N	1	5.6	17	77.3	0.000*
	↑	17	94.4	5	22.7	
HB	N	4	22.2	17	77.3	0.001*
	↑	14	77.8	5	22.7	
Platelet	N	6	33.3	21	95.5	0.000*
	↑	12	66.7	1	4.5	
WBCs	N	13	72.2	22	100	0.008*
	↑	5	27.8	0	0	
Uric acid	N	12	66.7	22	100	0.003*
	↑	6	33.3	0	0	

Down regulation of p27 was significantly associated with B symptoms with P value 0.0001. Significant relations were found between down regulation of p27 and the presence of respiratory manifestations, bleeding manifestations, bone ache and splenomegaly with p values of 0.047; 0.047, 0.002, 0.033 and 0.001 respectively. No significant relation was found between p27 down regulation and the presence of hepatomegaly (P=0.065), abdominal manifestations (P=0.13) or neurological manifestation (P=0.097) (Table 9).

Table (9): P27 expression in relation to clinical data

		P27				*P.* Value
		Normal expression		Down regulation		
		N	%	N	%	
Asymptomatic	**Presence**	6	46.2	0	0	0.000*
	Absence	7	53.8	27	100	
B symptoms	**Presence**	7	53.8	27	100	0.000*
	Absence	6	46.2	0	0	
Respiratory	**Presence**	0	0	7	25.9	0.047*
	Absence	13	100	20	74.1	
Bleeding	**Presence**	0	0	13	48.1	0.002*
	Absence	13	100	14	51.6	
Bone ache	**Presence**	1	7.7	11	40.7	0.003*
	Absence	12	92.3	16	59.3	
splenomegaly	**Presence**	2	15.4	21	77.8	0.000*
	Absence	11	84.6	6	22.2	
Hepatomegaly	**Presence**	0	0	6	22.2	0.065
	Absence	13	100	21	77.8	
Abdominal	**Presence**	3	23.1	13	48.1	0.130
	Absence	10	76.9	14	51.9	
CNS	**Presence**	0	0	5	18.5	0.097
	Absence	13	100	22	81.5	

Over expression of cyclin E was significantly associated with B symptoms (*P* =0.004), the presence of respiratory manifestation (*P*=0.024), bleeding manifestations (*P*=0.0001), bone aches (*P*=0.013), the presence of hepatomegaly (*P*=0.003), abdominal manifestations (*P*=0.014) and neurological manifestations (*P*= 0.008) (Table 10).

There was a significant inverse relation between the expression of cyclin E and p27 in NHL patients with p value of 0.004 (Table 11).

After the follow up period, when the fate of the patients was correlated with the level of expression of p27 and cyclin E, the following results were obtained:

Group Ia (Stage I): Significant relation associated between good prognosis and the normal expression of P27 and vice versa with a *p* value of 0.004. Over expression of cyclin E associated significantly with bad prognosis and vice versa with a *p* value of 0.013.

Group Ib (Stage II): A borderline significance could be detected between prognosis and the expression of p27 and cyclin E with a *p* value of 0.061 and 0.058 respectively.

Group Ic (Stage III): Significant relation associated between bad prognosis and the down regulation of p27 and vice versa with a p value of 0.045. Over expression of cyclin E associated with bad prognosis but it was of a borderline significance with a *p* value of 0.051.

Group Id (Stage IV): Significant association was found between bad prognosis and the down regulation of p27 and vice versa with a *p* value of 0.042. Over expression of cyclin E associated significantly with bad prognosis with a *p* value of 0.045.

Table (10): Cyclin E expression in relation to clinical data

		Cyclin E				*P.* value
		Over expression		Normal expression		
		N	%	N	%	
Asymptomatic	**Presence**	0	0	6	27.3	0.16*
	Absence	18	100	16	72.7	
B symptoms	**Presence**	18	100	14	63.6	0.004*
	Absence	0	0	8	36.4	
Respiratory	**Presence**	6	33.3	2	9.1	0.024*
	Absence	12	66.7	20	90.9	
Bleeding	**Presence**	12	66.7	1	4.5	0.000*
	Absence	6	33.3	21	95.5	
Bone ache	**Presence**	9	50	3	13.6	0.013*
	Absence	9	50	19	86.4	
Splenomegaly	**Presence**	17	94.4	6	27.3	0.000*
	Absence	1	5.6	16	72.7	
Hepatomegaly	**Presence**	6	33.3	0	0	0.003*
	Absence	12	66.7	22	100	
Abdominal	**Presence**	11	61.1	5	22.7	0.014*
	Absence	7	38.9	17	77.3	
CNS	**Presence**	5	27.8	0	0	0.008*
	Absence	13	72.2	22	100	

Table (11): Relation between cyclin E expression and the expression of p27 in NHL patients

		Cyclin E					
		Over expression		Normal expression		Total	
		N	%	N	%	N	%
P27	**Normal expression**	1	5.6	12	54.5	13	32.5
	Down regulation	17	94.4	10	45.5	27	67.5
Total		18	100	22	100	40	100
P* = 0.004							

Table (12): Fate according to NHL staging in relation to cyclin E and P27 expression

Groups	Fate & Expression		Remission	relapse	Died	*P* value
Group Ia	**P27**	Normal expression	6	1	0	0.044*
		Down regulation	1	3	1	
	Cyclin E	Over expression	0	3	1	0.013*
		Normal regulation	7	1	0	
Group Ib	**P27**	Normal expression	5	1	0	0.061
		Down regulation	1	3	2	
	Cyclin E	Over expression	1	3	2	0.058
		Normal regulation	5	1	0	
Group Ic	**P27**	Normal expression	0	0	1	0.045*
		Down regulation	0	0	14	
	Cyclin E	Over expression	0	0	10	0.051*
		Normal regulation	0	0	5	
Group Id	**P27**	Normal expression	0	0	0	0.042*
		Down regulation	0	0	10	
	Cyclin E	Over expression	0	0	8	0.045*
		Normal regulation	0	0	2	

Kaplan-Meier analysis of overall survival in NHL patients in relation to p27 expression shows significant short overall survival with p27 down regulation with (P=0.004) as shown in fig. 2.

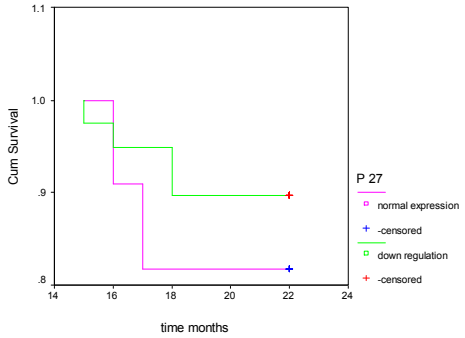

Fig. (2): Overall survival in NHL patients in relation to p27 expression at the end of the study

Kaplan-Meier analysis of overall survival in NHL patients in relation to Cyclin E expression show significant short overall survival with Cyclin E over expression with (P=0.011) as shown in fig.3.

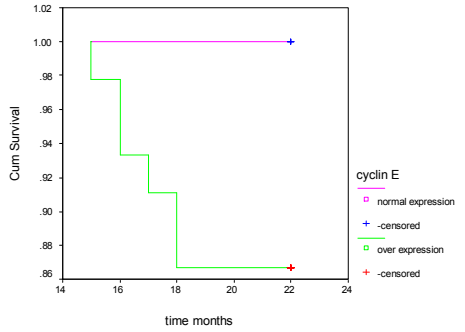

Fig (3): Overall survival in NHL patients in relation to Cyclin E expression at the end of the study

Kaplan-Meier analysis of overall survival in NHL patients in relation to Cyclin E expression shows significant short event free survival with Cyclin E over expression with (P=0.041) as shown in fig. 4.

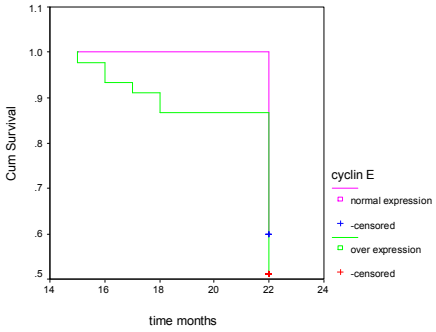

Fig. (4): Event free survival in NHL patients in relation to Cyclin E expression at the end of the study

Kaplan-Meier analysis of event free survival in NHL patients in relation to P27 expression shows significant short event free survival with P27 over expression with (P=0.03) as shown in fig.5.

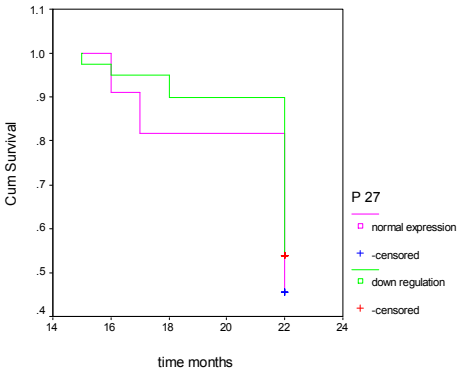

Fig. (5): Event free survival in NHL patients in relation to P27 expression at the end of the study

4. Discussion

A re-evaluation of parameters of cell cycle kinetics in view of our increasing knowledge of the molecular pathways of cell cycle control may provide more prognostic information for the management of patients with malignant lymphomas. Precise staging of NHL is prerequisite for the selection of a suitable therapeutic regiment and influence the likelihood of its success [1, 12].

The aim of the present work was to study the expression of the cell cycle regulators Cyclin E and P27 proteins by Western blot analysis on 40 newly diagnosed patients with NHL in different disease stages and to correlate their expression with the laboratory data, clinical manifestations, staging of NHL, and patient's survival.

In relation to the staging of NHL the present study showed that over expression of Cyclin E was significantly correlated with worsening of NHL outcome.

In agreement with this study **Keyomarsi *et al.*,** [13] and **Qi *et al.*,** [10] had observed a correlation between cyclin E expression, proliferation and aggressive staging. In contrast to this study **Farleya *et al.*,** [14] showed that expression of cyclin E was not associated with tumor stage.

In this study, the cyclin E expression in relation to sex was statistically not significant. On the other hand, the advancement of age of NHL patients was highly significantly associated with over expression of cyclin E. In agreement with this study **Xiangming *et al.*,** [15] showed that expression of cyclin E was related to the age of patients. In contrast, **Farleya *et al.*,** [14] showed that expression of cyclin E was not correlated with age.

As regards cyclin E expression and its relation to clinical data and laboratory findings, it was found that cyclin E over expression is significantly associated with worsening of all clinical and laboratory results. We agree in our results with **Ferreri *et al.,*** [16] who showed that high levels of cyclin E have been associated with advanced NHL manifestation.

Regarding the fate of the patients in relation to expression of cyclin E, our study demonstrated that over expression of cyclin E associated significantly with bad prognosis as delayed response to treatment, high rate of relapse and high incidence of mortality. The overall survival using Kaplan-Meier analysis in NHL patients in relation to cyclin E expression, showed significant short overall survival with Cyclin E over expression.

Event free survival showed significantly shorter event free survival with cyclin E over expressed patients. We agree in this with **Hayashi *et al.,*** [17] and **Keyomarsi *et al.,*** [13] who reported that all patients with a high level of cyclinE died. Also, **Keyomarsi *et al.,*** [13] and **Porter *et al.,*** [18] reported that the overall survival and the event-free survival were significantly longer among patients with low levels of cyclin E than among patients whose tumors had high levels of this protein. However, **Porter *et al.,*** [18] reported that high expression of cyclin E protein was not statistically significantly associated with either overall survival or event-free survival.

As regards p27 expression; down regulation of p27 in NHL patients was significantly associated with advanced staging of the disease. In agreement with our study, is the study of **Yasui *et al.,*** [19], who reported that down regulation of p27 expression significantly correlated with advanced stage, depth of tumor invasion and lymph node metastasis. These findings coincide also with those reported by **Xiangming *et al.,*** [15]; **Chiarle *et al.,*** [20]; **Khoo *et al.,*** [21]; **Moreira *et al.,*** [22] and **Porter *et al.,*** [18]. However, **Xiangming *et al.,*** [15]; **Gelen *et al.,*** [23] and **Filipits *et al.,*** [24] reported that p27 expression did not correlate with any of the clinic-pathological parameters examined including stage of the tumors, but **Xiangming *et al.,*** [15] reported that down regulation of p27 protein expression is age related and age has been reported as an important prognostic factor for malignant lymphomas.

Other authors as **Gelen *et al.,*** [23]; and **Filipits *et al.,*** [24] investigated the relationship between the expression of p27 and a series of clinico-pathological parameters including age and did not correlate p27 expression with any of the clinic-patholgical parameters examined.

Regarding the fate of the patients in relation to expression of p27, our study demonstrated that p27 down regulation is associated significantly with bad prognosis as delayed response to the treatment, high rate of relapse and high incidence of mortality.

Analysis of the overall survival in NHL patients in relation to p27 expression showed significant short overall survival with p27 down regulation. Analysis of event free survival also showed significant short event free survival with p27 down regulation.

In agreement with our results, are the results of **Yasui *et al.,*** [19], **Erlanson *et al.,*** [25] and **Moller *et al.,*** [26], who reported that reduced expression of p27 had been associated with aggressive tumor growth and predicted poor survival of patients. **Chiarle *et al.,*** [20] reported that total or partial loss of p27 appears to be a consistent feature of mantle cell lymphoma (MCL) and one that differentiates MCL from low-and even high-grade lymphomas, because p27 expression appears to correlate inversely with the cell proliferation index and the overall survival times.

Moreover, **Gelen *et al.,*** [23] reported that the presence of recurrence and relapse was more frequent in tumors with low p27. **Boudova *et al.,*** [3] reported that loss of p27 expression is a negative prognostic factor in the majority of B-Cell lymphomas. Also **Paik *et al.,*** [27] reported that p27 down regulation has been seated to be indicative of a poor prognosis in malignant lymphoma. **Porter *et al.,*** [18] reported that the expression of p27 protein was statistically significantly associated with overall survival and with event-free survival, and lower expression associated with poorer survival.

This work revealed that when p27 down regulation is accompanied with cyclin E overexpression this indicates advancing stage of the disease, delayed response to the treatment, increased possibility of relapse and recurrence and finally increased risk of death as indicated by short overall and event free survival among the patients. Several other authors reported that a high cyclin E level was shown to be a prognostic marker for poor prognosis, particularly when correlated with a low p27 level [10, 16, 18, 28, 29]

Conclusion:

P27 and cyclin E expression are important prognostic markers for lymphomas and, when combined with serum LDH levels, distinct prognostic subgroups of NHL patients can be defined. Modulation of p27 and cyclin E expression may be a potential therapeutic strategy to improve clinical outcome in patients with NHL in the future.

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Effect of Seasonal Temperature Changes on Thyroid Structure and Hormones Secretion of White Grouper (*Epinephelus Aeneus*) in Suez Gulf, Egypt

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Abstract: The thyroid is the largest and one of the phylogenetically oldest endocrine glands in vertebrate species. It is the first endocrine structure to become recognizable during an animal's development. Although the thyroid gland is structurally conserved in all vertebrate species, exhibiting a similar follicular structure and function. Seasonal temperature changes on the thyroid gland structure and hormones secretion was examined in white grouper; *Epinephelus aeneus* in Seuz Gulf, Egypt. 60 male of white grouper; *E. aeneus* (138.5±6.05 g) were netted from Suez Gulf during a year from July 2008 to June 2009. Water temperature and salinity were ranging from 12 to 34°C and 39 to 40 ppt during cold and warm seasons, respectively. Blood samples were collected from the caudal vein for thyroid hormones analysis. Samples of *E. aeneus* were dissected to expose the internal organs, histological examination and measuring the cell height of the thyroid epithelium. Thyroid gland composed of follicles scattered around the ventral aorta, near the gills. Follicular cells varied according to secretion of the gland during warm and cold seasons. Thyroid hormones [Triidothyronine (T_3) and Thyroxin (T_4)] were detected in the fish serum in levels ranged from 1.28-4.08 ng/ml for T_3 and 0.22-1.11 ng/ml for (T_4) in the warm and cold seasons, respectively. The results showed that the height of thyroid epithelium and plasma concentration of thyroid hormones (thyroid activity) in *Epinephelus aeneus* increased significantly during spring and summer. The peak of these factors occurred in midsummer (August). Then, the thyroid activity decreased significantly during autumn and early winter from October to December according to the decreasing of temperature. T_3 and T_4 increased significantly from January to April.
[Hossam H. Abbas, Mohammad M. Authman, Mona S. Zaki and Gamal F. Mohamed. **Effect of Seasonal Temperature Changes on Thyroid Structure and Hormones Secretion of White Grouper (*Epinephelus Aeneus*) in Suez Gulf, Egypt.** Life Sci J 2012;9(2):700-705]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 105

Key Words: White grouper, *Epinephelus aeneus*, Thyroid Gland, Triidothyronine,Thyroxin, Histology, Serum.

1. Introduction

The thyroid is the largest and one of the phylogenetically oldest endocrine glands in vertebrate species **(Dickhoff and Darling, 1983)**. It is the first endocrine structure to become recognizable during an animal's development. Although the thyroid gland is structurally conserved in all vertebrate species, exhibiting a similar follicular structure and function, there are some gross morphological differences among species, and the responses of this structure to environmental influences are also differ across the phylum **(Rupik, 2011)**.

Thyroid hormones (THs) include triiodothyronine (T_3) and thyroxin (T_4); are essential for regulating normal growth, development, differentiation, metabolism, and maintenance of normal physiological functions (e.g., homeostasis) in vertebrates **(Szisch *et al.*, 2005; Zoeller *et al.*, 2007; Schnitzler *et al.*, 2012)**. In all vertebrates embryogenesis, organogenesis and growth acutely depend on thyroid hormones **(Power *et al.*, 2001)**.

In fish, thyroid hormones are involved in the control of osmoregulation, metabolism, somatic growth and post-hatching metamorphosis **(Power *et al.*, 2001; Yamano, 2005; Schnitzler *et al.*, 2012)**. Although there is an extensive diversity in teleosts, developmental stages in most of them include larva, juvenile, and adult, which appear to regulate by THs **(Wright and Alves, 2001)**. As it seems, thyroid hormones (THs) involve in many physiological processes in teleosts. It has been suggested that photoperiod, temperature, and food intake may play species specific role in regulation of seasonal thyroid cycles **(Comeau *et al.*, 2000)**, and these seasonal changes may act to promote growth, migratory activity, and reproductive development **(Leatherland, 1994)**. It has been found that the changes of thyroid gland depend on species or population and are sensitive to food intake and diet composition models **(MacKenzie, 1998)**.

Groupers of the genus *Epinephelus* are widely distributed throughout the tropical and subtropical waters of the world. They are commercially important and highly regarded as a favorite marine food fish. The groupers possess excellent biological characteristics: they are fast-growing and disease resistant **(Yeh *et al.*, 2003)**. As no detailed study has been carried out on the thyroid patterns of *E. aeneus*, in Suez Gulf, Egypt, the present study was conducted on annual changes of the morphometric structure and hormones secretion of thyroid gland, triidothyronine (T_3) and thyroxin (T_4) in *E. aeneus*, in two seasons (cold and warm).

2. Material and Methods

Sampling:

60 male of white grouper; *E. aeneus* (138.5±6.05 g) were netted from Suez Gulf during a year from July 2008 to June 2009. Water temperature and salinity were ranging from 12 to 34°C and 39 to 40 ppt during cold and warm seasons, respectively.

Blood sampling:

Blood samples were collected from the caudal vein by a syringe with a little saturated solution of sodium citrate to prevent blood coagulation. The blood samples were kept on ice for up to 30 min and then, serum was separated using centrifuge (3000 rpm for 15 minutes) and frozen at -20°C for thyroid hormones analysis.

Thyroid gland histology:

E. aeneus was dissected to expose the internal organs and the jaws were cut at the corners to expose pharyngeal region. All tissues between the gills were fixed in Bouin's fixative for 72 hrs and then stored in 70% ethanol. Tissues were dehydrated using an ethanol series and embedded in paraffin **(Biswas *et al.*, 2006)**. Samples were then sectioned at 5-6µm and were stained with hematoxylin and eosin (H&E) for basic histological analyses. The cell height of the thyroid epithelium was measured under an Olympus microscope with a camera Lucida attachment according to **Halasz and Martin, 1985** in a total of 15 follicles per fish. Measurements were made at four points within each follicle at 90° from one another and reported as the mean ± SEM.

Thyroid hormones analysis

Triiodothyronine (T_3):

The gamma coat [^{125} I] T_3 Radioimmunoassay kit purchased from DiaSorin, Stillwater, Minnesota, USA was used for the quantitative determination of triiodothyronine (T_3) level in serum as previously described by **Van der Geyten *et al.* (2001)**.

Thyroxin (T_4):

The gamma coat [^{125} I] total T_4 Radioimmunoassay kit purchased from DiaSorin, Stillwater, Minnesota, and USA was applied for the determination of total thyroxin (T_4) levels in serum as previously described by **Van der Geyten *et al.* (2001)**.

Statistical analysis:

All values of thyroid hormone levels were represented as means±SE. The significant difference between warm and cold season values was analyzed using the t-test (Software Program of Statistical Analysis, **SPSS, 2008**).

3. Results

White grouper (*Epinephelus aeneus*) fish from Suez Gulf, Egypt is shown in figure (1) and inactive thyroid structure is shown in Photomicrograph (1) showing lobules, follicles and interlobular connective tissues of the thyroid gland.

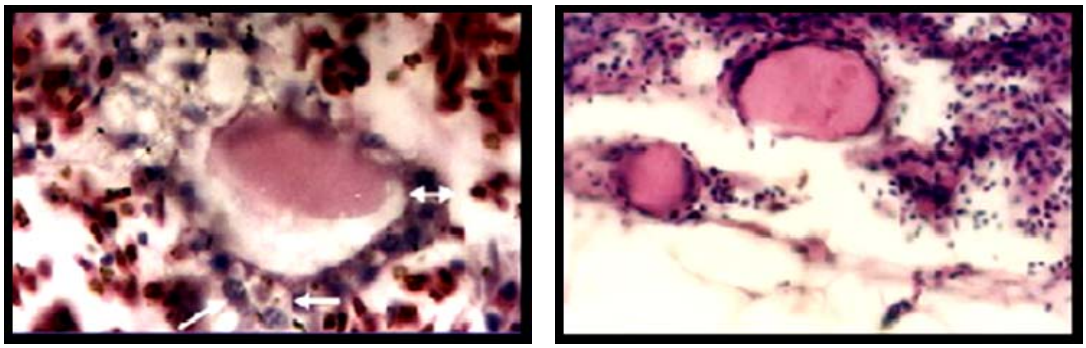
Structure of thyroid tissue:

The obtained results showed that the thyroid gland of *E. aeneus*, such as other teleosts is not capsulated. It was composed of follicles, which scattered throughout the pharyngeal region along with the dorsal surface of ventral aorta and bronchial arteries near the gills. The follicles were round and their walls were consisted of epithelial cells, include follicular cells and a few parafollicular cells, surrounding the central lumen full of colloid fluid. The epithelial cells were cuboidal to squamous during warm and cold seasons, respectively. The mean water temperature of Suez Gulf and the mean epithelial cell height for fishes during a year is shown in Table 1.

The results showed that there is 20% correlation between epithelial cell height and water temperature. Follicular epithelial cells had maximum height in August, then their height significantly decreased to January, after which it slowly increased throughout the winter (P<0.05). Fish thyroid gland was characterized by predominance of macrofollicles rich in colloid material during warm months (especially July to August) (Photomicrograph 1), whereas in cold months (especially October to December) thyroid gland showed some microfollicles with less colloid content and more interstitial connective tissue (Photomicrograph 1). There was a significant increase in ratio of parenchyma to stroma in summer in comparison with winter (P<0.05).

Seasonal changes of serum triidothyronine (T_3) and thyroxin (T_4): The results obtained with the RIA method are shown in Table 2. This method confirms that the serum level of T_3 and T_4 increased significantly from January to April, and again from April to June. This level was maintained up in summer and the peak of them in serum occurs during August (4.08±0.33 and 1.11±0.02 ng mL^{-1}, respectively), then declining significantly during autumn and early winter from October to December (P<0.05) to reach their lowest level in November (1.28±0.28 and 0.22±0.04 ng mL^{-1}, respectively). Both hormones varied similarly across seasons and there was 99% correlation (at the level of 0.01) between two hormones. The increasing of T_3 and T_4 were correlated with increase of temperature (98 and 82%, respectively) and with the height of thyroid epithelial cell.

Figure 1: Showing the white grouper (*Epinephelus aeneus*) from Suez Gulf, Egypt



Photomicrograph 1: Inactive thyroid follicle in December (left); Active thyroid follicle in August (right), Parafollicular cells (white arrows) and follicular epithelial height (two head arrows) (H&E 400X)

Table 1: Changes in heights of thyroid epithelial cells (Mean ± SE) according to the changes of water temperature during a year.

	Months											
	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sep.	Oct.	Nov.	Dec.
Water Temperature (°C)	12	14	19	25	28	31	33	34	32	24	21	15
Epithelial Height (µm)	1.75 ±0.7	1.95 ±0.6	2.25 ±0.4	2.50 ±0.5	2.80 ±0.8	3.30 ±0.7	3.62 ±0.6	3.71 ±0.4	3.41 ±0.4	2.51 ±0.5	1.58 ±0.8	1.37 ±0.4

-Data are represented as mean ± SE Y= 0.0491x + 2.8568 R^2 =0.0472

Table 2: Seasonal variations of the thyroid hormone concentrations (ng/ml) during a year in the White Grouper (Mean ± SE).

	Months											
	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sep.	Oct.	Nov.	Dec.
Water Temperature (°C)	12	14	19	25	28	31	33	34	32	24	21	15
T_3	2.57 ±0.23	2.77 ±0.30	2.94 ±0.31	3.38 ±0.21	3.51 ±0.33	3.62 ±0.25	3.96 ±0.21	4.08 ±0.33	3.78 ±0.18	1.44 ±0.19	1.28 ±0.28	1.64 ±0.26
T_4	0.62 ±0.05	0.73 ±0.03	0.79 ±0.06	0.81 ±0.02	0.86 ±0.08	0.87 ±0.04	1.04 ±0.02	1.11 ±0.02	0.96 ±0.03	0.35 ±0.04	0.22 ±0.04	0.37 ±0.03

-Data are represented as mean±SE Y= -0.0003x + 0.0018 R^2 =0.0018

4. Discussion

The synthesis of thyroid hormones (THs) occurs in the thyroid follicle, a single layer of epithelial cells enclosing a colloid-filled space and thyroxin (L-T_4) is the predominant hormone secreted. T_4 has few direct actions and is considered to act principally as a precursor for triiodothyronine (T_3), the biologically active form of the hormone **(Power *et al.*, 2001)**. The conversion of T_4 to T_3 occurs in the peripheral tissue by the enzymatic removal (5-monodeiodination of one of the iodide units of the outer ring of T_4. THs circulate in serum bound to thyroid hormone-binding proteins that include, albumin, transthyretin (TTR) and thyroxin-binding globulin in vertebrates **(Power *et al.*, 2000)**.

The present study showed that, thyroid gland of *E. aeneus* is not compact organ and is found in the subpharyngeal region, such as other teleosts. However, in some species thyroid follicles are found in heart, head kidney and kidney**.**

According to micrometric data, thyroid follicular cells of *E. aeneus* vary in size in cold and warm seasons. Also in Atlantic stingray, *Dasyatis Sabina*, follicular cells vary in size and shape, according to the activity of the gland **(Volkoff *et al.*, 1999)**. The surrounding epithelial cells are flattened, cuboidal, or columnar, depending on their activity. Tall, columnar epithelial cells with basophilic colloid containing vacuole-like spaces, characteristics of an active thyroid gland, were seen in warm season. In *Solea senegalensis*, thyroid represented colloid-filled follicles surrounded by a cuboidal epithelium during summer, suggesting a high activity state of this organ **(Ortiz-Delgado *et al.*, 2006)**.

Although seasonal cycle of thyroid hormones have been observed in numerous fish species, but the seasonal changes in thyroid hormones in *E. aeneus* have not been studied. Circulating thyroid hormone concentrations represent just one component of the multilevel control of target tissue metabolism by the hypothalamic-pituitary-thyroid axis. In the present study, significant monthly changes were observed in circulating levels of thyroid hormones in *E. aeneus* during a year. Thyroid hormones are a component of a large complex network of responses to a number of environmental and physiological factors, many of which also influence growth, development, and metabolism **(Hadley, 2000)**. They are involved in the regulation of energy management, functioning primarily to help control basal metabolic rate by regulating lipid metabolism **(Hadley, 2000)**.

Levels of thyroid hormones can be influenced by many factors including age, gender, diet, nutritional status, season and physiological condition **(Rolland, 2000; Schnitzler *et al.*, 2012)**. Stimuli such as the lunar cycle, rainfall, turbid water, temperature shock, chemicals, water quality, and swimming activity induce an increase in serum thyroid hormones concentration **(Iwata *et al.*, 2003)**. **Swift (1960)** suggested that the seasonal changes in thyroidal activity in many teleosts are regulated primarily by water temperature. This relationship of glandular activity and water temperature is interpreted as further evidence that the basic function of the thyroid is concerned in the control of the animal's metabolism, to compensate for changes in the environmental temperature. Thus the release of thyrotropic hormone from the pituitary would seem to be influenced by the environmental temperature. Serum levels of thyroid hormones were sensitive to temperature in starved eels *Anguilla anguilla* L. **(Leloup and De Luze, 1985)** and also in trout fed specific diets **(Latherland *et al.*, 1980)**. In the present study, mean serum T_3 and T_4 showed similar seasonal changes patterns. Both hormones decreased significantly during autumn and early winter from October to December according to decrease of temperatures, feed consumption and somatic growth.

In general, fasting and food restriction decrease both T_3 and T_4 levels in most animals **(Janan *et al.*, 1995)**. **Loter *et al.* (2007)** also reported minimum thyroid hormones in cold months. T_3 and T_4 increased significantly from January to April, and again from April to July. Thyroid activity increase in the winter corresponds with intermediate temperatures and feed consumption during rapid reproductive development and spawning period of *E. aeneus*.

E. aeneus spawns during late winter and early spring **(Abou-Seedo *et al.*, 2003)**. In normal diploid catfish, *Heteropneustes fossilis*, a general inverse relationship between thyroid hormone levels and advanced reproductive state has been observed **(Cyr *et al.*, 1988)**, which suggested involvement of thyroid hormones in reproductive maturity. **Weber *et al.* (1992)** found that accumulation of thyroid hormones into oocytes of tilapia, *Oreochromis mossambicus*, was against its concentration gradient, which could be a reason for depletion of thyroid in serum of normal diploid female specimens during the spawning period.

Increase of T_3 and T_4 serum concentrations in spring coincides with increasing ambient temperature but the results of the present study showed that the peak activity occurs during midsummer when temperature increase precipitously from July to September with elevating of feed consumption and somatic growth. These requirements vary seasonally in a poikilothermic animal such as a fish, increasing with the rising temperature of the water in summer and decreasing in winter **(Swift, 1955)**. **Loter *et al.* (2007)** reported that increased both T_4 substrate availability (higher serum T_4 levels) and increased temperature would lead to much greater enzyme activity and T_3 production in summer. In summary, the activities of the hepatic thyroid hormones deiodination pathways appear to be regulated to provide a much greater availability of T_3 in summer, when fish are eating and

growing most actively, than in winter **(Loter *et al.*, 2007)**. Decreased food consumption during cold season may depress thyroid hormone cycles in many fishes. The seasonal trend is consistent with the hypothesis that thyroid hormone production is activated during periods of increased nutrient assimilation **(MacKenzie *et al.*, 1998)**.

Conclusion

All together, high magnitude seasonal changes of thyroid hormones in *E. aeneus* suggest that this species provides an excellent opportunity to examine the relative contributions of the generation mechanisms of dynamic cycles in circulating thyroid hormone levels. This study was designed to determine basal concentrations of thyroid hormone in *E. aeneus*, utilizing assays which have been validated for this species. The relationships between these hormones and food deprivation, reproductive state, other circulating hormones, immunoglobulins and contaminants can now be identified by further investigations.

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Metabolic syndrome and risk of Coronary Artery Disease in west of Iran

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Abstract: A major concern about MS (Metabolic Syndrome) and CAD (Coronary Artery Disease) is that patients with these defects are at higher risks of mortality and morbidity due to a combination of MS risk factors. The purpose of study was to examine the differences between CAD and non-CAD patients regarding their MS components and selected lifestyle behaviors (i.e., dietary intake, physical activity patterns, and smoking habits) and there was an attempt to determine whether MS was an independent risk factor for CAD among the patients. The study used case-control methodology for collection and analysis of the data. 600 participants recruited for study. CLR was applied to quantify the odds Ratio (OR) of CAD associated with MS and its components and other life style risk factors of CAD. MS increased the risk of CAD 4.19 times significantly (OR=4.19, 95%CI=2.603-6.47, P= 0.0001). Multivariate analysis showed that MS conveyed no additional predictive information beyond its components (odds Ratio=0.81, p=0.6). The focus of physicians should be treatment of individual CAD risk factors, using the metabolic syndrome will not improve prediction of CAD as compared with detailed information on individual CAD risk factors.

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Keywords: Metabolic syndrome; Coronary artery disease; Risk factor; Iran

1. Introduction

Metabolic Syndrome (MS) also known as syndrome X, is comprised of hypertension, glucose intolerance, high triglycerides, and decreased level of high density lipoproteins (HDL). These defects in the body are considered as risk factors of cardiovascular diseases (Reaven, 1988). MS is also at time referred to as the Deadly Quartet, the Dysmetabolic Syndrome, and Insulin Resistance Syndrome. The above mentioned MS risk factors are considered as the main causes of development and progression of atherosclerosis, which eventually results in a higher risk for coronary artery diseases. MS components have also been associated with a higher risk for diabetes type 2. Generally, there is no single cause for MS; however, abdominal obesity and insulin resistance are the most important risk factors for this disease. Other underlying risk factors that can increase the risk of MS include physical inactivity, aging, hormonal imbalance, and race (Grundy, 2005). Previous research has shown that the MS confers an approximately twofold increase in relative risk for coronary vascular events. This implies that MS brings a relatively high risk for both CVD and diabetes (Stern et al., 1995). For the sake of brevity and clarity, I have classified the previous studies on MS into five categories. The first group of studies have highlighted the wide range of MS prevalence globally (e.g., Ford, 2002) and regionally including the latest statistics of the disease in Iran by Azizi (2004) and Ghayour-Mobarhan (2007). A second group of studies have emphasized the independent CAD prediction role for MS (e.g., Hunt, 2004; Lakka, 2002; Sadeghi, 2006; Chen, 2008). A third group of studies on MS show the biomolecular basis of MS components and their atherogenicity (Villena, 2004; Ginsberg, 2006; Szapary, 2004). The effect of dietary factors, as well as physical factors on the risk of MS have been emphasized by these studies (see Clark SD, 2000; Maki, 2004; Haffner, 2007). A fourth stream of studies (e.g., Laaksonen, 2002; Lee, 2005) has included life style factors (e.g., physical

activity, smoking habit, etc.) as influential on the risk of being involved in MS. Finally, the fifth category of studies (e.g., Ford, 2005; Ghayour, 2007; Azizi, 2004; Sadeghi, 2006) has suggested the need to explore the problem in different populations as a multivariable model.

The results of these studies have shown that patients with MS have a higher risk of developing CVD mortality & morbidity (Isoma et al. 2001, Chen et al., 2008). The main reason for this association is that a combination of risk factors concomitant with MS interacts synergistically with several other factors thereby causing or accelerating the progression of atherosclerosis (Isoma et al., 2002). It has been found that individuals with one or two of the MS components are at a two-fold greater risk of CAD and CAD mortality (Gorter, 2004; MacNeill et al., 2004). Similar studies have shown that the coronary artery disease is the leading cause of death in general population in Iran (Azizi et al., 2004) Although a strong link between MS and coronary artery disease (CAD) has not been well documented (Petra et al., 2004); studies investigating MS (Maki et al., 2004; Lee et al., 2005; Niaura et al., 2000; Raikkonen et al., 2002) have shown that MS can be influenced by biological, behavioral, and social factors which have already been identified as factors affecting the development of CAD. Likewise, The results of a few studies conducted on prevalence of MS and its risk factors in general population in Iran (Zabetian et al., 2007; Azizi et al., 2003) have suggested that MS factors can trigger CAD development although there is little knowledge determination of risk factors of MS in high risk patents in Iran.

Azizi et al., (2003) and Zabetian et al. (2007) note that over the past years general population in Iran have experienced rapid life style changes with drastic reductions in physical activity and increase in consumption of processed food, resulting in an epidemic of obesity and diabetes. The results of these studies indicate that lifestyle changes and dietary habits that carry the risk of MS, can also lead to aggravation of CAD patients' condition; however, research on life style screening for MS risk factors in CAD patients in the setting of this study-Iran has been scarce. Review of the literature on metabolic syndrome demonstrates that MS is a prevalent syndrome both throughout the world and in Iran (Azizi et al., 2004). As discussed earlier in this chapter, the noteworthiness of the epidemiologic studies on MS in Iran is that the prevalence of this syndrome is considerably higher in this region than the world rate. As a result of the combined effect of risk factors of MS, we know that the prevalence of MS is increasing parallel to the trend in overweight and obesity (Azizi et al., 2004). Generally, the prevalence of MS increases with age and its prevalence are considerably different among races and ethnic groups, which supports the probable impact of genetic predisposition. Based on the reviewed literature, it becomes evident that the genetic factors as well as the social, environmental, psychological, and behavioral variable are linked to this clinical syndrome to some extent although the direct association of these factors with CAD is still in need of further research. Previous studies (Azizi et al., 2004; Zabetian et al., 2007) have recommended a strong need for more studies to explain the interrelation between MS components and their association with CAD.

Numerous studies have focused on MS in CAD patients. Most of these studies consider MS as an independent predictor of CAD. Lakka (2002) states that individuals with metabolic syndrome are at increased risk for CAD. Similarly, Kragelund et al., (2007) showed that patients with positive exercise electrocardiogram or myocardial radionuclide imaging can suggest ischemia. They also concluded that the metabolic syndrome in women with stable coronary heart disease provides considerable prognostic information on all-cause mortality; however they did not find the same results in men. It is noticeable that this association was independent of diabetes. The invasive measurements of severity of coronary artery disease and left ventricular function, made this study unique in comparison with previous studies of metabolic syndrome and prognosis. In Iran, Zabetian and colleagues (2007) extended the previous knowledge about the value of MS as a risk factor in the general population to patients with stable coronary disease by emphasizing the prognostic significance of MS in women.

Chen and colleagues (2008) investigating the relationship between MS and CAD in elderly concluded that the CAD–MS patients showed a higher prevalence of multivessel disease, unstable lesions and needed more revascularization procedures than the simple CAD patients. Their study also determined that the prevalence of CAD and the number of blocked coronary vessels were directly correlated with MS their findings suggested that MS as a predictor of the prevalence and extent of future CAD in the elderly. Iribarren (2006) examining the association between the metabolic syndrome (MS) and early-onset coronary artery disease (CAD) concluded that the presence of ATP-III MS without diabetes and with diabetes was a strong independent determinant of early-onset CAD, but neither definition of MS remained significantly associated with early-onset CAD in multivariate models adjusting for individual components. Thus, Iribarren (2006) concluded that the MS is a risk factor of early-

onset clinical CAD, but the prognostic information associated with the syndrome was not greater than the sum of its parts. Numerous studies by other investigators have reached similar conclusions. For example, blood pressure, HDL cholesterol, and diabetes, but not presence of MS, were significant multivariate predictors of prevalent CAD in an analysis of the Third National Health and Nutrition Examination Survey (Alexander et al., 2003). In the Caerphilly and Speedwell population studies, the excess of ischemic heart disease risk associated with MS was no greater than that can be explained by individual effects of the defining variables in a multiple logistic model (Yarnell et al., 1998).

2. Material and Methods

The National Cholesterol Education Program (NCEP)-Adult Treatment Panel (ATP) has introduced one of the most widely accepted diagnostic criteria for the diagnosis of MS. This criteria works on some measurements including waist circumference, serum triglyceride (TG), HDL-C, Blood pressure (BP), and fasting blood sugar (FBS). Recently, as illustrated below the American Heart Association/National Heart Lung & Blood Institute (AHA/NHLBI) has made some minor modifications on the NECP criteria which is currently used in research focusing on MS and its association with Coronary Artery Diseases (CAD) (Grundy, 2005).

1. Waist circumference > 88 cm in women and >102 in men
2. Fasting triglycerides ≥ 150 mg/dL or medication for treatment
3. HDL cholesterol < 50 mg/dL in men and <40 in women or medication for treatment
4. Hypertension (systolic blood pressure ≥ 130 mm Hg, diastolic blood pressure ≥ 85 mm Hg or medication for treatment
5. Fasting glucose ≥100 mg/dL or medication for treatment

The elective coronary angiography was performed using Judkin's approach which has been used by several studies (e.g., Chen, et al., 2007; Jong-youn et al., 2010; Ertek et al., 2010). CAD was defined as > 50% luminal diameter stenosis of at least one major epicardial coronary artery.

All statistical analyses were performed using SPSS version 17 and STATA11. Descriptive statistics such as frequencies, percentages, means, ranges and standard deviations were used to describe the data. The Coronary artery disease was considered as the dependent variable, while all other variables were analyzed as independent variables. After checking for normality, for normally distributed continuous variables Paired Samples t-test was used to determine the differences between the case and control groups. For non-normally distributed continuous variables, the Wilcoxton matched pairs signed rank test was used. McNemar's test (sometimes called McNemar's test of symmetry or McNemar symmetry chi-square) was used to determine the association between categorical variables with the CAD. $p < 0.05$ was used as level of significance. Conditional logistic regression was then applied to quantify the relative odds of CAD associated with MS and its components and other life style risk factors of CAD (The Mantel-Haenszel test could be assessed in the normal stratified analysis method but with the many strata, stratification produces sparse data the CLR algorithm is designed to handle sparse data). Conditional logistic regression (CLR) was used to decide whether MS is a risk factor of CAD independently and quantify the association between CAD and its risk factors via multivariate modeling .seven sequential models were fitted: first a model with no adjustment for covariates (gender and age are adjusted for by design); second a model adjusting for covariates external to MS including smoking and body mass index. In model 3, to ascertain the prognostic importance of the MS above and beyond its components, we then added each of the 5 individual components categorically defined (i.e., all components included in the same model) with CAD. In model 4, we added smoking and BMI to model 3. In model 5,6 and 7, we used (step wised automatically technique), so all the variables added in model if they have related to CAD with p-value <0.2, one time without and other time with BMI.

3. Results

Patients suffering from metabolic syndrome (cases) were identified and compared with the controls. As shown in table 1, abdominal obesity is prevalent in 59.9% (178) of the cases and 46.5%) (138of controls have. High blood pressure was seen among 90.6% (269) of the cases and 79.1 % (234) of controls. High fasting blood glucose or using medication for that were in Sixty five percent (193) of the cases and 45.4% (134) of the controls had high fasting blood glucose or were using medications for that. High triglycerides were observed in the medical profile of 240(80.8%) of the cases and 193(65.6%) of the controls. Low HDL-cholesterol were seen in 236(80.0%) of the cases and 179(60.5%) of the controls. None of the cases showed zero components while only 6(2.0%) of the controls were found with this characteristic. A total of 258(87.5%) of the cases and 190(64.6%) of the controls were found to have metabolic syndrome disease. Seven (2.4%) of cases and 32(10.9%) of the controls had one component of MS. Thirty (10.2%) of the cases and 66(22.4%) of the controls had two MS components. Seventy-one (24.1%) of the cases and 79 (26.9%) of the controls had three components of MS. There were four MS

components in 107(36.3%) of the cases and 83(28.2%) of the controls. There were five MS components in 80(27.1%) of the cases and 28(9.5%) of the controls.

Table 1. Distributionof cases and controls according to Biochemical Data (N=594)

Biochemical analysis	Case		Control	
	n	%	n	%
Total Cholesterol:				
Desirable (<200 mg/dL)	218	73.4	242	81.5
Borderline High (200-239)	50	16.8	43	14.5
High (≥240)	29	9.8	12	4.0
Triglyceride Serum level:				
Normal (<150 mg/dL)	75	25.3	126	42.4
Borderline High (150-199 mg/dL)	105	35.4	95	32.0
High (200-499)	115	38.7	76	25.6
Very high ≥ 500	2	7	0	0
HDL cholesterol:				
Low(<40MG/dL in men,<50 women)	210	70.7	159	53.5
Between low and high	74	24.9	115	38.7
High(>60 mg/dL)	13	4.4	23	7.7
FBS:				
High(≥100)	185	58.7	130	41.3

3.1 MS Components and CAD Risk

Odds ratios of MS components related to CAD are shown in Table 2.

3.1.1 Waist circumference

There were significant positive association between increased waist circumference (OR=2.48, 95% CI =1.56-4.03, P=0.00) and risk of CAD.

3.1.2 Triglycerides and Drug Use

Increased TG (or using medication for that) increased the risk of CAD significantly (OR=2.41, 95% CI =1.57-3.80, P=0.00).

3.1.3 Blood pressure and Drug Use

Increased BP level or using medication for that (OR=2.47, 95% CI=1.50-4.21, P=0.00) increased the risk of CAD significantly.

3.1.4 HDL cholesterol and Drug Use

Decreased HDL-cholesterol or using medication for that increase the risk of CAD significantly (OR=2.70, 95% CI =1.80-4.13, P=0.00).

3.1.5 Fasting blood sugar and Drug Use

Increased FBS level or using medication for that (OR=2.23, 95% CI =1.56-3.22, P=0.00) increased the risk of CAD significantly.

3.2 Number of MS Components

It was also found out that patients with 3, 4, or 5 components of MS increased the risk of CAD significantly with odd ratios of (OR=2.68, 95% CI =1.56-4.61, P=0.00), OR=4.47, 95% CI =2.58-7.74, P=0.00), and (OR=13.08, 95% CI =6.37-26.83, P=0.00), respectively. Finally, MS with ATPIII criteria increased the risk of CAD 4.19 times significantly (OR=4.19, 95%CI=2.603-6.47, P=0.0001).

3.3 MS as an Independent Risk Factor for CAD

Another research question asked if MS was an independent risk factor for CAD among the patients. Test of Independency of Metabolic Syndrome as CAD risk factor was conducted through multivariate modeling, the results of which are presented in the following sections. Conditional logistic regression was conducted to quantify the risk of CAD associated with metabolic syndrome after adjusting for other potential risk factors that were assessed in this study.

Seven sequential models were developed: In the first model, with no adjustment for covariates (age and gender adjusted for by design), before adjusting for external risk factors, the presence of the MS by ATP-III (2005) conferred an almost 4-fold increase in the risk of CAD (odds ratio=4.19, p=0.00).

In model 2, adjustments were made for the two main covariates external to MS which included smoking and body mass index. After adjusting for these external risk factors, the presence of MS by ATP-III conferred an almost 3.5-fold increase in the odds of CAD (odds ratio=3.52, p=0.00).

In model 3, to ascertain the prognostic importance of the MS above and beyond its components, each of the 5 individual components were added with CAD. Multivariate analysis showed that MS conveyed no additional predictive information beyond its components (odds Ratio=0.81, p=0.6).

Table 2. Association between Daily Calorie and Nutriens Intake and Coronary Artery Disease (N=594)

Cal and nutrients	percentile	Odds Ratio	95%CI	P	Prob>Chi2
Energy(kcal)	<25	1.00			
	25-50	0.74	0.46-1.21	0.23	
	50-75	0.69	0.43-1.13	0.14	0.460
	>75	0.74	0.46-4.18	0.20	
Protein (g)	<25	1.00			
	25-50	0.92	0.58-1.46	0.74	
	50-75	0.73	0.45-1.16	0.18	0.190
	> 75	1.19	0.77-1.83	0.42	
Carbohydrate(g)	<25	1.00			
	25-50	**0.41**	0.25-0.69	**0.000****	
	50-75	**0.68**	0.42-1.11	**0.01***	0.006
	> 75	**0.55**	0.33-0.89	**0.01***	
Fat(g)	<25	1.00			
	25-50	0.79	0.50-1.24	0.31	
	50-75	0.69	0.43-1.11	0.12	0.330
	> 75	0.67	0.42-1.06	0.09	
Cholesterol(mg)	<25	1.00			
	25-50	1.07	0.68-1.69	0.75	
	50-75	1.16	0.73-1.85	0.52	0.781
	> 75	0.92	0.59-1.42	0.71	
SFA(g)	<25	1.00			
	25-50	0.90	0.56-1.44	0.67	
	50-75	1.06	0.66-1.69	0.79	0.909
	> 75	0.92	0.58-1.46	0.74	
MUFA(g)	<25	1.00			
	25-50	1.07	0.68-1.70	0.75	
	50-75	0.84	0.53-1.32	0.46	0.674
	> 75	0.89	0.56-1.42	0.63	
PUFA(g)	<25	1.00			
	25-50	1.24	0.78-1.98	0.35	
	50-75	0.72	0.45-1.13	0.16	0.025
	> 75	0.65	0.41-1.04	0.07	
Fiber(g)	<25	1.00			
	25-50	0.96	0.60-1.52	0.86	
	50-75	0.66	0.41-1.05	0.08	0.288
	> 75	0.82	0.53-1.26	0.38	
Na(mg)	<25	1.00			
	25-50	0.75	0.48-1.18	0.22	
	50-75	0.92	0.56-1.51	0.75	0.230
	> 75	**1.21**	0.75-1.94	**0.02***	
K(mg)	<25	1.00			
	25-50	0.95	0.60-1.49	0.83	
	50-75	0.81	0.51-1.27	0.36	0.820
	> 75	0.93	0.59-1.47	0.77	
Iron(mg)	<25	1.00			
	25-50	0.65	0.41-1.04	0.07	
	50-75	0.64	0.39-1.04	0.07	0.220
	> 75	0.83	0.52-1.33	0.43	
Zn(mg)	<25	1.00			
	25-50	1.23	0.79-1.92	0.94	
	50-75	0.76	0.47-1.22	0.63	0.050
	> 75	1.42	0.91-2.22	0.68	

Vit E(µg)	<25	1.00			
	25-50	0.98	0.62-1.54	0.94	
	50-75	1.11	0.71-1.73	0.63	0.860
	> 75	0.91	0.59-1.41	0.68	
Vit A(µg)	<25	1.00			
	25-50	0.89	0.56-1.43	0.65	
	50-75	1.08	0.69-1.69	0.72	0.838
	> 75	0.9	0.57-1.41	0.65	
Vit C(mg)	<25	1.00			
	25-50	1.12	0.71-1.78	0.60	
	50-75	1.12	0.70-1.78	0.61	0.907
	> 75	0.99	0.62-1.56	0.97	
Oleic Acid(g)	<25	1.00			
	25-50	0.83	0.51-1.35	0.46	
	50-75	0.75	0.45-1.23	0.26	0.630
	> 75	0.76	0.48-1.21	0.25	
Linoleic Acid(g)	<25	1.00			
	25-50	1.17	0.74-1.85	0.98	
	50-75	0.70	0.44-1.11	0.13	0.069
	> 75	0.68	0.43-1.08	0.10	
Linolenic Acid(g)	<25	1.00			
	25-50	1.35	0.84-2.17	0.26	
	50-75	1.09	0.69-1.72	0.70	0.497
	> 75	0.97	0.61-1.54	0.90	

*Correlation is significant at the 0.05 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed).

In model 4, smoking and BMI were added to model three. MS odds ratio still remained with no additional predictive information (odds ratio=0.68, p=0.3).

In model 5 and 6, step wise automatically was used, and all the variables with significant (p-value <0.2) associations with CAD were added to the model once with and once without considering BMI. In both models (with and without BMI), the presence of MS by ATP-III conferred an almost 3.5 till 3.7-fold increase in the odds of CAD (with BMI odds ratio=3.52, p=0.00, and without BMI odds ratio=3.76, p=0.00).

In model 7, to ascertain the prognostic importance of the MS above and beyond its components, each of the 5 individual components were added to the last model with stepwise variables. Multivariate analysis showed that MS conveyed no additional predictive information beyond its components (Odds Ratio=0.53, p=0.3).

In addition to the previous models, hierarchical Conditional (fixed-effects) logistic regression models were also add to these models. In these two hierarchical models (see Hierarchy 1 & 2, next pages), all the variables with significant (p-value <0.2) associations with CAD were added to previous models. Additionally, in the second model, to ascertain the prognostic importance of the MS above and beyond its components, each of the five individual components were added to the first model. The analysis showed that in first model the presence of MS by ATP-III conferred an almost 3.67 -fold increase in the odds of CAD, p=0.00. Multivariate analysis showed that MS conveyed no additional predictive information beyond its components (odds Ratio=0.33, p=0.1) in second model.

4. Discussions

4.1 MS in CAD Patients

Based on the ATP III (revised by the AHA/NHLBI, 2005) guidelines, abdominal obesity was prevalent in 59.9% (178) of the cases and 46.5% (138) of the controls. High triglycerides were observed in the medical profile of 240(80.8%) of the cases and 193(65.6%) of the controls. Low HDL-cholesterol were seen in 236(80.0%) of the cases and 179(60.5%) of the controls. High blood pressure was evident among 90.6% (269) of the cases and 79.1 % (234) of the controls. High fasting blood glucose or using medication for that was observed in 65% (193)of the cases and 45.4%(134) of the controls had high fasting blood glucose or were using medications for that (as shown in Table 3).

Table 3. Metabolic Syndrome Data (ATPIII, AHA/NHLBI* 2005, N=594)

Metabolic Syndrome	Case(n=297)		Control(n=297)	
	n	%	n	%
Abdominal obesity**	178	59.9	138	46.5
Blood pressure ≥130/85 mmHg or using drugs	269	90.6	234	79.1
Fasting blood glucose ≥100 mg/dl or using drugs	193	65.0	134	45.4
Triglycerides ≥150 mg/dl or using drugs	240	80.8	193	65.6
Low HDL-cholesterol <50 mg/dl in women,<40 mg/dl in men or using drugs	236	80.0	179	60.5
Zero component	0.0	0.0	6	2.0
One component	7	2.4	32	10.9
Two components	30	10.2	66	22.4
Three components	71	24. 1	79	26.9
Four components	107	36.3	83	28.2
Five components	80	27.1	28	9.5
Defined metabolic syndrome	258	87.5	190	64.6

*NCEP/ATP The National Cholesterol Education Program-Adult Treatment Panel modified by AHA/NHLBI American Heart Association/National Heart Lung & Blood Institute .

** Abdominal obesity: waist circumference >88 cm for women,>102cm for men

The reasons for the occurrence of these abnormalities and the possible causes for their creation have been discussed in the preceding sections. Among the cases no one was found with zero components while only 6(2.0%) of the controls were found to be characterized as zero-component. Overall, a total of 258(87.5%) of the cases and 190(64.6%) of the controls were found to have metabolic syndrome disease. participants with one component comprised 7 (2.4%) of the cases and 32(10.9%) of the controls. Thirty (10.2%) of the cases and 66 (22.4%) of the controls had two MS components. Seventy-one (24.1%) of the cases and 79 (26.9%) of the controls had three components of MS. Four-component participants comprised 107(36.3%) of the cases and 83(28.2%) of the controls. Finally, 80(27.1%) of the cases and 28(9.5%) of the controls were identified as having five MS components.

The analysis showed that the highest number among both cases and controls were involved with all five components of MS and fewer patients were identified suffering from a single component. Thus, it might be wise to conclude that MS components are less often developed independently of each other. In other words, MS victims are more likely to be interacted with more than one of its components. These results might also be indicative of a close interaction between different components of MS and their possible synergistic effects on the target patients. Such an understanding of the behavior of MS components among susceptible CAD patients can provide insights into the study of MS and CAD as clinical entities in general and shed light on timely diagnosis and treatment of coronary artery diseases, in particular.

4.2 Individual MS Components and CAD Risk

One of the most widely accepted diagnostic criteria for the diagnosis of MS has been introduced by the National Cholesterol Education Program (NCEP) also called Adult Treatment Panel (ATP), which works on some measurements including waist circumference, serum triglyceride (TG), HDL-C, Blood Pressure (BP), and Fasting Blood Sugar (FBS). Recently some minor modifications have later been made to the NECP criteria by the American Heart Association/National Heart Lung & Blood Institute (AHA/NHLBI), on which is currently used in research focusing on MS and its association with Coronary Artery Diseases (CAD) (Grundy, 2005). The ATP III criteria for the metabolic syndrome have been widely used in both clinical practice and epidemiological studies. An advantage of these diagnostic criteria is that it avoids emphasis on a single cause. The AHA and NHLBI affirm the overall utility and validity of the ATP III criteria and suggest that they continue to be used with minor modifications and clarifications. These modifications and clarifications include allowing for adjustment of waist circumference to lower thresholds when individuals or ethnic groups are prone to insulin resistance; allowing triglycerides, HDL-C levels, and blood pressure to be counted as abnormal when a person is taking drug treatment for these factors; clarifying that the definition of elevated blood pressure is a level that exceeds the threshold for either systolic or diastolic pressure; and reducing the threshold for counting elevated fasting glucose from 110 mg/dL to 100 mg/dL, in accordance with the American Diabetes Association's (ADA's) revised definition of impaired fasting glucose (IFG).

The results of applying these criteria in measurement of MS components this study revealed that the prevalence of these components among the cases were: BP (90.6%), TG (80.8%), HDL-C (80.0%), FBS (65%) and WC (59.9 %) while the frequency of observed MS components in controls were: BP (79.1%), TG (65.6%), HDL-C (60.5%), WC (46.5%), and FBS (45.4%). These results indicate that BP is the most prevalent MS component among the patients followed by TG and HDL-C; however, BP is more prominent among the cases than the controls. This can be due to the fact that there are several factors that may be the cause for high BP among the participants. The participants' low socioeconomic status, lack of knowledge about healthy diet, low levels of literacy and education, and large numbers kids in the family can give rise to unhealthy diet and obesity among the participants. Since hypertension is a major risk factor for CAD, its control in the community should be integrated into a comprehensive preventive program for CAD control. Therefore, screening for high blood pressure, in addition to patients' adherence to the medical management of controlling blood pressure can also is effective in reducing the incidence of the development of CAD.

More specifically, the results of examining the association of PB to CAD showed that increased BP level or using medication for that (OR=2.47, 95% CI =1.50-4.21, P=0.00) increased the risk of CAD significantly. Based on ATPIII criteria, 65.75% (195) of cases and 54.2 % (161) of controls had high Systolic BP, while 42% (125) of the cases and 16% (48) the controls had high Diastolic BP. Increased systolic (OR=1.78, 95%; CI=1.26-2.51; P=0.00) and diastolic blood pressure (OR=3.5, 95% CI=2.22-5.50, P=0.00) increased the risk of CAD significantly.

The data also showed that having history of using drugs for blood pressure significantly increases the risk of CAD. Although patients who used to take medication for lowering PB had normal BP at the time of blood collection, indeed they used to have elevated BP based on their medical profile. The odds ratio (OR=1.93) and 95% CI (1.34-2.77) for the use of blood pressure drugs in association with their risk of CAD development were both statistically significant (P=0.001).

Additionally, the effects of having high triglycerides (TG) or using medication for that on the risk of CAD observed through the medical profile of the patients indicated that increased TG or using medication for that increased the risk of CAD significantly (OR=2.41, 95% CI =1.57-3.80, P=0.00). It is worth noting that 35% of cases and 22% of controls used lipid lowering medications that decreased TG. The highest risk of CAD was recorded for high and very high categories (OR=2.85, 95% CI =1.82-4.47, P=0.0001) of TG suggesting that the higher the level of TG in patients, the greater the risk of CAD. Similarly, history of using blood lipid drugs that increase HDL as well as previous history of using blood lipid drugs that decrease TG put the patients at significantly greater risk of CAD both (OR=1.80, 95% CI=1.25-2.58, P=0.001). The findings are also supported by the studies by Washio et al. (2001) and Fava et al. (2008) who indicated that elevated fasting triglycerides level is a risk factor for CAD and CVD, and it works independently from other risk factors. Therefore, from a public health perspective it is not enough to focus only on serum triglyceride levels to decrease the burden of CAD in any population. It appears that reduction and /or modification in serum lipids and lipoproteins all together in addition to other risk factors could beneficial.

Low HDL-cholesterol or using medication for that were seen in 236(80.0%) of the cases and 179 (60.5%) of the controls. Lower HDL cholesterol concentrations were 70.7% and 4.4% and higher HDL cholesterol levels were 53.5% and 7.7% in cases and controls, respectively. It was found that decreased HDL-cholesterol or using medication for that increases the risk of CAD significantly (OR=2.70, 95% CI =1.80-4.13, P=0.00). More specifically, the results showed that decreased HDL-C approximately doubled the risk of CAD.

High fasting blood glucose or using medication for that were observed in 65%(193) of the cases and 45.4%(134) of the controls. The results indicated that increased FBS level (more than 100 mg/dL) or using medication for that (OR=2.23, 95% CI =1.56-3.22, P=0.00) increased the risk of CAD significantly. High FBS levels were 185(58.7%) and 130(41.3%) in the cases and controls, respectively (See Table 4). The odds ratio and 95% CI for the use of blood sugar drugs in association with their risk of CAD development were OR=5, 95% CI =2.69-9.29, P=0.001 respectively. Increased FBS is caused due to several factors including obesity. In this study the relatively high prevalence of high FBS may be due the impact of increased sedentary lifestyle and high prevalence of obesity. Based on the fact that the cause of CAD is multifactorial, diabetes mellitus is just one of the contributors. Therefore, there is a need to promote health awareness among the population with an emphasis on controlling and carrying out periodic check up of blood sugar.

Table 4. Biochemical Data (N=594)

Biochemicals	Case			Control			z or t	P
	Mean	95% CI	SD	Mean	95% CI	SD		
Total cholesterol mg/dl	178.48	173.54-183.41	42.67	163.92	159.05-168.80	42.67	t= -4.20	**0.000****
Triglyceride mg/dl	195.09	186.31-203.88	76.93	169.16	160.63-177.68	74.66	Z= -4.94	**0.000****
HDL mg/dl	39.71	38.25-40.89	10.40	44.10	42.92-45.29	10.35	Z= -5.09	**0.000****
FBS mg/dl	124.32	118.67-129.45	46.80	106.28	100.98-111.59	46.46	t= -4.76	**0.000****

*Difference is significant at the 0.05 level (2-tailed).**Difference is significant at the 0.01 level (2-tailed)

In sum, a brief look at the mean values of the five MS components (i.e., BMI, fasting glucose, HDL cholesterol, TG, and BP discussed above) shows diversified values of each component among the patients. This diversity seems a common trend on similar research results. For instance, in Mottillo et al.'s (2010) study in Canada, the 5 components of the metabolic syndrome in different studies related to MS and CAD were reported as: 1) BMI ranged from 22 to 33 kg/m2; 2) fasting glucose ranged from 82 to 196 mg/dl; 3) HDL cholesterol ranged from 37 to 64 mg/dl; 4) triglycerides ranged from 88 to 199 mg/dl; and 5) systolic blood pressure ranged from 117 to 174 mm Hg. In the same vein, Chen et al (2008) in a comparison of the prevalence of MS symptoms in CAD and non-CAD groups (by using NCEP ATP III, 2002 criteria) showed that in CAD group the value of BMI, prevalence of hypertension and hyperglycemia were higher than those of non- CAD group ($p < 0.05$, $p < 0.05$ and $p < 0.01$, respectively) whereas TG and HDL-C did not differ significantly between the two groups. Similar results were achieved by Irribaren et al. (2006) in a case control study comparing CAD and non-CAD patients regarding their MS components. The study, which used the AHA/NHLBI criteria, showed that hypertension followed by low HDL cholesterol and large waist circumference were the most common components among case subjects; low HDL cholesterol and hypertension were also the most common components among control subjects.

The study used two criteria to assess the values of each component. It was found that while 31% of case subjects and 8% of control subjects had high FBS by ATP-III criteria, 49% and 15%, respectively, did by AHA/NHLBI criteria. The case/control prevalence ratio was highest for ATP-III fasting glucose (3.9) and lowest for high triglycerides (Irribaren et al. (2006). The study further discusses that that the components of MS that were more strongly associated with early-onset CAD were hypertension, low HDL cholesterol, and increased FBS. Notably, FBS defined by the lower AHA/NHLBI threshold was more strongly associated with the outcome than FBS defined by the ATP-III criteria (100 and 110 mg/dl respectively). It was concluded that waist circumference and triglycerides were not independently related to early-onset CAD (Irribaren et al, 2006).

Consistent with these studies, in Chen et al.'s (2008) study, of all the aged CAD-patients with MS, obesity, hypertension, hyperglycemia, disorder of TG and hyperfibrinogenemia were more common than the CAD- patients without MS. Obesity, especially abdominal obesity, was found to be the initiating factor of IR (Insulin Resistance), and the hyperglycemic patients corresponding to IR, could had atherosclerosis much more easily. This could be due to the accumulation of fat in the pancreas which causes the dysfunction of b cells and IR accelerates the apoptosis of b cells. The authors concluded that both these procedures evoke the inflammation and thereafter atherosclerosis through the change of the endothelium. Chen et al. (2008) further maintained that because of the smaller stature of Chinese population comparing with the Western people, BMI rather than waist nor was hip circumference chosen as the threshold level for obesity. Moreover, they found that although MS patients only had low to medium grade hypertension, it developed heart disease as in a target organ, while it was known as the main factor to cause CAD. The disorder of serum lipids increases the risk of atherosclerotic cardiovascular disease in MS patients. The hyperglycemic patients showed an increased level of TG and TG-rich remnant-like particles, considered to be the mainly dangerous factors of CAD as contended by Akayanagi et al. (2004). The study also reports that LDL-C is easier to be oxidized and deposited in the vessel wall, and is difficult to be diminished by the classic metabolic ways, which gives rise to easier development of the atherosclerosis. Research also shows that decreased

HDL-C might reduce the clearance of the peripheral cholesterin and diminish the efficacy of anti-atherosclerotic effects.

4.3 MS and CAD Correlation

A total of 258(87.5%) of the cases and 190(64.6%) of the controls were found to have metabolic syndrome. The results indicate that individuals suffering from MS, as measured by ATPIII criteria, had about four-fold increased risk of CAD. (OR=4.19, 95%CI=2.603-6.47, P=0.0001). These results suggest that patients who show symptoms of MS are also at greater risks of coronary artery diseases and therefore need to take necessary actions to uphold their health. Hence, certain health-maintaining actions such as controlling dietary intake, receiving appropriate and timely medication, avoiding smoking, and performing regular physical exercises, which were shown in this study to improve the conditions of CAD patients, are recommended. Previous research (e.g., Chen et al., 2008; Irribaren et al., 2006; Mottillo et al., (2010) has also documented that patients with MS symptoms are exposed to higher risk of CAD. Chen et al. (2008) using NCEP ATP III, 2002 criteria showed that the cumulated occurrence of MS symptoms was significantly higher in the CAD group than in the non-CAD group (48.7% versus 23.4%, $p < 0.01$). The study also demonstrated through a logistic regression analysis, that the risk of having future CAD was in direct correlation with MS (b = 1.475, 0.670 (S.E.), Wald = 4.852, p = 0.028, exp (b) = 4.371). Similarly, Irribaren et al (2006), using the AHA/NHLBI criteria, found a 32% prevalence of MS among case subjects and 11% among control subjects. In the same study, it was seen that the presence of the MS by ATP-III criteria in the absence of diabetes conferred an almost 5-fold increase in the odds of early-onset CAD. Irribaren et al (2006) held that the AHA/NHLBI definition resulted in a slightly better prediction of outcome. Likewise, in a study by Mottillo et al.(2010) in Canada, the prevalence of the metabolic syndrome ranged from 1% in a study of women without type 2 diabetes mellitus (Takahashi et al., 2007) and 78% in a study of patients with type 2 diabetes mellitus (Butler et al., 2006). The study also showed that the point estimates for cardiovascular risk were consistently higher in women compared with men. Overall, consistent with previous research, results of the current study reconfirm the fact the metabolic syndrome is strongly linked to the increased risk of CVD.

4.4 CAD and MS as an Independent Risk Factor

Having discussed the correlation of individual MS component with severity of CAD, the study also intended to find out whether adding all the components of MS together will have different effects on severity of CAD among the patients. In other words, the association of MS as an independent risk factor for CAD was assessed using multivariate modeling programs. To quantify the risk of CAD associated with metabolic syndrome after adjusting for other potential risk factors conditional logistic regression was conducted. It is also assumed that if the metabolic syndrome operates independently of its individual components, its potential interventions may modify the risk of CAD above and beyond intervening on individual risk factors, such as hypertension. In this study all the designed models show that MS is a significant predictor of CAD even in the presence of the other confounders and potentially CAD risk factors (Odds Ratio between 3.5 till 4.15, $p<0.000$), however by adding MS components to ATPIII(2005) criteria MS conveyed no additional predictive information (Odds Ratio=0.33 up to 0.81 , $p>0.1$). Previous research shows that although both the presence of multiple risk factors and MS itself (Gami et al.,2007) confer an increased risk of CAD, it is unclear whether the adverse impact on health by the MS is greater than those related to the sum of its components (Grundy et al. , 2004). It has also been argued that the concept of syndrome implies that the risk associated with having the syndrome ought to be greater than the sum of its parts, and that all the factors should have a common underlying physiology which is responsible for their clustering (Kahn et al., 2005). Alexander et al. (2003) in a large cross-sectional study conducted in the US population aged over 50 (participants of the NHANES III survey), pointed out that MS does not improve prediction of CAD events in the presence of its components considering the ATPIII definition.

On the contrary, in a prospective study on older individuals, the MS by the ATPIII definition but not by the WHO criteria appeared to be an independent predictor of cardiovascular events after adjusting for its components and the traditional cardiovascular risk factors. Additionally, Ford (2003) found that the MS defined by the ATPIII and WHO definitions only had the modest job of predicting cardiovascular disease (estimated summary relative risk of 1.7 to 1.9). Iribarren (2006) examining the association between the metabolic syndrome (MS) and early-onset coronary artery disease (CAD) concluded that the presence of ATP-III MS without diabetes and with diabetes was a strong independent determinant of early-onset CAD, but neither definition of MS remained significantly associated with early-onset CAD in multivariate models adjusting for individual components. Thus, Iribarren (2006) concluded that the MS is a risk factor of early-onset clinical CAD, however, the prognostic information associated with the syndrome was not

greater than the sum of its parts. Hadaegh et al.'s (2009) findings also confirmed MS as an independent predictor of CAD over and above its individual components in this study. Numerous other studies have not been able to establish the role of MS as an independent risk factor for CAD severity. For example, Alexander et al. (2003) pointed out that blood pressure, HDL cholesterol, and diabetes, but not the presence of MS, were significant multivariate predictors of prevalent CAD in an analysis of the Third National Health and Nutrition Examination Survey. Similarly, Yarnell et al. (1998) reported that the excess of ischemic heart disease risk associated with MS was no greater than that can be explained by individual effects of the defining variables in a multiple logistic model. This finding implies that MS conveyed no additional predictive information beyond its components. A logical interpretation of this finding is that clinicians would be better off addressing the individual risk factors rather than "treating the syndrome." It is also arguable that the identification of a condition like MS, even if it does not provide incremental value over its components, may better motivate physicians to treat the condition and its accompanying risk factors. Previous investigators have reached similar conclusions.

Irrespective of the fact that the result of this study indicate a significant association between MS as an independent risk factor for CAD, the overall results of the above cited investigations are equivocal. Hence, without evaluating whether or not specific biological mechanisms may explain the association between the metabolic syndrome and CAD, it would still remain unclear if the metabolic syndrome should be viewed as an etiologic entity above and beyond its individual components.

In an attempt to offer a logical solution regarding the uncertainty of research over MS as an dependant risk factor, Orchard et al. (2005) suggest that more intensive efforts should be directed toward prevention and management of the individual risk factors than to "diagnosing" the syndrome assert based on the strength of associations observed for the individual components and the fact that the association of the MS with CAD was greatly attenuated and no longer significant after adjustment for its components.. From the standpoint of primary prevention, interventions designed to increase exercise and achieve weight loss have been shown to effectively reduce these risk factors. Finally, the present study in line with previous studies (e.g., Hadaegh et al., 2009; Iribarren et al., 2006; Alexander et al., 2003; Yarnell et al., 1998) provides important information and adds useful insights regarding MS and CAD severity mostly . Based on this body of research, it is recommended that to reduce the risk of CAD, physicians target individual CAD risk factors for modification and/or treatment. Therefore, it might seem that using information on the metabolic syndrome will not help prediction of CAD as compared with detailed information on well-established individual CAD risk factors.

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Beneficial effects of the burdock ferment liquid on diabetic disorders in STZ-induced diabetic rats

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Abstract: The present study was undertaken to characterize the effects of the burdock (*Arctium lappa* L.) ferment liquid (BFL) on diabetic disorders employing streptozotocin-induced diabetic rats (STZ-diabetic rats) as a type-1 diabetic model. There was a tendency towards a reduction in hypercholesterolemia after oral administration of BFL in diabetic rats for 2 consecutive weeks. BFL with abundant inulin was capable of alleviating significantly the hypertriglyceridemia and hyperglycemia. Diabetic-dependent alterations in serum creatinine concentrations, blood urea nitrogen and creatinine clearance were ameliorated after 2-week treatment with BFL in a dose-dependent manner. Additionally, BFL with polyphenolic components was able to scavenge 1,1-diphenyl-2-picrylhydrazyl radical as well as to attenuate the oxidative stress, evidenced by the reduction of hyperactivity in antioxidants including superoxide diamutase and glutathione peroxidase in plasma of diabetic rats. Thus, BFL has the ability to decrease the hyperlipidemia and hyperglycemia, and alleviate the hyperglycemia-associated oxidative stress in STZ-diabetic rats.

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1. Introduction

Diabetes, which ranks highly among the top ten causes of mortality around the world, often leads to disability from the vascular complications of coronary artery disease, cerebrovascular disease, renal failure, blindness, and limb amputation in addition to neurological complications and premature death [1]. It has been demonstrated that the use of pharmacological intervention in combination with lifestyle modifications that include diet and moderate exercise is particularly useful in the management of diabetes [2]. Accordingly, novel treatment with fewer side effects is feasible for the control of diabetic disorder, indicating the merit of additional medication for diabetic patients. In fact, diabetics and experimental diabetic animal models exhibit high oxidative stress due to persistent and chronic hyperglycemia, which thereby depletes the activity of antioxidative defense system and thus promotes de novo free radicals generation [3]. As a new strategy for alleviating the oxidative damage in diabetes, interest has grown in the usage of natural dietary antioxidants.

Burdock (*Arctium lappa* L.) has long been cultivated as a vegetable in Taiwan for dietary use. Burdock is also used as a folk medicine as a diuretic and antipyretic. It has become a popular health drink in Taiwan in the last decade. Several studies have reported that the root of burdock possesses various pharmaceutical activities including antibacterial activity [4-5], desmutagenic activity [6], antioxidant ability [7-10], hepatoprotective effect [11-12], gastroprotective activity [13-14] and anti-inflammatory activity [8], among which the gastroprotective activity, hepatoprotective efficacy, anti-inflammatory activity, and antioxidant activity are associated with the free radical scavenging activity. Additionally, burdock is claimed to be helpful for improve glycemic control in hyperglycemic subjects [15]; however, the related research on diabetes is not clear-cut.

Fermentation using yeast or lactic acid bacteria has long been applied in food industry due to its beneficial effects in flavor development, in inhibition of spoilage bacteria and pathogens, in intestinal health and other health benefits related to

cancer prevention, blood cholesterols levels and immune competence, which could be resulted from the modification and/or creation of nutrient, botanically-active components and microbial metabolites [16-19]. The streptozotocin induced-diabetic rats (STZ-diabetic rats) serve as an excellent model to study the molecular, cellular and morphological changes in tissue induced by stress during hyperglycemia [20]. The present study is thus to examine the efficacy of a burdock (*Arctium lappa* L.) ferment liquid (BFL) on diabetic disorders employing STZ-diabetic rats as a type-1 diabetic model.

Material and Methods

Materials

The root of burdock (*Arctium lappa* L.) with Good Agriculture Practice (GAP) certification cultivated at Gueilai Area, Pingtung, Taiwan was applied as materials for preparation of burdock ferment liquid (BFL). BFL was kindly supplied by Dong Yuan Biotech Pharmaceutical Co., Ltd. (Kaohsiung, Taiwan). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), inulin, gallic acid, hydroxymethylfuraldehyde (HMF) and streptozotocin (STZ) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Folin-Ciocalteu reagent was from Merck (Darmstadt, Germany). Cosmosil 5C18-AR-II column was purchased from Nacalai Tesque (Kyoto, Japan). Acetonitrile and methanol were LC grade from Tedia (Fairfield, USA). The diagnostic kits for determinations of glucose (Cat. No. COD12503), cholesterol (Cat. No.COD11539) and triglyceride (Cat. No. COD11529) in plasma were purchased from BioSystem (Barcelona, Spain). Nephrat II enzyme-linked immunosorbent assay (ELISA) kit (Cat. No. NR002) was obtained from Exocell, INC. (PA, PUA). The diagnostic kits for determinations of creatinine concentration in serum or urine (Cat. No. 221-30), and kinetic reagent for measurement of blood urea nitrogen (BUN) (Cat. No. 283-30) were purchased from Diagnostic Chemicals Limited (Connecticut, USA). The colorimetric assay kits for measurements for superoxide dismutase (SOD, Cat. No. 706002) and glutathione peroxidase (GSH-Px, Cat. No.703102) activities in plasma were purchased from Cayman Chemical (Michigan, USA). All other reagents were from standard sources.

Determination of total polyphenol and inulin in BFL

Total polyphenols in BFL were determined spectrophotometrically using the Folin-Ciocalteu reagent based on a colorimetric oxidation/reduction reaction. To 0.2 ml of diluted aqueous acetone sample, 1 mL of Folin-Ciocalteu reagent (diluted 10 times with distilled water) was added. After that, 0.8 mL of 7.5 % Na_2CO_3 was added and mixed thoroughly. After 30 min of standing, the absorbance was measured at 765 nm. The amount of total polyphenols was calculated as a gallic acid equivalent based on a calibration curve of gallic acid standard, and expressed as mg gallic acid/mL BFL. All measurements were done in triplicate.

Inulin in BFL was measured using a HPLC method as described by Dall'Amico *et al.*, with minor modifications [21]. Sample preparation for HPLC measurement was performed by diluting BFL with distilled water and then mixed with 200 μl of 70% $HClO_4$. After boiling for 10 min to hydrolyze inulin to fructose and to convert fructose to hydroxymethylfuraldehyde (HMF), the sample was cooled on ice for 5 min and aliquots of 20 μl were subjected to HPLC analysis. A Hitachi (Tokyo, Japan) L-2130 HPLC pump system equipped with an L-2450 diode array detector, and an L-2200 autosampler was used to analyze HMF on a Cosmosil 5C18-AR-II column (5 μm; 4.6 × 250 mm i.d.) with a flow-rate of 1 ml /min and monitored at 280 nm. Mobile phase system A was 3.2 mM HCl, pH 2.8, and B was acetonitrile/3.2 mM HCl (60:40, v/v). The HMF in sample was eluted chromatographically at ambient temperature with a gradient program: 0 % B (0-1 min), 0 %-35% B (1-9 min) and 0 % B (9.1-10 min). The amount of inulin in BFL was calculated based on a calibration curve constructed by plotting the HMF response area vs. inulin concentration. All measurements were done in triplicate.

Evaluation of free radical-scavenging activity of BFL

The free radical-scavenging activity of BFL was evaluated using DPPH free radical-scavenging assay as described previously [22]. BFL was diluted with methanol and an aliquot of 50 μL of each dilution was transferred into a 96-well microplate (NUNC, Roskilde, Denmark). A working solution of DPPH (250 μM) in methanol was freshly prepared and then an aliquot of 150 μL was added to each well. After incubation for 30 min, the remaining percentage of DPPH was measured at 490 nm on an ELISA reader (ThermoLabsystems, Cheshire, UK). Each dilution was performed at least in triplicate.

Animal Models

Male Wistar rats, weighing 200-250 g were obtained from the Animal Center of National Cheng Kung University Medical College (Tainan, Taiwan). They were maintained in a temperature-controlled room (25 ± 1°C) and kept on a 12:12 light-dark cycle (light on at 06:00 h). Standard rat chow and water were available *ad libitum*. STZ-diabetic rats were

prepared by intravenously (i.v.) injecting STZ (60 mg/kg) into male Wistar rats. Animals were considered to be diabetic if they had plasma glucose concentrations of 350 mg/dL or greater in addition to polyuria and other diabetic features. All studies were carried out 2 weeks after the injection of STZ. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) with IACUC approval number 9616.

Treatment protocols

BFL solution were prepared by dilute with distilled water to have the tested solution at the indicated concentration as following: one part of original BFL diluted with one part of distilled water (1:1, v/v) to have solution with 50% BFL; one part of original BFL diluted with three part of distilled water (1:3, v/v) to get solution with concentration of 25% BFL; one part of original BFL diluted with five part of distilled water (1:5, v/v) to get solution with 17% BFL. A metabolism coefficient of 6.25 was employed to convert the recommended daily oral dosage of BFL (20 ml) for adult into rats, assuming that average body weight of an adult is 60 kg. Thus, diluted BFL solution was given by oral gavage at the indicated concentration for 2 mL/kg, twice a day, to the separate groups of the STZ-diabetic rats. Another group of STZ-diabetic rats was received the equivalent volume of distilled water used to dissolve the preparations of interest. All animals were administered twice a day via gastric tube. The standard rat diet and water were available *ad libitum* throughout the entire treatment period. Two weeks after the treatment, rats were weighed and blood samples were collected from a tail vein; meanwhile, individual rats were placed in metabolic cages (Shineteh Instruments Co., Ltd, Taipei, Taiwan) to obtain 24-hour urine collections for measurements of urine creatinine (Cr). The systolic blood pressure (SBP) of the tail artery was also measured at weekly intervals.

Blood sampling and analysis

Blood sample of rats were centrifuged at 2,000 *g* for 10 minutes at 4°C, plasma was removed and aliquot for the respective analytical determinations. Plasma glucose concentration was measured by glucose oxidase method. Levels of cholesterol and triglycerides in total plasma were analyzed enzymatically. The serum Cr concentration was determined by the modified Jaffe´ method. BUN was determined according to the urease procedure. Plasma SOD (E.C.: 1.15.1.1) activity was determined by commercial kit for measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) in absorbance at 450 nm. One unit of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50%. The activity assay for GSH-Px (E.C.: 1.11.1.9) determined by commercial kit was based on the oxidation of NADPH to NAD+, catalyzed by a limiting concentration of glutathione reductase, with maximum absorbance at 340 nm. All analyses were performed in accordance with the manuals provided by the manufacturers.

Analysis of urine parameters

The 24-h urine collected from each diabetic rat and age-matched control was centrifuged at 2,000 *g* for 10 min. Urinary albumin concentrations were measured by ELISA assay using an anti-rat albumin antibody. The Cr concentration in pooled urine samples was determined by the modified Jaffe´ method using the commercial assay kit. All analyses were performed in accordance with the manuals provided by the manufacturers. Creatinine clearance (Ccr) was calculated using the following equation: Ccr (mL/min/kg) = [urinary Cr (mg/dL) x urinary volume (mL)/serum Cr (mg/dL)] x [1000/body weight (g)] x [1/1440 (min)].

Blood pressure measurement

SBP of the tail artery was measured by non-invasive blood pressure system (MODEL BP-6, Diagnostic & Research Instruments Co., Ltd., Taoyuan, Taiwan). The measurements for SBP were recorded in quadruplicate for each rat and the average blood pressure was calculated.

Statistical analysis

Data are presented as the mean ± SD. The statistical significance between groups was analyzed by an analysis of variance (ANOVA) test, a *p value* of less than 0.05 was considered to be significant.

Results

Contents of total polyphenol and inulin in BFL

The content of total polyphenol and inulin in various dilutions of BFL was shown in Table 1. The total polyphenol and inulin in original BFL was 5.10 ± 0.27 mg/mL and 304.0 ± 26.7 (mg/mL), respectively. Dilutions were in inverse proportion to its content of total polyphenol and inulin in BFL.

DPPH radical-scavenging activity of BFL

BFL was able to scavenge significantly DPPH radical with concentration-dependant manner (Table 2). Additionally, the DPPH radical-scavenging activity of BFL was reached a plateau within 30 min

and maintained for 120 min (Table 2). In comparison of free radical scavenging activity of BFL with that of vitamin E (40 ppm), the dilution of BFL required to achieve similar effect was 1:10 (v/v) (data not shown).

Table 1. The contents of total polyphenols and inulin in dilutions of BFL

Dilutions (v/v)	Total polyphenol (mg gallic acid/mL)	Inulin (mg/mL)
100 % BFL (1:0, v/v)	5.10 ± 0.27	304.0 ± 26.7
50 % BFL (1:1, v/v)	2.92 ± 0.27	160.6 ± 2.0
25 % BFL (1:3, v/v)	1.49 ± 0.06	66.9 ± 7.9
17 % BFL (1:5, v/v)	0.76 ± 0.05	44.1 ± 1.0

Values (mean ± SD) were obtained for each group of 3 experiments.

General characteristics of STZ-diabetic rats repeatedly treated with BFL

Each diluted BFL solution made influences neither on body weight nor on SBP in STZ-diabetic rats at the end of the 2-week treatment period (Fig. 1A; Fig. 1B). The plasma glucose in STZ-diabetic rats receiving for 2-week treatment with higher concentration of BFL was lower than the corresponding values for vehicle-treated group (Fig. 2A). The higher plasma level of cholesterol was reduced in STZ-diabetic rats receiving for 2-week treatment with 50% BFL solution, but the values did not achieve statistical significance as compared to that of vehicle-treated counterparts (Fig. 2B). At the termination of 2-week treatment, the plasma level of triglyceride in STZ-diabetic rats tended to be reduced by 17% BFL solution. The action of BFL on the alleviation of hypertriglyceridmia was markedly in STZ-diabetic rats treated with 2-week 50% BFL solution (Fig. 2C).

Changes in renal function related parameters in STZ-diabetic rats repeatedly treated with BFL

Following 2-week treatment, STZ-diabetic rats received 17% BFL solution exhibited lower levels of serum Cr, whereas the difference did not achieve statistical significance as compared to those of vehicle-treated group at the corresponding time (Fig. 3A). The levels of BUN in STZ-diabetic rats received 17% BFL solution was also tended to be reduced at the end of 2 weeks treatment, but did not differ from their vehicle-treated counterparts (Fig. 3B). Both values for serum Cr and BUN of STZ-diabetic rats were significantly (p<0.05) lower by 50% BFL solution as relative to their vehicle-treated counterparts at the end of 2 weeks treatment (Fig. 3A; Fig. 3B).

Furthermore, treatment with BFL form 2 weeks onward reduced the increase in urine volume of STZ-diabetic rats in a concentration manner (Fig. 3C). Also, treatment STZ-diabetic rat with 50% BFL solution significantly (p<0.05) reduced the increase in Ccr as compared with their vehicle-treated counterparts at the end of 2 weeks treatment (Fig. 3D).

Changes on plasma antioxidant enzyme activity in STZ-diabetic rats repeatedly treated with BFL

The plasma SOD activity in STZ-diabetic rats was gradually reduced by 2 weeks treatment with dilutes BFL solution in a concentration manner. Similarly, 2-week BFL administered diabetic rats displayed significantly lower activity of plasma GSH-Px as compared to their vehicle-treated counterparts (Fig. 1C; Fig. 1D).

Discussion

Diabetics and experimental diabetic animal models exhibit high oxidative stress due to persistent and chronic hyperglycemia, which thereby depletes the activity of antioxidative defense system and thus promotes de novo free radicals generation（23,24）. Although adequate control of blood glucose levels may prevent the development of complications, it is difficult to achieve strict blood glucose control, leading to a year-by-year increase in the number of patients with diabetes . Therefore, strategies to reduce oxidative stress in diabetes mellitus may exert favourable effects on the progression of diabetic complication [25]. Among diabetic complications, nephropathy is the most common cause of end-stage renal disease in developed countries and a major cause of morbidity and mortality in patients with diabetes [26]. In assays utilizing unnatural model radicals, the DPPH radical scavenging assay, BFL was shown to scavenge the DPPH radical significantly and concentration-dependently. Hence DPPH assay is one of the most common methods of assessing antioxidant activities [27], the in vitro antioxidant activity of BFL could be considered. STZ-Diabetes provides a relevant example of endogenous chronic oxidative stress and hyperglycemia [20]. Thus, we evaluated the oxidative stress and nephropathy induced by STZ in Wistar rats and examined the potential protective effects of BFL against the changes induced by STZ.

Table 2. The free radical scavenging activity in dilutions of BFL

Dilutions (v/v)	The remaining DPPH radicals (%)			
	30 min	60 min	90 min	120 min
Blank	95.25 ± 2.22	91.55 ± 2.60	91.06 ± 2.70	90.63 ± 2.65
1:45	73.23 ± 1.35*	68.13 ± 2.07*	64.85 ± 2.10*	62.18 ± 2.07*
1:40	70.06 ± 1.28*	64.98 ± 1.77*	61.44 ± 1.71*	58.27 ± 2.07*
1:35	65.19 ± 1.35*	59.64 ± 1.26*	55.67 ± 1.24*	52.15 ± 1.25**
1:30	64.64 ± 2.23*	58.04 ± 2.62*	53.02 ± 2.51*	48.74 ± 2.49**
1:25	63.19 ± 2.09*	56.79 ± 2.21*	51.73 ± 2.15**	47.34 ± 2.18**
1:20	52.92 ± 2.31**	44.15 ± 2.90**	37.65 ± 2.62**	32.07 ± 2.73**
1:15	30.93 ± 1.93**	19.94 ± 1.96**	14.65 ± 1.23**	13.22 ± 0.70**
1:10	15.37 ± 0.54**	13.83 ± 0.58**	13.53 ± 0.58**	12.87 ± 0.68**

Values (mean ± SD) were obtained for each group of 3 experiments. * $p < 0.05$ and ** $p < 0.01$ compared to the values of blank at the indicated times, respectively.

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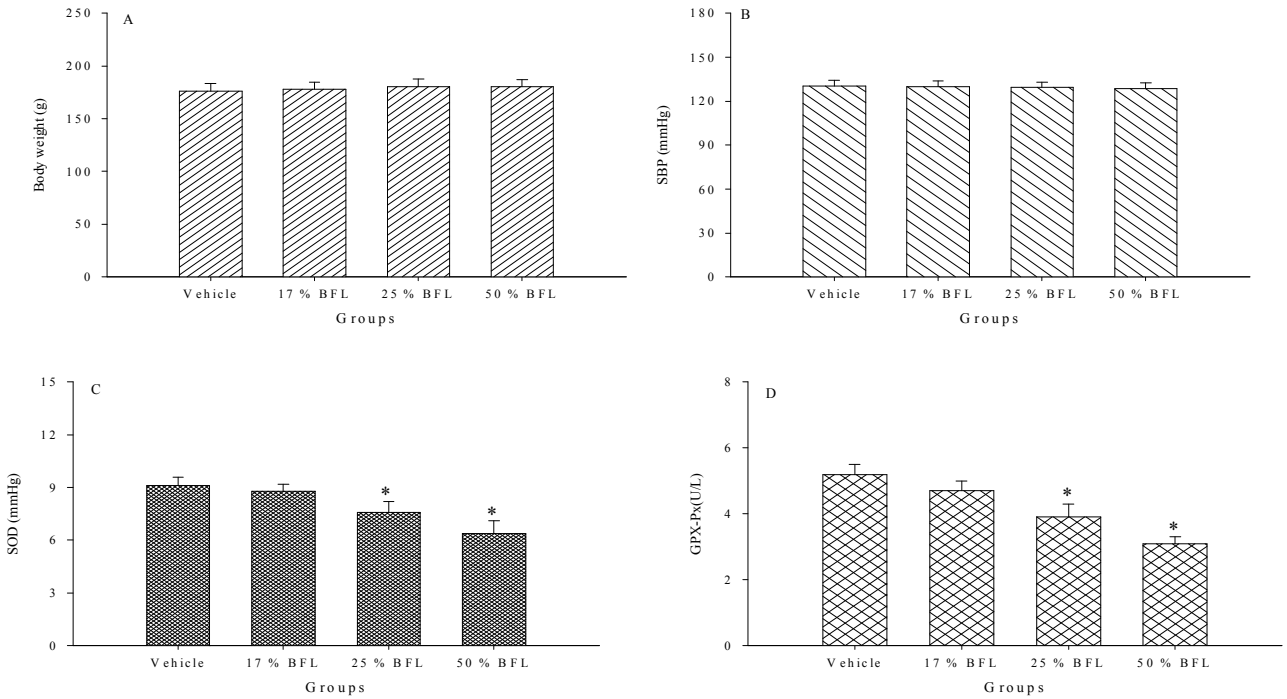

Fig. 1. Changes of the body weight, systolic blood pressure (SBP) and the antioxidant enzyme activity in STZ-diabetic rats receiving 2 weeks of BFL administration. Values (mean ± SD) were obtained for each group of 6 animals. * $p < 0.05$ compared to the values of vehicle-treated STZ-diabetic rats.

We observed that 2 week-administration of diluted BFL solution reduced the higher level of serum Ccr and BUN as well as creatinine clearance in STZ-diabetic rats significantly and dose-dependently. Otherwise, the typical characteristics of diabetes with large increase of urine output and the less body weight gain in STZ-diabetic rats were ameliorated by 2-week of BFL administration, implying the product be with the capacity to modify the renal hyperfiltration. After 2 week-treatment with diluted BFL solution, the results revealed significant reduction in hyperglycemia of diabetic animals. The beneficial effect of BFL on diabetic nephropathy may be associated with reduce severity of hyperglycemia.

It has long been known that hypertension is an aggravating factor in increased vascular pressure and may be the key hemodynamic determinant of diabetic renal injury as well; it is therefore well recognized that blood pressure control is important in diabetic patients [28]. Actually, BFL at any concentration was observed to make no influence on the blood pressure in STZ-diabetic rats, indicating that the beneficial effect of BFL on diabetic nephropathy was not linked with the amelioration of hypertension. Besides hyperglycemia and high blood pressure, other risk factors have been identified in the development or progression of diabetic kidney disease; hyperlipidemia has been associated with occurrence of severe renal failure secondary to development of glomerulosclerosis and tubulointerstitial disease [29-30]. The increased lipid peroxidation in the kidney implies the level of susceptibility to diabetic oxidative stress, leading to diabetic complications. From this view point, prevention of hyperlipidemia and/or lipid peroxidation resulting from oxidative stress is considered to play a crucial role in protection from disorders associated with diabetes [29-30]. We observed that BFL, especially at the higher concentration, be effective in the modification of hypertriglyceridemia as well as alleviation of hypercholesterolemia in STZ-diabetic rats. The beneficial effects of BFL on the amelioration of diabetic renal function are thought to be linked to the observed control of lipid metabolism.

Some dietary components that completely escape glucide digestion, such as resistant starch and oligofructose, have been demonstrated to exert systemic effects by modifying lipid metabolism [31-32]. In contrast to starch, inulin is fermentable dietary fiber, resistant to hydrolysis by pancreatic amylase and saccharidases in the upper gastrointestinal tract. Previous study have been demonstrated that inulin was produced enzymatically from sucrose, and that supplementing inulin be helpful for reduced elevated hepatic levels of triacylglycerols in rats fed with a high-fat and high sucrose diet [33]. Actually, inulin is rich in BFL. It is possible to anticipate that the reduction in elevated levels of hepatic lipids in diabetes was associated with the inulin of BFL.

As established clearly, reactive oxygen species (ROS) are generated in augmented amounts in diabetes, the main mechanisms being increased glucose autooxidation and advanced protein glycation, activation of poliol pathway and attenuated antioxidant defence system. It is known that oxygen radical scavenging enzymes can respond to conditions of increased oxidative stress with compensatory increases in activity [34], our findings are consistent with this fact. SOD is reported to be the first induced enzyme; its higher activity could be due to its induction by increased superoxide anion production. The induction of SOD in turn leads to protection of GSH-Px against inactivation by superoxide anion the net effect being a higher GSH-Px activity [35]. However in BFL administered STZ-diabetic rats, the activities of both SOD and GSH-Px in plasma were lower in comparison to diabetic controls, indicating the lower levels of oxidative stress in these rats by BFL treatment. The above observation shows that BFL possesses antioxidant activity, which could exert a beneficial action against pathological alterations caused by the presence of free radicals in STZ diabetes. Taking the above results into consideration, the renoprotective effects of BFL in diabetes were not only attributable to improved dyslipidemia alone, but also likely reflected its antioxidant activity. The combined antioxidant and reducing hyperlipidemia and hyperglycemia actions of BFL should be particularly advantageous and perhaps even synergistic in preventing renal injury and other diabetic complications.

Phenolic compounds from fruits and vegetables have been receiving increasing interest from consumers and manufacturers because numerous epidemiological studies have suggested associations between consumption of polyphenol-rich foods or beverages and the prevention of certain chronic diseases dependent of their antioxidant properties[36]. Indeed, polyphenolic compounds were abundant in this burdock product. Application of the burdock product proved the positive influence of polyphenols on oxidative stress and hyperlipidemia through the antioxidants could thus be considerable.

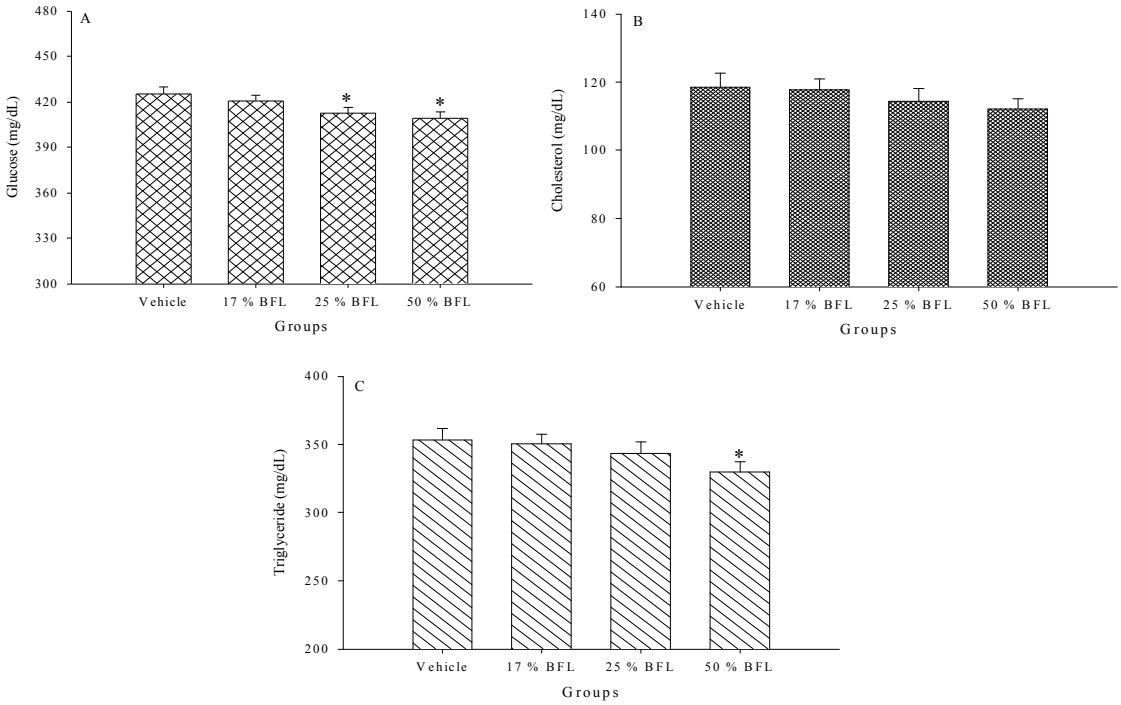

Fig. 2. Changes of the plasma parameters in STZ-diabetic rats receiving 2 weeks of BFL administration. Values (mean ± SD) were obtained for each group of 6 animals. * $p < 0.05$ compared to the values of vehicle-treated STZ-diabetic rats.

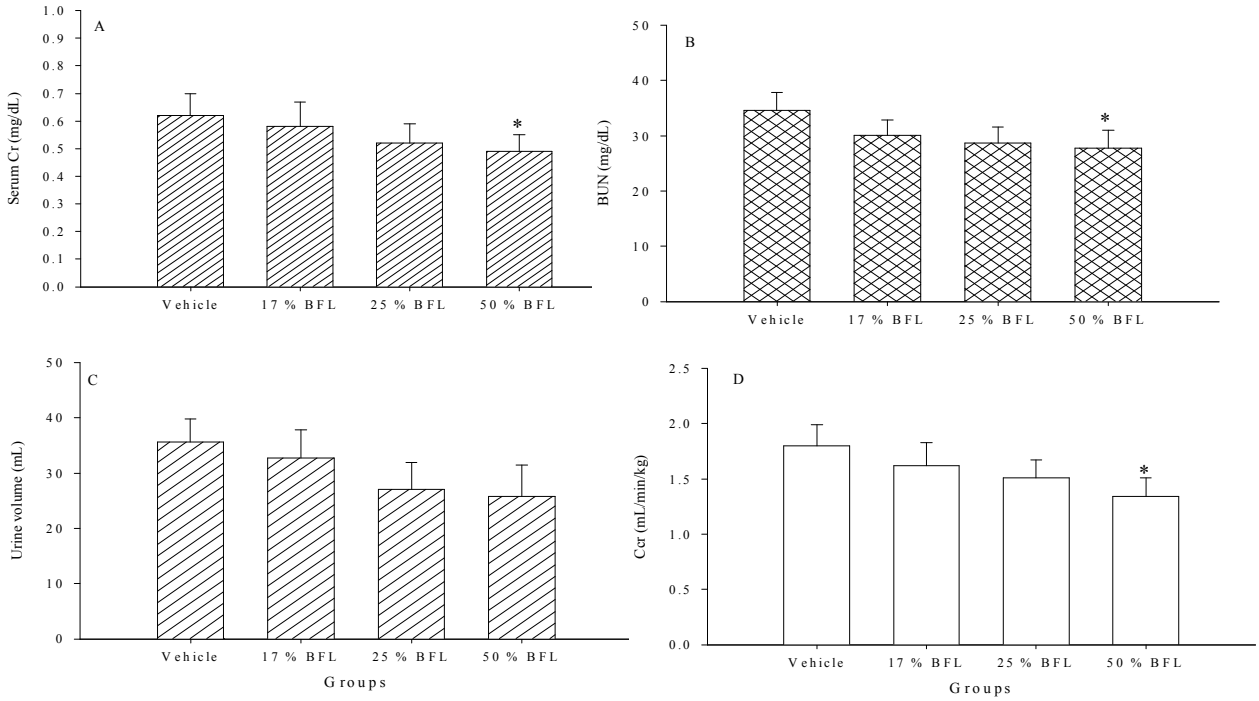

Fig. 3. Changes of the renal function related parameters in STZ-diabetic rats receiving 2 weeks of BFL administration. Values (mean ± SD) were obtained for each group of 6 animals. * $p < 0.05$ compared to the values of vehicle-treated STZ-diabetic rats.

The antioxidant responsiveness mediated by burdock may also be anticipated to have biological significance in eliminating reactive free radicals that may otherwise affect the normal cell functioning.

In conclusion, burdock showed an antidiabetic effect *via* reducing hyperlipidemia and hyperglycemia in STZ-diabetic rats. Besides, BFL possesses antioxidant activity which could exert a beneficial action against pathological alterations and diabetic renal complications caused by the presence of free radicals in hyperglycemia. The obtained data strengthen the basis for recommending BFL as an adjuvant for diabetic individuals.

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Chemical Characteristics and Antioxidant Capacity of Egyptian and Chinese Sunflower Seeds: A Case Study

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Abstract: In the last few years Chinese sunflower seed has invaded our Egyptian market increasingly. We took it and the Egyptian sunflower seed as a comparable case study to characterize and investigate them as a source of effective natural antioxidants, oil and protein. Chemical characteristics of the two seeds revealed that protein, oil, ash, moisture and total phenolic contents (TPC) increased significantly ($P<0.05$) after dehulling with pronounced larger amount of these parameters in the Egyptian sunflower seed compared to the Chinese one. Fatty acid analysis showed that Egyptian sunflower oil contains more than 86% and Chinese sunflower oil contains more than 80% unsaturated fatty acids which give these oils a relative advantage. Chlorogenic acid was the major phenolic compound present in TPC as measured by HPLC. Antioxidant activity (AA %) of the phenolic extracts was followed up by measuring radical scavenging activity (RSA %) of the stable DPPH• radical, the degradation rate of β-carotene-linoleic acid o/w emulsion, and the oxidation stability measured by the fully automated active oxygen method (Rancimat). Egyptian sunflower seed have more AA % than Chinese seed as revealed by the higher RSA%, less degradation rate of β-carotene-linoleic acid color and higher induction period measured by Rancimat. Results also demonstrated the suitability of Egyptian and Chinese sunflower seed to be an effective source of protein, with some good functional properties such as solubility, dispersibility water absorption capacity, and emulsifying capacity. Contrary, it showed poor foaming and gelling abilities. Egyptian sunflower oil can also be used as an effective source of unsaturated fats and natural antioxidants (TPC). Hence, can be supplemented in many foods and can replace the synthetic antioxidant with their remarkable hazards.
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Key words: sunflower seed, chlorogenic acid, total phenolic content, antioxidant activity.

1. Introduction

Sunflower is one of the major oilseed crops ranking fourth with a worldwide production of about 10.6 million metric tons in 2006 [(1)]. Sunflower is an annual plant native to the Americas belonging to the family Asteraceae. Per 100 g the seed enclose protein up to 20.78 g, total lipid (fat) up to 51.46 g, ash up to 3.02 g, fiber up to 8.6 g with total energy of 2445 kj. The oil accounts for 80% of the value of the sunflower crop, as contrasted with soybean which derives most of its value from the meal. Sunflower oil is generally considered a premium oil because of its light color, high level of unsaturated fatty acids and lack of linolenic acid, bland flavor and high smoke points. The primary fatty acids in the oil are oleic and linoleic (typically 90% unsaturated fatty acids), with the remainder consisting of palmitic and stearic saturated fatty acids. The primary use is as a salad and cooking oil or in margarine. In the USA, sunflower oils account for 8% or less of the market, but in many sunflower-producing countries, sunflower is the preferred and the most commonly used oil [(2)].

Egypt's production of edible vegetable oils suffers several problems nowadays. During the early sixties, Egypt used to be self-sufficient in edible vegetable oils, where self-sufficiency ratio reached 95%. Such ratio followed a declining trend until reaching as low as 31.6% in 2007, which led to increasing volume of oil imports that reached 5.6 thousand tons worth L.E 1.992 billion in 2007 [(3)]. The problem is further complicated by the reliance of the edible oils industry in Egypt on imported raw materials, where private sector's dependency ratio is estimated at 85% [(3)] and according to Egyptian-British Chamber of Commerce Egypt imported 92% of edible oil consumed in 2010 [(4)]. As a result of such a gap between consumption and production Chinese sunflower seed has increasingly invaded the Egyptian market during the last few years.

Lipid oxidation is well known to cause deterioration of fats or fat-containing foods. Also, reactive oxygen species (ROS) produced during natural biological activity in the human body tends to accumulate therein. Antioxidants are needed to retard or delay lipid oxidation and to scavenge ROS. Antioxidants can act by the following mechanisms in lipid peroxidation: (1) decreasing localized oxygen concentrations, (2) preventing chain initiation by scavenging initiating radicals, (3) binding catalysts, such as metal ions, to prevent initiating radical generation, (4) decomposing peroxides so they

cannot be reconverted to initiating radicals, and (5) chain-breaking, to prevent continued hydrogen abstraction by active radicals [5]. Commonly utilized chain-breaking antioxidants include butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), tert- butylhydroquinone (TBHQ), propyl gallate (PG) and the naturally occurring tocopherols. There are some serious problems concerning the safety and toxicity of BHA, BHT and TBHQ related to their metabolism and possible absorption and accumulation in body organs and tissues [6]. Therefore, the search for natural antioxidants is highly desirable. The phenolic components and tocopherols are the most important antioxidants for storage stability, as well as, nutritional quality of food made from sunflower seeds. Cells contain a complex system of antioxidant defenses to protect against the harmful consequences of activated oxygen species. When such a complex mechanism inside the cell fail to get rid of ROS it may cause many dangerous diseases such as inflammation, cardiovascular-diseases, cancer and aging [7, 8]. Numerous scientific articles refer to several natural phenols delaying the in vitro oxidation of simple or complex lipid matrices [9].

So, the aim of this work was to take Egyptian and Chinese sunflower seeds as a comparable case study for investigating their suitability as effective sources for natural antioxidants, oil and protein.

2. Materials and Methods

Materials

Two samples of sunflower seed, an Egyptian and a Chinese, were purchased from the local market, Dokki district, Cairo, Egypt. Chlorogenic acid (CGA) and TBHQ were purchased from Sigma. All reagents were BDH or of analytical grade.

Methods

Proximate composition of sunflower seeds

Sunflower seeds were divided into two parts. Half of the seeds were used as such with hull and designated whole seed (WS) while the rest half was dehulled manually and designated (DS). Hulls were separated by aspiration. WS and DS were ground and sieved to pass an 80 mesh screen and then analyzed for their proximate composition that is moisture, crude protein, oil content, crude ash, and crude fiber as recommended by A.O.A.C. [10]. Oil content was measured using a soxthlet extractor and n-hexane as a solvent. Hexane was evaporated using a rotary evaporator (Buchi Rotavapor Switzerland) at 40ºC. The oil was dried over anhydrous sodium sulphate then placed in a vacuum oven until constant weight. The oil was kept at -20ºC until analyses.

The defatted meal resulting from WS and DS was spread to dry at room temperature and designated defatted meal of whole seeds (DMWS) and defatted meal of dehulled seeds (DMDS). The meal was kept in closed containers at -20ºC until further work.

Phenolic extract preparation

Preparation of phenolic extracts was carried out following scientific literature regarding this subject [11-13]. Briefly, ground samples of whole (WS) or dehulled (DS) sunflower seeds (20 g) were extracted with 200 ml of solvent consisting of methanol, 0.16 M hydrochloric acid and water, mixed in proportion 8:1:1, respectively, for 2 h. The above mentioned procedure was repeated on the residue and extracts were combined and washed three times with 15 ml hexane to remove escaped oil using separatory funnel. The combined methanol layer was dried using rotary evaporator at 40ºC and stored in darkness at -20ºC.

Total phenolic content (TPC)

Total phenolics were determined colorimetrically using Folin-Ciocalteau reagent according to Hung et al. [14]. The absorbance was measured at 725 nm using a UV – 1601 PC UV-visible spectrophotometer (Shimadzu, Japan). Total phenolics were quantified by calibration curve obtained from measuring the absorbance of a known concentration of chlorogenic acid and the results were expressed as milligrams chlorogenic acid equivalent (CAE) per 100 g extract.

Chemical characteristics and fatty acid composition

The chemical characteristics of the oils used for the experiment have been determined according to A.O.A.C. [10]

For determination of fatty acid composition sunflower oil methyl esters were prepared according to A.O.A.C. method [10]. Determination of fatty acids composition was performed using a Hewlett Packard HP 6890 gas chromatograph, operated under the following conditions: Detector, flame ionisation (FID); column, capillary, 30.0 m X 530 µm, 1.0 µm thickness, polyethylene glycol phase (INNO Wax); N2 with flow rate, 15 ml/ min with average velocity 89 cm/s (8.2 psi); H2 flow rate, 30 ml/min; air flow rate, 300 ml/min; split ratio, 8:1, split flow, 120 ml/min; gas saver, 20 ml/min. Detector temperature, 280ºC; column temperature, 240ºC; injection temperature, 280ºC. Programmed temperature starting from 100ºC to reach a maximum of 240ºC was used for eluting the fatty acid methyl esters. The identification of the peaks was made as compared

with chromatograms of standard fatty acids methyl esters (Sigma, USA).

Antioxidant Activity (AA%)

Antioxidant activity was determined by three methods: Radical scavenging activity [15], by the β-carotene/ linoleic acid method described by Al-Shaikhan et al. [16] and the fully automated active oxygen (i.e. Rancimat) method. The later method was carried out using the Rancimat 679® (Metrohm AG, Herisau, Switzerland) instrument at 110°C with the air flow rate of 20 L/hr [17]. The oxidative stability was expressed as induction time (hr).

All antioxidant activity experiments were performed using 500 ppm of phenolic extracts in purified (stripped**)** sunflower oil**.**

Sunflower oil stripping (purification)

Sunflower oils were stripped from antioxidants and from trace metals and other prooxidants according to Fuster et al., 1998 [18] via adsorption chromatography to yield purified sunflower triacylglycerols fraction. A glass column (40 × 2.5 cm i.d.), plugged with glass wool, was packed with 250 g of alumina (activated at 100°C for 8 h and then at 200°C for 12 h) suspended in n-hexane, capped with sea sand, and conditioned by prewashing with 200 mL of n-hexane. The oil (100 mL) was dissolved in an equal volume of hexane and passed through the column, which was then washed with 200 mL of n-hexane. The chromatographic column was wrapped with aluminium foil to prevent light-induced oxidations during the purification process, and triacylglycerols were collected in an aluminum foil wrapped flask. Analysis of the purified oils by thin-layer chromatography (Merck precoated silica gel 60 thin-layer chromatographic plates, 0.25 mm layer thickness and chloroform diethyl ether; 90:10, vol/vol) showed that they are composed mainly of triacylglycerols (data not shown).

Functional properties of sunflower defatted meals

Nitrogen solubility index (NSI), protein dispersibility index (PDI) were determined as described by Smith and Circle [19]. Water absorption capacity (WAC) was estimated according to Huber [20]. Oil holding capacity (OHC) according to Childs and Forte [21]. Emulsifying Capacity (EC) as indicated by Shahidi *et al*. [22]. Foam Stability (FS) as described by A.A.C.C. [23]. Gelation according to Circle et al.[24].

HPLC Analysis

Methanolic extracts (5 µL) were injected in An Agilent 1100 Series HPLC system with a quaternary solvent delivery system, an online degasser, an autosampler, a DAD detector was used for the analysis. The column was a Phenomenex Luna C18 (5 µm, 250 mm X 4.6 mm) and column temperature was maintained at 30 °C. Two mobile phases, A 0.1% phosphoric acid and B acetonitrile were used in a gradient elution at a flow of 1 ml/min with the following gradient profile: 20 min from 10-22% B, 20 min with a linear rise to 40% B, 5 min reverse to 10% B, and additional 5 min equilibration time [34]. The system was controlled and data analysis was performed by Agilent Chemstation Software. All the calculations concerning the quantitative analysis were performed with external standardization by the measurement of peak areas.

Statistical analysis

All chemical analyses were performed in three replicates and the results were statistically analysed. Statistical analysis was performed using the GLM procedure with SAS (25) software. Duncan's multiple comparison procedure was used to compare the means. A probability to $p \leq 0.05$ was used to establish the statistical significance.

3. Results and Discussion

Proximate composition of sunflower seeds

Results in Table 1. Revealed that Moisture content in WS (9.2±0.85%) and in DS (8.9±0.24%) of Chinese seeds was higher than that of the Egyptian ones (7.02± 0.66% and 7.45±0.35 in whole and dehulled seeds, respectively. Protein content in WS (22.96±1%) and DS (28.46±0.5%) of Egyptian sunflower were higher compared to the Chinese ones (21.22±0.99% and 26.69±1.99%, for WS and DS, respectively). The same trend was recorded for oil content where Egyptian sunflower seeds had higher oil content both in WS and DS than the Chinese seeds. The former recorded oil content of 22.11 ±1.01% and 29.09 ±0.99% while the latter recorded oil content of 16.33 ±0.96% and 20.36 ±0.89% in WS and DS, respectively. Whereas, the ash content of Chinese seeds recorded higher percentages both in whole (7.36±0.14%) and dehulled (8.3±0.33%) seeds than whole (3.95±0.22%) and dehulled (5.53±0.62%) seeds of the Egyptian type. Chinese sunflower seeds showed lower crude fiber content (31.51±2.1%) in WS than that of Egyptian WS (34.91±2.1%) but, higher crude fiber (18.81±0.36%) in Chinese DS than Egyptian DS (16.36±0.34%) were recorded. Nitrogen free extract (calculated) was higher in DS than WS, also higher in Chinese seeds than the Egyptian ones.

From the results we notice that after dehulling there is a significant increase in all constituents except crude fiber which is high in the seed hulls. Similar finding were also found by some other scientists [26, 27]. Bhagya and Sastry [28] also reported similar effects of dehulling on Niger seeds.

Table 1. Proximate composition of Egyptian and Chinese sunflower seeds

Parameter	Egyptian		Chinese	
	WS*	DS*	WS	DS
Moisture (%)	7.02±0.66[a]	7.45±0.35[b]	9.2±0.85[d]	8.9±0.24[c]
Protein (%)	22.96±1.2[b]	28.46±0.5[d]	21.22±0.99[a]	26.69±1.99[c]
Oil Content (%)	22.11 ±1.01[c]	29.09 ±0.99[d]	16.33 ±0.96[a]	20.36 ±0.89[b]
Ash (%)	3.95±0.22[a]	5.53±0.62[b]	7.36±0.14[c]	8.3±0.33[d]
Crude Fiber (%)	34.91±2.1[d]	16.36±0.34[a]	31.51±2.1[c]	18.81±0.36[b]
Nitrogen Free Extract	9.05±0.86 [a]	13.11±0.59 [b]	14.38±0.75 [c]	16.94±0.98 [d]

Means followed by the same letter within the same row are not significantly different (P<0.05) *WS: Whole seed; DS: Dehulled seeds. Values are mean ± SD.

Total phenolic content (TPC)

According to the results (Table 2.) the total phenolic content (TPC), expressed as chlorogenic acid equivalent (mg/100g), was significantly higher (P<0.05) in dehulled seeds (DS) than in whole seeds (WS) of both Egyptian and Chinese sunflowers. The TPC was lower by about 35% in WS than DS. The highest TPC in WS (772 ±3.3 mg CAE/100 g) and in the DS (1088 ±3.95 mg CAE /100 g) was in Egyptian seeds. Chinese seeds showed less phenolics content both in WS (625 ± 2.1 mg CAE /100 g) and in the DS (886 ±3.5 mg CAE /100g). Various scientists have investigated the content of phenolic compounds in sunflower seeds [29-32]. De Leonardis and coworkers [30] reported that TPC in sunflower was in the range of 1.11 to 1.15 mg/mL chlorogenic acid equivalent which is in good agreement with our findings, while Fisk et al.[31] determined the TPC in sunflower seeds and found it to be 2700 mg/100 g. Comparison is hardly possible because of differing analytical methodologies, and differences in the sample material and origin.

Table 2. Total phenolic compounds (mg/100g CAE*) in Egyptian and Chinese sunflower seeds

Parameter	Egyptian		Chinese	
	WS*	DS*	WS	DS
Total Phenolics (mg CAE*/100g, (spectrophotometric)	772±3.3[b]	1088±3.95[d]	625±2.1[a]	886±3.5[c]
Chlorogenic acid (mg/100gm, HPLC)	501.8[b]	728.96[d]	400.2[a]	602.3[c]

Means followed by the same letter within the same row are not significantly different (P<0.05), CAE *: chlorogenic acid equivalent *WS: whole seed; DS: dehulled seeds. Values are mean ± SD.

In the two (Egyptian and Chinese sunflower oils) samples, chlorogenic acid was the most abundant phenolic compound, 602.3-728.96mg/100gm for DS and 400.2- 501.8 for WS, respectively constituting ≈ 65% of total phenolics (Table2.) as measured by HPLC. Other phenolic constituents were present in too small amounts to be detected by HPLC. Results of Žilić et al.[33] were comparable to our results where they found that TPC of sunflower oil comprised principally of chlorogenic acid, while caffeic acid, ferulic acid, rosmarinic acid, myricetin, and rutin were found in very small percentages. De Leonardis et al.[30] showed that phenolic spectrum of sunflower seeds included seven components (chlorogenic acid, protocatechuic, caffeic acid, o-cinnamic acid, ferulic acid, syringic acid and an unidentified phenolic compound). These authors also reported that the chlorogenic acid was the most abundant phenolic compound (≈79.4% of total phenols). Phenolic compounds in seeds and kernels of sunflower deserve much more attention because the total phenolic content, strongly correlate (r=0.93, P<0.05) with total antioxidant activity [30,33].

Chemical characteristics and fatty acid composition

The chemical characteristics of the Egyptian and Chinese sunflower oil samples are shown in Table 3. Results revealed that acid and peroxide values of the two samples were moderate and comparable to each other and were not significantly different (P< 0.05). Iodine value (IV) which represents the degree of unsaturation indicated that Egyptian sunflower oil has higher IV (129.41) compared to IV of the Chinese oil (115.18), hence Egyptian sunflower oil had significantly (P< 0.05) higher total unsaturation than the Chinese one. Regarding the ester value, saponification value, and unsaponifiable matters, results showed that the Egyptian sunflower oil had higher values than the Chinese one (P< 0.05).

Table 3. Chemical characteristics of Egyptian and Chinese sunflower oils

Parameter	Egyptian	Chinese
Acid value (mg KOH /g oil)	3.23±0.22[a]	3.8±0.31[a]
Peroxide value (meq.O_2/kg oil)	0.71±0.33[b]	0.88±0.23[b]
Iodine value	129.41±1.44[d]	105.18±1.54[c]
Saponification value (mg KOH / g oil)	189.4±2.1[b]	187.6±2.3[a]
Unsaponifiable matter (%)	1.85±0.15[b]	1.74±0.13[a]
Ester value	186.17±1.53[b]	183.8±0.99[a]

Means followed by the same letter within the same row are not significantly different (P<0.05), Values are mean ± SD.

Regarding fatty acid profile, Table 4 shows that palmitic acid contents ranged from 6.50 to 9.53%, palmitoleic acid contents from 1 to 1.3%, stearic acid contents from 7.25 to 9.78%, oleic acid contents from 32.95 to 27.8% and linoleic acid contents seeds ranged from 52.3 to 51.56% for Egyptian and Chinese sunflower oils, respectively. As seen from Table 4. almost 86% of Egyptian sunflower oil and 80% of Chinese sunflower oil are of good unsaturated type. Clinical studies show that higher unsaturated fat diets may be preferable even to low-fat diets because they lower total cholesterol, low density lipoprotein (LDL) or bad cholesterol and triglycerides, while maintaining beneficial high density lipoprotein (HDL) cholesterol, which is needed to carry the "bad" cholesterol away [21].

Table 4. Fatty acid composition of Egyptian and Chinese sunflower oils

Fatty Acid	Egyptian	Chinese
Palmitic acid	6.5±0.77[a]	9.53±0.98[b]
Palmitoleic	1±0.39[a]	1.33±0.64[a]
Stearic	7.25±0.44[a]	9.78±0.86[b]
Oleic	32.95±0.79[b]	27.8±0.69[a]
Linoleic	52.3±0.85[b]	51.56±0.8[a]
Total Saturation (SFA)	13.75[a]	19.31[b]
Total unsaturation (UFA)	86.25[b]	80.69[a]
SAT/USAT	0.16[a]	0.24[b]

Means followed by the same letter within the same row are not significantly different (P<0.05), Values are mean ± SD

Antioxidant activity (AA%)

The antioxidant property of sunflower seed extracts influenced by the presence of phenolic compounds was followed up by measuring the capacity of scavenging DPPH• (RSA %), the oxidation of β-carotene- linoleic acid o/w emulsion and as well as measuring oxidation stability by the automated active oxygen method (Rancimat).

Radical scavenging activity (RSA%)

The stable DPPH• is scavenged by accepting a hydrogen atom or an electron from the antioxidant and DPPH• transforms into its reduced form, DPPH-H [34-36]. DPPH• has a maximum UV–Vis absorbance at 516 nm. Decreasing the absorbance of DPPH solution indicates an increase in DPPH radical scavenging in terms of hydrogen-donating ability. The solution of the purple-colored DPPH radical changed to yellow-colored DPPH-H after reduction. The time taken for the initial DPPH• concentration to reach 50% is called TC_{50}. Decrease of TC_{50} indicates high RSA% and vice versa. TC_{50} of Egyptian and Chinese were shown in Fig. 1. As shown in Fig.1 the RSA% of dehulled seed extracts were higher (TC_{50} ranged 7.5-15.33 min) than whole seed extracts (TC_{50} ranged 10.5-18.5 min) for Egyptian and Chinese sunflower, respectively. Egyptian sunflower seeds revealed significantly (P<0.05) higher RSA% both in WS and DS than Chinese seeds.

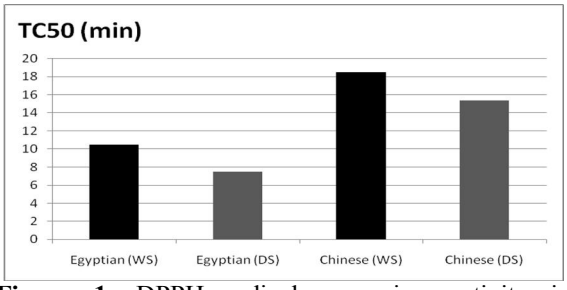

Figure 1. DPPH radical-scavenging activity in Egyptian and Chinese sunflower seed extracts expressed as TC_{50}; WS: whole seed; DS: dehulled seeds.

β-Carotene – linoleic acid assay

The oxidation stability of WS and DS of Egyptian and Chinese sunflower TPC extracts in emulsions, was assessed by the coupled oxidation of β-carotene and linoleic acid in o/w emulsion. The test is based on the fact that β-carotene undergoes rapid discoloration in the absence of antioxidant and during oxidation an atom of hydrogen is abstracted from the active methylene group of linoleic acid located on carbon-11 between the two double bonds[37, 38]. The pentadienyl free radical so formed then attacks highly unsaturated β-carotene molecules to reacquire an hydrogen atom. As the β-carotene molecules lose their conjugation, they lose their characteristic orange color. This process can be monitored spectrophotometrically[39]. The presence of phenolic antioxidant can hinder the extent of β-carotene degradation by neutralizing the linoleate free radical and any other radicals formed within the system. The rate of β-carotene bleaching by Egyptian (WS and DS), Chinese (WS and DS) sunflower seeds, TBHQ and α-tocopherol was shown in Fig.2. Among the six tested samples the least β-carotene bleaching (i.e. highest antioxidant activity) was recorded for TBHQ while highest β-carotene bleaching (i.e. least

antioxidant activity) was that of Chinese WS. The order of decreasing antioxidant as shown in Fig.2 was TBHQ>Egyptian DS> Egyptian WS > α-tocopherol >Chinese DS > Chinese WS.

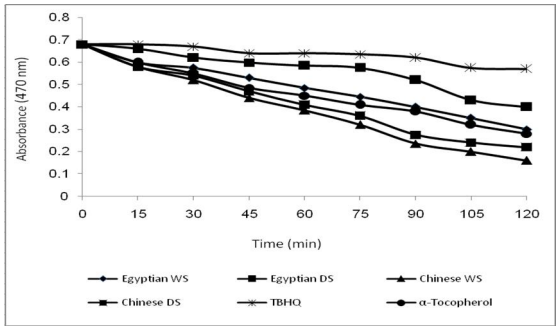

Figure 2. Effect of Egyptian and Chinese sunflower seed extracts on bleaching of β-carotene/linoleic acid w/o emulsion bleaching β-carotene/linoleic acid, WS: whole seed; DS: dehulled seeds.

Active oxygen method (Rancimat)

The measurement of fat and oil oxidation stability is commonly assessed by the fully automated version of active oxygen method available in Rancimat apparatus (Metrohm Ltd, Herisau, Switzerland) and is accepted as a standard method by American Oil Chemists' Society (AOCS Cd 12b-92) [10, 40-42].

Rancimat method determines the induction period by measuring the increase in volatile acidic by-products released from the oxidizing fat at 100-110 °C. The concentration of degradation products which are transferred into distilled water is monitored by measuring the conductivity. Longer induction periods suggest stronger activity of the added antioxidants.

It is clear that TBHQ revealed the highest protection as indicated by its longest induction period (12.3 hr) among all the tested samples, whereas the control (stripped sunflower oil without any addition) showed the least induction period (3.28 hr). The descending order of antioxidant capacity was TBHQ>Egyptian DS> Egyptian WS ≥ Chinese DS >Chinese WS >control.

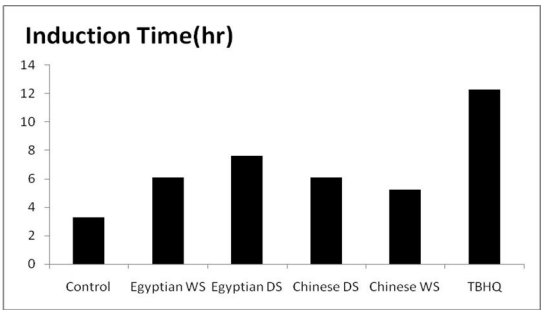

Figure 3. Effect of Egyptian and Chinese sunflower seed extracts on oxidation stability of stripped sunflower oil measured by Rancimat, WS: whole seed; DS: dehulled seeds.

Functional properties of defatted sunflower meals.

Proximate composition of the defatted meal of whole seed (DMWS) and defatted meal of dehulled seeds (DMDS) for both Egyptian and Chinese seeds are represented in Table 5.

Removal of the oil from WS meal and DS meal resulted in concentration of almost all other constituents specially protein.

Protein content was raised from 22.96 and 28.46% (Table 1) for Egyptian WS, and DS, respectively, to 45.36 and 51.43% protein for DMWS and DMDS (Table 5), respectively. While the Chinese WS and DS contained 21.22 and 26.69% protein respectively (Table 1), which increased upon defatting to 43.89 and 49.72% protein for DMWS and DMDS, respectively (Table 5). Other values in table 5 are self explanatory.

Apart from their nutritional properties, the functional properties of protein and protein products must be taken into account as stated by Finch [43]. Pour –El [44] had broadly defined functionality as any property of a food or food ingredient except its nutritional ones that affected its utilization. The range of desirable and attractive functional properties that should be looked for is almost as broad as the range of foods themselves. Some of the important functional properties were chosen and investigated for DMWS and DMDS protein, and their results are illustrated in Table 6.

Table 5. Proximate composition of defatted meal of whole seed (DMWS) and defatted meal of dehulled seed (DMDS) of both Egyptian and Chinese sunflower seeds

Parameters (%)	Egyptian		Chinese	
	DMWS*	DMDS*	DMWS	DMDS
Moisture	8.23±0.56	7.69±0.66	7.99±0.85	9.01±0.23
Protein	45.36±0.62	51.43±0.55	43.89±0.98	49.72±0.42
Oil	0.5±0.16	0.4±0.35	0.2±0.57	0.4±0.62
Ash	6.7±0.52	8.04±0.43	9.01±0.72	10.12±0.29
Crude fiber	35.91±0.64	18.23±0.86	34.69±0.39	20.12±0.65
Nitrogen free extract	3.3±0.01	14.21±0.34	4.22±0.41	10.63±0.33

*DMWS= defatted meal of whole seeds; DMDS= defatted meal of dehulled seeds

Table 6: Functional properties of defatted meal of whole seed (DMWS) and defatted meal of dehulled seed (DMDS) proteins of both Egyptian and Chinese sunflower seeds, as well as soybean meal for comparison.

Functional Properties	Egyptian		Chinese		Soya bean* meal
	DMWS	DMDS	DMWS	DMDS	
NSI (%)	5.5±. 23	7.9 ±.11	5.6 ±.35	6.9 ±.27	15.48 ±.66
PDI (%)	6.0 ±.44	9.4 ±.45	4.0 ±.36	15.5 ±.55	16.25 ±.51
WAC (%)	480 ±.35	450 ±.26	480 ±.16	460 ±.36	300 ±.32
OHC (%)	5.4 ±.72	6.3 ±.56	6.3 ±.32	7.14 ±.14	1.875 ±.71
EC (%)	20.0 ±.	20.8 ±.31	20.0 ±.55	20.8 ±.46	20.8 ±.42
GE (%)	1.0 ±.42	1.0 ±.37	1.0 ±.53	1.0 ±.12	3. ±.41
Foam Stability After					
40 Second	18.3 ±.34	12.85 ±.51	19.76 ±.31	10.86 ±.43	32.5 ±.33
50 Second	18.4 ±.25	15.67 ±.17	21.7 ±.11	14.13 ±.44	130.5 ±.54
60 Second	20.03 ±.55	20.03 ±.55	21.35 ±.53	8.7 ±.62	160.3 ±.66

*Taha and Ibrahim [46]. N.S.I: Nitrogen Solubility Index E.C.: Emulsifying Capacity
P.D.I.: Protein Dispersibility index O.H.C : Oil Holding Capacity ml oil to mg sample
W.A.C: Water absorption Capacity G.E: Gelation

Nitrogen Solubility Index (NSI)

NSI is a very important measure of the functionality of the proteins in different food systems, especially in fortifying nutritious beverages, instant foods, bakery products , salad dressings, soups and others. The American Dairy Products Institute emphasized that a high value of the NSI indicates that the product is less soluble. NSI of sunflower protein products indicate very good solubility of protein compared to soybean meal protein. NSI values for DMWS(Egyptian), DMWS (Chinese), DMDS (Chinese), DMDS (Egyptian),and soybean meal were 5.5, 5.6, 6.9, 7.9, and 15.48%, respectively.

Protein Dispersibility Index (PDI):

PDI is another criterion similar to NSI. It confirms the good solubility of sunflower protein. Soybean meal possessed 16.25% PDI, while DMDS (Chinese) had a close PDI 15.5% to soybean. On the other hand sunflower products, namely DMDS (Egyptian), DMWS (Egyptian), DMWS (Chinese) had 9.4, 6.0, and 4.0 % PDI, respectively, showing superiority to soybean protein.

Water absorption capacity (WAC):

It is the ability of a product to absorb water or swell. This property is important in the manufacture of bakery products, pastas, doughnuts and others. Sunflower protein products show better WAC than soybean meal protein thus is even more suitable to fortify the above mentioned products . DMDS (Chinese), DMWS (Egyptian), DMWS (Chinese), DMDS (Egyptian) showed 480, 480, 460, and 450% WAC, respectively, compared to 300 % WAC of soybean meal.

Oil Holding Capacity (OHC)

OHC is the ability of a protein to bind with oil. It is an important criterion in the meat industry (sausages, hamburgers etc.) OHC % for DMDS (Egyptian), DMDS (Chinese), DMWS (Egyptian), DMWS (Chinese), and soybean meal were 6.3, 7.14, 5.4, 6.3, and 1.875, respectively.

Emulsifying capacity (EC)

Emulsifying and film forming ability of plant proteins is essential for those proteins to perform well in meat systems. Also a protein's ability to form emulsion is critical to their application in mayonnaise, salad dressing, milks, and frozen desserts. EC of whole seed proteins is 20ml oil/100g sample which means less than soybean meal. EC of defatted meals is comparable to that of soybean meal (20.8ml oil/100g) sample. González –Pérez and Vereijken [46] reported that the emulsifying properties of sunflower protein, show very interesting perspectives to enhance their usage, as they seem at least comparable to those of soy protein.

Gelling Ability or Gelation (GE)

It is an important criterion as a protein's EC in comminuted meat systems. It is reported as the lowest concentration of protein that remained as a stable gel after 30 min at room temperature. Soybean meal gelled at 3% protein concentration while sunflower protein products gelled at 1% protein concentration which indicates better gelling properties. On the other hand González –Pérez and Vereijken ([46] reported that gelling properties of sunflower were not as promising as the EC.

Foam Stability (FS)

FS Is the capacity to form stiff, stable foam and is a requirement of proteins to be incorporated into gel cakes, whipped toppings, desserts and soufflé like products. Results in Table 6. reveal low foam stability of all sunflower protein products compared to soybean meal. Poor foaming properties of sunflower protein was in agreement with González – Pérez and Vereijken ([46]) concluded poor foaming properties for sunflower protein.

In conclusion Egyptian and Chinese sunflower seed and meals did not show much difference between the functional properties of their meal proteins.

Conclusion

This work assessed that dehulling of sunflower seeds either Egyptian or Chinese increase significantly total proteins, total fats (of which the majority is unsaturated), and total phenolics. Egyptian seed and oil was found to be superior to the Chinese ones in most chemical characteristics and in its content of protein, fat and antioxidant activity. Although, its production is insufficient to meet the consumption of edible oils Egyptian sunflower seed can be used efficiently in supplementation of many foods due to its superior protein, unsaturated fat, and natural antioxidants contents. On the other hand, Egyptian and Chinese sunflower seeds and meals did not show much difference between the functional properties of their meal proteins.

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Synthesis and molecular modeling study of novel pyrrole Schiff Bases as anti-HSV-1 agents

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Abstract: A series of novel pyrrole Schiff bases were synthesized by reaction of 2-amino-1,5-diaryl pyrrole-3-carbonitrile **1a-h** with different aromatic aldehydes using P_2O_5 as a catalyst to obtain **2a-p**, which were evaluated against herpes simplex virus type1 (HSV-1). The compounds **2d**, **2h**, **2m**, and **2n** were found to reduce the virus yield by 94-99 %, while compounds **2g**, **2k** and **2o** showed moderate activity (65-70%) compared to ACV (96%). The rest of the compounds were found to be inactive against HSV-1. Molecular modeling studies were carried out through docking the compounds in its most stable conformation into the active site of HSV-1 TK. The study revealed that the best fitted conformer was **2n** with a higher docking score (-7.32) and a better binding mode than ACV. The O-atom of the OCH_3 group formed two H-bond interactions with Arg^{176} and Tyr^{101}, hydrogen bonds were also formed with Arg^{163} and Tyr^{132} all of which are crucial amino acid residues for the enzyme activity, in addition to a H-bond interaction between the N^2-pyrazole and Gly^{59}. It could be suggested that the van der Waal interactions demonstrated by 1, 5-diaryl pyrrole located the molecule in close proximity to the active site Arg^{176}, Tyr^{101}, Arg^{163} and Tyr^{132}.

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Keywords: Pyrrole Schiff bases/HSV-1/HSV-Thymidine Kinase/Molecular modeling studies.

1. Introduction

Herpes simplex virus (HSV) causes herpes labialis, herpes keratitis, genetic herpes and life-threatening herpes encephalitis. HSV infections are more severe in immunocompromised patients, which are characterized by chronic and extensive lesions of the mucous membranes [1]. Most therapies directed against HSV infections are nucleotides, nucleosides or pyrophosphate analogues, such as acyclovir (ACV), valacyclovir, penciclovir and famciclovir. After uptake by virus-infected cells, these drugs are phosphorylated by virus-encoded thymidine kinase (TK), compete with the nucleotides to inhibit the viral DNA polymerase and subsequently cause the termination of growing viral DNA chains [2]. Although these drugs are effective in the treatment of many acute infections, the intensive use of these drugs has led to the emergence of resistant viral strains, mainly in immunocompromised patients. Inspite of the substantial advance in HSV therapy through the introduction of ACV, this anti-HSV compound and most of the other compounds under pharmaceutical development are substrate analogs of thymidine kinase [3]. It is important to note that TK is not a direct target of antiviral therapy because it is not required for virus replication; however viral TK activity may be required for reactivation of the virus from latency in the nerve cells [4]. Since antiviral drug resistance has become an issue of increasing clinical importance, the need for structurally unrelated agents which incorporate novel mechanisms of viral inhibition is apparent. Therefore, search for new non-nucleoside compounds as anti-HSV could be useful TK inhibitors. Pyrrolo[2,3-*d*]pyrimidines are purine analogs that display remarkable biological activities such as ant-inflammatory [5], anticancer[6-10], antimicrobial [11], antibiotics containing this moiety [12], antiasthmatic [13] and antiviral [14-15]. In addition, Tubercidine and Sangivamycin are pyrrolo[2,3-*d*]pyrimidine nucleoside antibiotics isolated from *Streptomyces* species that inhibit HSV replication [16]. Many bioactive natural products and synthetic drugs contain a pyrrole moiety as their key skeleton [17,18], subsequently highly substituted pyrroles has been one of the major targets in synthetic chemistry. Virtually, they were found to possess a wide variety of biological activities such as antibacterial[19], anticancer [20-23], antifungal [24], anti-inflammatory [25-28] and anti-oxidants [29].Many studies have reported on the antiviral activity of pyrroles [30-33], interestingly it was established that Congocidine and Distamycin are pyrroleamidine antibiotics isolated from *Streptomyces chromagens* that inhibits the multiplication of DNA virus HSV [34,35].Moreover, Schiff bases derived from pyrrole were effective antiviral agents [35].Schiff bases also deserve great interest due to their biological properties [36].The

above mentioned facts encouraged us to undertake the synthesis of some new Schiff bases of 2-amino-3-cyano-1,5-disubstitutedpyrrole to be evaluated as anti HSV-1.

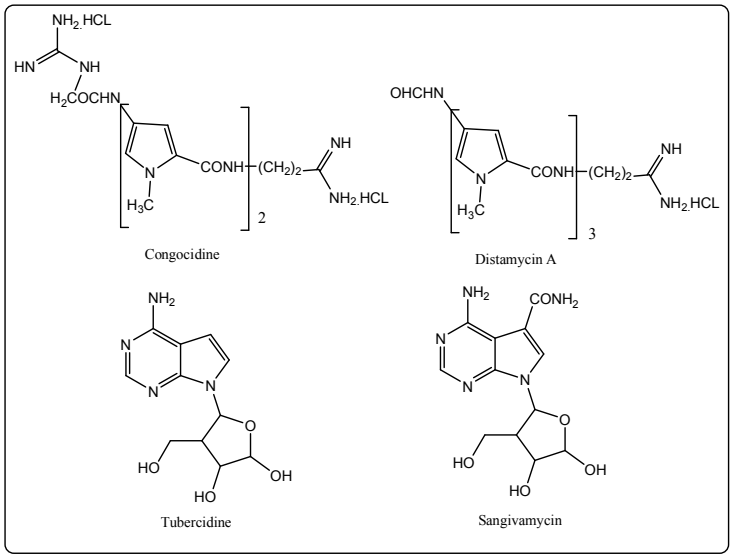
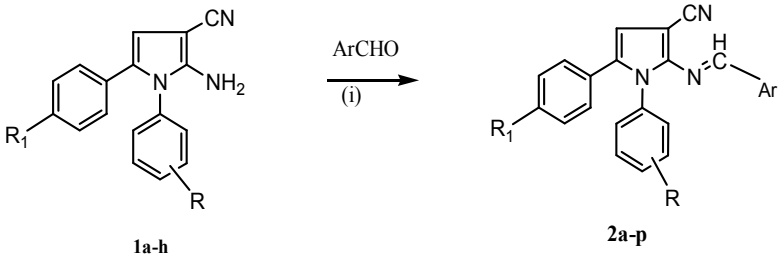


Figure 1. Structures of selective natural pyrrole and pyrrolopyrimidine lead compounds used as anti HSV-1.

2. Results and Discussion

The synthetic route utilized for the synthesis of the target compounds is outlined in Scheme 1. Condensation of 2-amino-3-cyano-1,5-diarylpyrroles 1a-h [37] with various aryl aldehydes in the presence of a catalytic amount of phosphorous pentoxide (P_2O_5)for 2-12 hrs yielded the Schiff bases 2a-p. The structures of the new compounds 2-arylideneamino-1,5-diaryl-1H-pyrrole-3 carbonitriles2a-p were confirmed by spectral data. The main feature characterizing their IR spectra is the presence of CN band at 2202-2212 cm^{-1}, the disappearance of the band corresponding to NH_2 group and the detection of a strong C=N stretching band at 1580-1645 cm^{-1} evidenced the formation of the Schiff base. Moreover, ^{1}H-NMR of the synthesized compounds revealed a singlet at around δ6.6 attributed to the pyrrole hydrogen, another singlet characterized the ^{1}H-NMR spectra at approximately δ 9.1 corresponding to the imine hydrogen. The ^{13}C{1H} NMR spectrum of 2j in DMSO-d_6 showed downfield signals at δ 162.7 ppm (C_2-naphthyl), 159.8 ppm (C-imine), 157.8 ppm (C_4'-phenyl) as well as a signal to up field at δ 55.5 ppm (OCH_3). It is worth mentioning here that, the use of the strong dehydrating agent phosphorous pentoxide (P_2O_5) in catalytic amount allowed the reaction to proceed forward in much less time (TLC monitored) and significantly higher yields. Different structural aldehydes were used to conduct a comparative study on the time of the reaction as well as its yield. The reaction of O-vanillin and salisaldehyde took place easily with good yield especially when pyrrole derivatives with substitution on two phenyl group were in para position. The reactions of cinnamaldehyde were faster and gave better yield than the pyrazole-4-carbaldehyde derivatives.



Compound No.	R	R_1
1a	4-Cl	H
b	3-CF_3	H
c	4-OCH_3	4-Cl
d	H	4-Br
e	4-CH_3	H
f	H	H
g	4-Br	H
h	4-OCH_3	H

(i)Absolute ethanol, P_2O_5, reflux.

Scheme1. Synthesis of Schiff bases of 2-amino-3-cyano-1,5-diaryl-pyrroles.

3. Antiviral Screening

The newly synthesized compounds **2a-p** were evaluated for their *in vitro* antiviral activity against herpes simplex virus type 1. The antiviral screening was performed using the plaque reduction assay against HSV-1 in vero cell; ACV was used as the standard drug showing 96% reduction in the number of virus plaques. Table 1 summarizes the anti HSV-1 activity of these compounds with respect to Acyclovir. Compounds **2d**, **2h**, **2m**, and **2n** reduced the virus yields by 99-94 %. Both **2d** and **2n**proved to be the most active among the tested compounds and even more than ACV by reducing the number of HSV-1 plaques by 99% and 97% respectively. The antiviral activity of compounds **2h** and **2m** was comparable to the standard ACV, 96% and 94% respectively. Compounds **2g**, **2k** and **2o** showed moderate activity (65-70%) compared to ACV (96%). The rest of the compounds were found to be inactive against HSV-1.Extensive SAR exploration of this novel series showed that the 5-pyrrole position of the most active compounds **2d** and **2n** was occupied by a 4-methoxyphenyl, the highest activity was achieved by compound **2d** which also had a 4-methoxybenzylideneamino in position 2 of the pyrrole ring. However, a total loss in the activity was conferred when the 4-methoxybenzylideneamino was replaced by 2-hydroxy-4-methoxybenzylideneamino. The high activity of **2n** could be attributed to the combination of the 4-methoxyphenyl and the pyrazole ring which itself has reported antiviral activity [38].The Schiff base derived from cinnamaldehyde **2g** expressed moderate antiviral activity thus, we aimed to enhance the activity by replacing the 5-phenylpyrrole with a 4-methoxyphenl counterpart, unfortunately this resulted in an inactive compound **2i**, while 5-(4-bromophenyl) pyrrole**2h** showed the same activity as ACV. The moderate activity of compounds **2k** and **2o** could be attributed to the reported antiviral activity of quinoline and pyrazole [38,39]. Bulky rigid structures as **2j** and **2l** were devoid of antiviral activity. The results indicate that there is no main substituent on the pyrrole that is responsible for the activity, instead all those substituents work together to bring up activity and help position the compound in the best fit into the enzyme.

The experimental concentrations of the 1,5-diaryl-2-(ylideneamino)-1*H*-pyrrole-3-carbonitriles (**2a-p**) were 50 µM and ACV concentration was 10 µM.

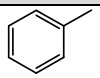
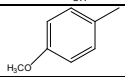
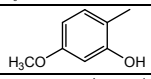
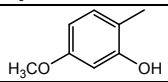
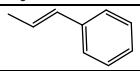
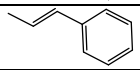
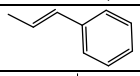
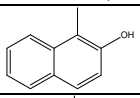
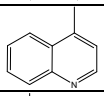
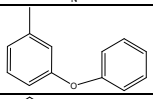
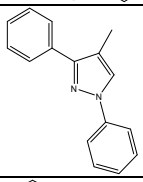
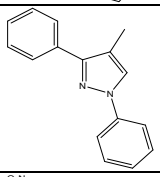
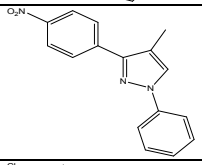
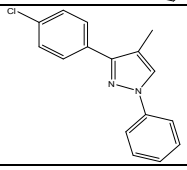
a) % per cent of reduction = [1-(t/c)] x 100
b) NA: Non active
c) ACV: acyclovir has been included for comparison purposes

1. Docking Studies

Thymidine kinase acts catalytically to phosphorylate thymidine, getting it ready for further phosphorylation and eventual incorporation into DNA [40].Thus, the research into the binding capabilities of TK and other ligands can serve to develop more effective HSV treatment that would act as inhibitors of this enzyme rather than substrates. In the present study, the ligand-receptor interactions of compounds **2d, 2h, 2m** and **2n** with herpes simplex virus type-1 thymidine kinase were investigated by performing docking studies using Molecular Operating Environment (MOE)version 2008.10[41].The crystal structure of herpes simplex virus type-1 thymidine kinase in complex with acyclovir (PDB code 1KI5) [42] was retrieved from the Protein Data Bank [43] and the accuracy of MOE docking protocol was validated and confirmed by docking the co-crystallized acyclovir inside the active site of thymidine kinase where the docked acyclovir showed 1.7841 Å root-mean-squared deviation fig.2.The compounds were docked in their most stable conformation in the active site of HSV-1 TK and subjected to energy minimization. The docking scores are presented in Table 2.

The docking interaction of compound **2d** with the active site of thymidine kinase is represented in figure 3. It was observed that the oxygen of the two methoxy groups formed H-bond acceptor interactions with Lys^{62} (2.8 Å) and Tyr^{132} (3.2 Å) two of the amino acids in the active site of the enzyme [40, 42, 44, 45], Thr^{63} (2.7, 3.6 Å) in addition to H-bond acceptor interaction between the cyano nitrogen and Arg222 (2.8 Å). Hydrophobic interactions between pyrrole and His^{58} as well as the phenyl ring with Arg^{222} and Lys^{62} appear to constrain the molecule in close proximity with the amino acids forming the forementioned hydrogen bonding. Moreover, compound **2d** formed van der Waals interactions with non polar atoms of (Trp^{88}, Ile^{100}, Tyr^{101}, Gln^{125}, Met^{128}, Tyr^{132}, Asp^{162}, Arg^{163}, Ala^{168}, Tyr^{172}, Arg^{176} and Arg^{222}) similar to those reported for the ligands deoxythymidine (dT) and ACV [40,42,45], in addition to hydrophobic interactions with (Glu^{83}, Glu^{225}, Gly^{59}, Gly^{61}, Ile^{97}).

Table 1. The Anti-HSV-1 activity of the 1,5-diaryl-2-(ylideneamino)-1*H*-pyrrole-3-carbonitriles (2a-p)

Compound No.	R	R_1	Ar	Cytotoxicity of compound	% Reduction in cytopathic effect of HSV-1[a]
2a	4-Cl	H		10^{-2}	NA [b]
2b	4-Cl	H		10^{-2}	NA
2c	3-CF_3	H		10^{-2}	NA
2d	4-OCH_3	4-Cl		10^{-2}	99
2e	H	4-Br		10^{-2}	NA
2f	4-CH_3	H		10^{-2}	NA
2g	H	H		10^{-2}	65
2h	4-Br	H		10^{-2}	96
2i	4-OCH_3	4-Cl		10^{-2}	NA
2j	4-OCH_3	4-Cl		10^{-2}	NA
2k	H	4-Br		10^{-2}	67
2l	H	H		10^{-2}	NA
2m	H	H		10^{-2}	94
2n	4-OCH_3	H		10^{-2}	97
2o	H	H		10^{-2}	70
2p	4-Br	H		10^{-2}	NA
ACV[c]	-	-	-	10^{-2}	96

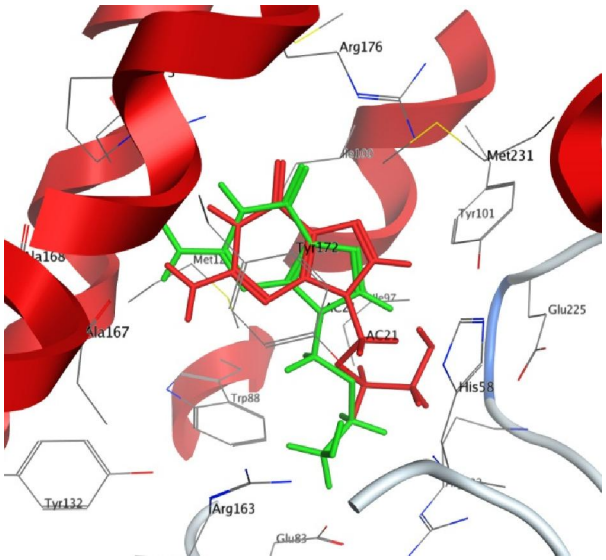


Figure 2. Comparison between the co-crystallized acyclovir (red) and Docked acyclovir (green).

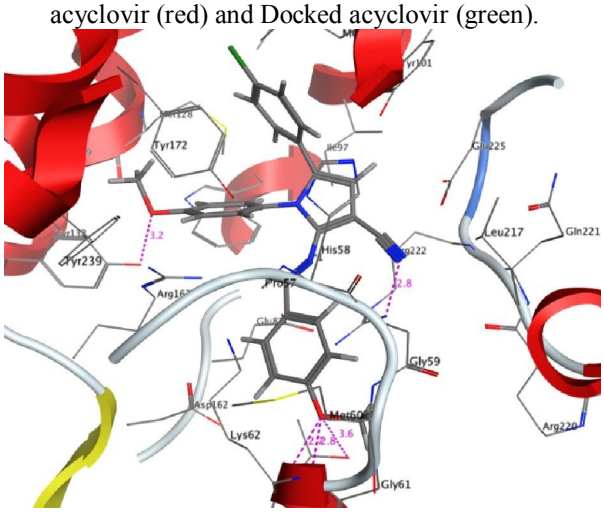


Figure 3. Interaction of compounds **2d** with thymidine kinase.

Table 2. **Docking scores of the acyclovir, 2d, 2h, 2m and 2n.**

Compound	Docking Score
Acyclovir	-12.74
2d	-11.30
2h	-11.44
2m	-8.98
2n	-7.32

The binding mode observed for compound **2n** shows that it binds within the deoxythymidine binding pocket with a docking score better than ACV (-7.32), the oxygen of the OCH_3 forming two hydrogen bonds, one with Arg^{176}(2.8Å), a crucial residue in the TK binding site, and another one with Tyr^{101} (2.8 Å) that resembles the bond made by the 3' O of dT [42,46]. The imine nitrogen establishes a hydrogen bond with the residue Arg^{163}(2.6Å), which is responsible for making hydrogen bond interaction with the 5' O of dT [40, 46]. Additionally the cyano nitrogen forms a hydrogen bond with Tyr^{132}(3.2 Å) and another one is observed between theN^2-pyrazoleand with Gly^{59} (3.0Å). Further stabilization is obtained by hydrophobic interactions with His^{58} and by van der Waal interactions between (Tyr^{132}, Arg^{163}, Ala^{168}, Trp^{88}, Met^{231}, Met^{128},Tyr^{172}) as reported [40,42,45], other hydrophobic interactions were also observed withGly^{59}, Gly^{61}, Lys^{62}, Thr^{63}, Thr^{64}, Glu^{83}, Ile^{97}, Tyr^{101}, Gln^{125}, Asp^{162}, Arg^{176}, Arg^{220}andGlu^{225} (figure 4).It is worth mentioning here, that Arg^{222} formed strong hydrophobic interactions with 1,3-diphenyl pyrazole moiety that could be responsible for bringing the molecule into close proximity with the crucial active sites of the enzyme.

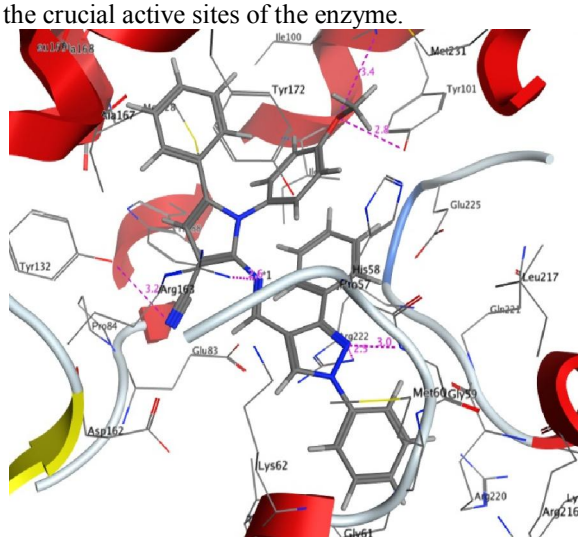


Figure 4. Interaction of compounds **2n** with thymidine kinase

Given that the pyrrole derivative **2n** presentation is similar to that of dT and Acyclovir, and that ACV is considered a weak substrate [47], the potential phosphate-binding function (the hydroxyl group) was replaced by a methoxy group. It would appear that its inhibitory properties arise from the much greater binding enthalpy that the van der Waals interactions of the additional hydrophobic moieties give rise to. The high affinity, arising, no doubt, from these interactions, may restrict the flexibility of the complex to allow phosphorylation of the ligand [42].In case of compound **2h** (supplementary data), only hydrophobic interactions were observed with His^{58}, Gly^{59}, Lys^{62}, Thr^{63}, Glu^{83}, Trp^{88}, Ile^{97}, Ile^{100}, Tyr^{101}, Met^{128}, Tyr^{132}, Asp^{162}, Arg^{163}, Ala^{167}, Ala^{168}, Tyr^{172}, Arg^{176}, Arg^{222} and Glu^{225}. Similarly, compound **2m** showed only hydrophobic interactions with His^{58}, Gly^{59}, Lys^{62}, Thr^{63}, Glu^{83}, Trp^{88}, Ile^{97}, Ile^{100}, Tyr^{101}, Gln^{125}, Met^{128}, Tyr^{132}, Asp^{162}, Arg^{163}, Ala^{167}, Ala^{168}, Tyr^{172}, Arg^{176}, Arg^{222} and Glu^{225}(supplementary data). Compounds **2h** and **2m**

although possessed a significant antiviral activity and their docking scores were comparable to ACV yet their docking into TK did not show any hydrogen bonding interactions in the binding site of the enzyme, suggesting that the compounds may have another mechanism for their antiherpetic activity.

Conclusion

In this article, a series of new pyrrole Schiff bases**2a-p** has been synthesized. The new pyrrole Schiff bases **2a-p** were tested for anti HSV-1 activity in vero cell. The tested compounds **2d** and **2n** demonstrated potent antiviral activity against HSV-1 more than the standard drug. Also, the compounds**2h** and **2m** exhibited antiviral activity comparable to the standard drug. However, the rest of the pyrrole Schiff bases didn't have such an influence on activity. Molecular modeling studies showed that the binding mode of compound **2n**, in spite of its size difference, was very similar to the previously reported ACV and dT. Thus, **2n** could be a potential ligand to target /inhibit TK of herpes simplex virus.

2. Experimental

2.1. General

Melting points (^{o}C uncorrected) were recorded with a Gallenkamp apparatus (Weiss-Gallenkamp, London, UK). The IR spectra were recorded on KBr pellets on a Jasco FT/IR 460 plus (Japan). ^{1}HNMR and ^{13}CNMR spectra were recorded on Varian Gemini spectrophotometer (200 MHz) in DMSO-d_6 or $CDCl_3$ as solvent, using tetramethyl-silane (TMS) as internal reference standard. The chemical shifts values are expressed in ppm (parts per million). Elemental analyses were performed by a Vario III CHN analyzer (Germany). All compounds were within ± 0.4% of the theoretical values. Mass spectra were performed on DI analysis Shimadzu QP-2010 plus mass spectrometer. All spectroscopic data and elemental analysis were made at the Micro analytical center, Cairo University, Egypt. The progress of the reaction and purity of the compounds were monitored by TLC analytical silica gel plates 60 F_{254} (E. Merck Germany) using the appropriate eluent. The chemical reagents used in synthesis were purchased from Fluka, Sigma and Aldrich.

2.2. Synthesis of compounds 1,2

2.2.1. General procedure for the synthesis of compounds 1a-h[37]

Derivatives of phenacylmalononitrile were refluxed with different anilines in ethanol in the presence of conc. HCl to give pyrrole derivatives **1a-h**.

2.2.2. General procedure for the synthesis of 2-(arylideneamino)-1,5-diaryl-*1H*-pyrrole-3 carbonitriles 2a-p

A mixture of **1a-h** (0.01mol) and various aromatic aldehydes (0.01mol) in ethanol (20 mL), in the presence of catalytically amount of P_2O_5 was refluxed for 2-12 hrs. The separated solid was filtered and recrystallized from ethanol.

2.2.2.1. 2-(Benzylideneamino)-1-(4-chlorophenyl)-5-phenyl-*1H*-pyrrole-3-carbonitrile (2a)

Yield 80%; m.p. 164-166 ^{0}C. IR (KBr) ν cm^{-1}: 2208 (CN); 1580 (C=N).^{1}HNMR spectrum ($CDCl_3$).δ ppm: 6.65 (s, 1H, $CH_{pyrrole}$); 7.11–7.75 (m, 14H, Ar-H); 9.12(s, 1H, -N=CH). MS m/z (%): 381(M^+, 19).Anal. Calcd for $C_{24}H_{16}ClN_3$ (381.86): C, 75.49; H, 4.22; N, 11.00.Found: C, 75.45; H, 4.25; N, 11.03.

2.2.2.2. 1-(4-Chlorophenyl)-2-(2-hydroxybenzylideneamino)-5-phenyl-*1H*-pyrrole-3-carbonitrile (2b)

Yield 81%; m.p. 176-178 ^{0}C. IR (KBr) νcm^{-1}: 2207 (CN); 3413 (OH); 1591 (C=N).^{1}HNMR spectrum ($CDCl_3$).δ ppm: 6.64 (s, 1H, $CH_{pyrrole}$), 7.10–7. 54 (m, 13H, Ar-H); 9.18 (s, 1H, -N=CH); 11.34(s, 1H, OH). MS m/z (%): 398 (M^+, 32); 400 (M^{+2}, 2).Anal. Calcd for$C_{24}H_{16}ClN_3O$ (397.86): C, 72.45; H, 4.05; N, 10.56. Found: C, 72.48; H, 4.08; N, 10.50.

2.2.2.3. 2-(2-Hydroxybenzylideneamino)-5-phenyl-1-3-(trifluoromethyl)-5-phenyl-*1H*-pyrrole-3-carbonitrile (2c)

Yield 78%; m.p. 178-180 ^{0}C. IR (KBr) νcm^{-1}: 2210 (CN); 3410 (OH); 1599 (C=N).^{1}HNMR spectrum ($CDCl_3$).δ ppm: 6.67 (s, 1H, $CH_{pyrrole}$); 7.11–7. 61 (m, 13H, Ar-H); 8.92 (s, 1H, -N=CH); 11. 29 (s, 1H, OH). MS m/z (%): 431 (M^+, 100).Anal. Calcd for $C_{25}H_{16}F_3N_3O$ (431.41): C, 69.60; H, 3.74; N, 9.74. Found: C, 69.57; H, 3.76; N, 9.72.

2.2.2.4. 5-(4-Chlorophenyl)-1-(4-methoxyphenyl)-2-(4-methoxybenzylideneamino)-1H-pyrrole-3-carbonitrile (2d)

Yield 83%; m.p. 220-222 ^{0}C. IR (KBr) νcm^{-1}: 2206 (CN); 1620 (C=N). ^{1}HNMR spectrum ($CDCl_3$).δ ppm: 3.92(s, 6H, $2OCH_3$); 6.68(s, 1H, $CH_{pyrrole}$); 7.00–7. 54 (m, 12H, Ar-H); 9.11(s, 1H, -N=CH). MS m/z (%): 441 (M^+, 32); 443 (M^{+2}, 21).Anal. Calcd for $C_{26}H_{20}ClN_3O_2$ (441.91): C, 70.67; H, 4.56; N, 9.51.Found: C, 70.70; H, 4.59; N, 9.48.

2.2.2.5. 5-(4-Bromophenyl)-2-(2-hydroxy-4-methoxybenzylideneamino)-1-phenyl-1H-pyrrole-3-carbonitrile (2e)

Yield 80 %; m.p. 218-220 ^{0}C. IR (KBr) νcm^{-1}: 2210 (CN); 3412(OH); 1643 (C=N).^{1}HNMR spectrum ($CDCl_3$).δ ppm: 3.89 (s,3H, OCH_3); 6.70 (s, 1H, $CH_{pyrrole}$); 7.00–7. 52 (m, 12H, Ar-H); 9.33(s, 1H, -N=CH); 11. 27(s, 1H, OH).MS m/z (%): 471 (M^+, 26); 473 (M^{+2}, 15).Anal. Calcd for $C_{25}H_{18}BrN_3O_2$ (472.33): C, 63.57; H, 3.84; N, 8.90. Found: C, 63.54; H, 3.87; N, 8.88.

2.2.2.6. **2-(2-Hydroxy-4-methoxybenzylideneamino)-5-phenyl-1-(p-tolyl)-*1H*-pyrrole-3-carbonitrile (2f)**

Yield 79 %; m.p. 230-232 ^{0}C. IR (KBr) νcm^{-1}: 2212 (CN); 3408 (OH); 1592 (C=N).^{1}HNMR spectrum (DMSO-d_6).δ ppm:2.37 (s, 3H, CH_3); 3.82 (s, 3H, OCH_3); 6.69 (s, 1H,$CH_{pyrrole}$); 7.00–7.26 (m, 12H, Ar-H); 9.26 (s, 1H, -C=NH); 11.29 (s,1H,OH). MS m/z (%): 407 (M^+, 100).Anal. Calcd for $C_{26}H_{21}N_3O_2$ (407.46): C, 76.64; H, 5.19; N, 10.31.Found: C, 76.63; H, 5.16; N, 10.29.

2.2.2.7. **1,5-Diphenyl-2-(3-phenylallylideneamino)-1H-pyrrole-3-carbonitrile (2g)**

Yield 75%; m.p. 184-186 ^{0}C. IR (KBr) νcm^{-1}: 2210 (CN); 1609 (C=N). ^{1}HNMR spectrum (DMSO-d_6).δ ppm: 6.65 (s, 1H, $CH_{pyrrole}$); 7.07(t, 1H, CH=CH); 7.10–7.41 (m, 15H, Ar-H); 7.70-7.72 (d, 1H, CH=CH- Ph); 8.77-8.83 (d, 1H, -N=CH). MS m/z (%): 373 (M^+, 100).Anal. Calcd for $C_{26}H_{19}N_3$ (373.45): C, 83.62; H, 5.13; N, 11.25. Found: C, 83.65; H, 5.10; N, 11.28.

2.2.2.8. **1-(4-Bromophenyl)-5-pheny-2-(3-phenylallylideneamino)-*1H*-pyrrole-3-carbonitrile (2h)**

Yield 74 %; m.p. 208-210 ^{0}C. IR (KBr) νcm^{-1}: 2210 (CN); 1622 (C=N). ^{1}HNMR spectrum (DMSO-d_6).δ ppm: 6.69 (s, 1H, $CH_{pyrrole}$); 7.08 (t, 1H, CH=CH); 7.11–7.54 (m, 14H, Ar-H); 7.70-7.72 (d, 1H, CH=CH-Ph); 8.75-8.80 (d, 1H, HC=N).MS m/z (%): 452 (M^+, 37); 454 (M^{+2}, 8).Anal. Calcd for $C_{26}H_{18}BrN_3$ (452.35): C, 69.04; H, 4.01; N, 9.29.Found: C, 69.00; H, 3.99; N, 9.27.

2.2.2.9. **5-(4-Chlorophenyl)-1-(4-methoxyphenyl)-2-(3-phenylallylideneamino)-*1H*-pyrrole-3-carbonitrile (2i)**

Yield 80 %; m.p. 252-254 ^{0}C. IR (KBr) νcm^{-1}: 2210 (CN); 1588 (C=N). ^{1}HNMR spectrum (DMSO-d_6).δ ppm: 3.80 (s, 3H, OCH_3); 6.91 (s, 1H, $CH_{pyrrole}$); 7.07(t, 1H, CH=CH); 7.11–7.40 (m, 13H, Ar-H); 7.70-7.72 (d, 1H, CH=CH- Ph); 8.61-8.70 (d, 1H, HC=N). MS m/z (%): 437 (M^+, 35), 439 (M^{+2}, 12).Anal. Calcd for $C_{27}H_{20}ClN_3O$ (437.92): C, 74.05; H, 4.60; N, 9.60.Found: C, 74.04; H, 4.63; N, 9.57.

2.2.2.10. **5-(4-Chlorophenyl)-2-[(2-hydroxynaphthalen-1-yl)methyleneamino]-1-(4-methoxyphenyl)-*1H*-pyrrole-3-carbonitrile (2j)**

Yield 79%; m.p. 237-239 ^{0}C. IR (KBr) νcm^{-1}: 2208 (CN); 3396 (OH); 1614 (C=N). ^{1}HNMR spectrum (DMSO-d_6).δ ppm: 3.83 (s, 1H, OCH_3); 6.72 (s, 1H, $CH_{pyrrole}$); 7.11-8.28 (m, 14H, Ar H); 10.11 (s, 1H, -N=CH); 12.93 (s, 1H, OH). ^{13}CNMR ($CDCl_3$) δ ppm: 55.53 (OCH_3); 111.94 (C_1-naphthyl); 114.84 (CN); 119.10, 120.00, 124.11, 128.01, 128.68, 129.31 (C-arom); 131.05 (N-C_1-phenyl); 132.87(C_4-pyrrole); 135.98(C-Cl); 157.89 (C-OCH_3); 159.86 (C=N); 162.73 (HO-C_2-naphthyl). MS m/z (%): 477 (M^+, 100); 479(M^{+2}, 33).Anal. Calcd for $C_{29}H_{20}ClN_3O_2$ (477.94): C, 72.88; H, 4.22; N, 8.79.Found: C, 72.86; H, 4.23; N, 8.81.

2.2.2.11. **5-(4-Bromophenyl)-1-phenyl-2-[(quinolin-4-yl)methyleneamino]-*1H*-pyrrole-3-carbonitrile (2k)**

Yield 72 %; m.p. 254-256 ^{0}C. IR (KBr) νcm^{-1}: 2211 (CN); 1609 (C=N). ^{1}HNMR spectrum ($CDCl_3$).δ ppm: 6.69 (s, 1H, $CH_{pyrrole}$); 7.22–8.10 (m, 15H, Ar-H); 8.34 (s, 1H, -N=CH); 9. 32 (s, 1H, N=$CH_{quinoline}$). MS m/z (%): 476 (M^+, 16); 478 (M^{+2}, 21).Anal. Calcd for $C_{27}H_{17}BrN_4$ (477.35): C, 67.93; H, 3.59; N, 11.74.Found: C, 67.90; H, 3.62; N, 11.70.

2.2.2.12. **1,5-Diphenyl-2-(3-phenoxybenzylideneamino)-*1H*-pyrrole-3-carbonitrile (2l)**

Yield 70 %; m.p. 186-188 ^{0}C. IR (KBr) νcm^{-1}: 2208 (C≡N); 1607 (C=N). ^{1}HNMR spectrum ($CDCl_3$).δ ppm: 6.70 (s, 1H, $CH_{pyrrole}$); 7.01–7. 74 (m, 19H, Ar-H); 8.32 (s, 1 H, - N=CH). MS m/z (%): 439 (M^+, 79).Anal. Calcd for $C_{30}H_{21}N_3O$ (439.51): C, 81.98; H, 4.82; N, 9.56.Found: C, 81.97; H, 4.80; N, 9.63.

2.2.2.13. **2-[(1,3-Diphenyl-1H-pyrazol-4-yl)methyleneamino]-1,5-diphenyl-*1H*-pyrrole-3-carbonitrile (2m)**

Yield 74 %; m.p. 204-206 ^{0}C. IR (KBr) νcm^{-1}: 2209 (CN); 1644 (C=N). ^{1}HNMR spectrum (DMSO-d_6).δ ppm: 6.66 (s, 1H, $CH_{pyrrole}$); 7.12-7.97 (m, 20H, Ar-H); 8.63 (s, 1H, -N=CH); 9.17 (s, 1H, $CH_{pyrazole}$). MS m/z (%): 489 (M^+, 91).Anal. Calcd for $C_{33}H_{23}N_5$ (489.57): C, 80.96; H, 4.74; N, 14.31.Found: C, 80.93; H, 4.70; N, 14.28.

2.2.2.14. **2-[(1,3-Diphenyl-1H-pyrazol-4-yl)methyleneamino]-1-(4-methoxyphenyl)-5-phenyl-*1H*-pyrrole-3-carbonitrile (2n)**

Yield 75%; m.p. 202-204 ^{0}C. IR (KBr) νcm^{-1}: 2209 (CN); 1599 (C=N). ^{1}HNMR spectrum (DMSO-d_6).δ ppm: 3.82 (s, 3H, OCH_3); 6.71 (s, 1H, $CH_{pyrrole}$); 7.11-7.67 (m, 19H, Ar-H); 8.42 (s, 1H, CH =N); 9.15 (s, 1H, $CH_{pyrazole}$). MS m/z (%): 519 (M^+, 30).Anal. Calcd for $C_{34}H_{25}N_5O$ (519.60): C, 78.59; H, 4.85; N, 13.48.Found: C, 78.56; H, 4.82; N, 13.44.

2.2.2.15. **1,5-Diphenyl-2-[(3-(4-nitrophenyl)-1-phenyl-1H-pyrazol-4-yl)methyleneamino]-*1H*-pyrrole-3-carbonitrile (2o)**

Yield 77%; m.p. 260-262 ^{0}C. IR (KBr) νcm^{-1}: 2209 (CN); 1645 (C=N). ^{1}HNMR spectrum (DMSO-d_6).δ ppm: 6.71 (s, 1H, $CH_{pyrrole}$); 7.11-7.97 (m, 19H, Ar-H); 8.23 (s, 1H, CH =N); 9.17 (s, 1H, $CH_{pyrazole}$). MS m/z (%): 534 (M^+, 100).Anal. Calcd for $C_{33}H_{22}N_6O_2$ (534.57): C, 74.14; H, 4.15; N, 15.72.Found: C, 74.16; H, 4.14; N, 15.69.

2.2.2.16. **1-(4-Bromophenyl)-2-[(3-(4-chlorophenyl)-1-phenyl-*1H*-pyrazol-4-yl)methyleneamino]-5-phenyl-1H-pyrrole-3-carbonitrile (2p)**

Yield 73%; m.p. 210-212 ^{0}C. IR (KBr) νcm^{-1}: 2209 (CN); 1602 (C=N).^{1}HNMR spectrum (DMSO-d_6).δ ppm: 6.81 (s, 1H, $CH_{pyrrole}$); 7.12-7.97 (m, 18H, Ar-H); 8.26 (s, 1H, -N=CH); 9.19 (s, 1H, $CH_{pyrazole}$). MS m/z (%): 603 (M^+, 27), 605 (M^{+2}, 8).Anal. Calcd for $C_{33}H_{21}BrClN_5$ (602.91): C, 65.74; H, 3.51; N, 11.62.Found: C, 65.71; H, 3.55; N, 11.65.

6.3. Antivirus activity

2.3. 1. Cells and virus

Herpes simplex Virus type-1 (HSV-1) was kindly supplied by The National Research Center, Dokki, Egypt. The virus was propagated in Vero cells (cells isolated from the African green monkey), which were commercially obtained from The Egyptian Organization of Serum and Vaccines. Minimal essential medium (MEM) was used as growth and maintenance medium for tissue culture with 10% or 2% fetal calf serum (FCS), respectively.

6.3.2. Titration of HSV-1 by plaque assay [48]

Cell suspension (3 ml) with the concentration 10^5 cells/ml GM was dispensed into each of the wells of 6-well plates (Falcon). Plates were incubated at 37^{o}C in a 0.5% CO_2 for 24–48 hrs to obtain a confluent sheet. Ten-fold dilution of the virus stock (10^{-1}–10^{-7}) was done as mentioned under titration of HSV by $TCID_{50}$ endpoint. Then growth medium was aspirated from the wells and 0.2 mL/well of each virus dilution was added in duplicate manner. For each plate, two wells were left uninoculated as cell control, each contained 0.2 mL MEM only; incubation at 37^{o}C in a 0.5% CO_2 incubator for one hour, to permit virus adsorption. Then 30 ml 1% agarose solution was melted at 48^{o}C in a water bath, and mixed with 2 x 30 mL MEM (supplied with 1% antibiotic solution and 2% FCS) in a ratio of 1:1 to prepare an overlay mixture. Then 3 ml of overlay mixture was added quickly to each well in the plate. After overlay solidification, plates were inverted and incubated at 37^{o}C in a 0.5% CO_2 incubator. Daily observation was carried out for early plaque detection; usually it takes 2–3 days for plaque detection. After plaques development, cells were fixed by flooding with 10% formalin solution for 1 h at room temperature. Agarose overlay was removed with forceps; cell monolayers were washed under tap water and stained with 10% crystal violet solution for 10 min. Plaques were recorded as clear unstained areas against a violet background of stained viable cells and counted either visually or using a stereomicroscope. The infectivity titer represented as number of plaque forming unit/mL (PFU/mL) of the stock virus suspension. It was calculated form the following equation: PFU/mL = No. of plaques x reciprocal of dilution x reciprocal of volume in mL.

6.3.3. Determination of the solvent Cytotoxicity

Growth medium was decanted from 96 well micro titer plate after a confluent sheet of cells was formed. Ten-fold serial dilutions of 2-(arylideneamino)-1,5-diaryl-1*H*-pyrrole-3-carbonitriles were made in MEM medium without FCS, starting from 5000 µm/mL till 10^{10} dilution, 0.2 ml of each dilution was tested in 3 different wells and leaving two wells/row as control receiving only maintenance medium. The plate was incubated in a CO_2 incubator at 37^{o}C and examined for up to 3 days. Cells were checked for any physical signs of toxicity, partial or complete loss of the monolayers, shrinkage, or cell granulation. Then, the non-toxic concentration of each substance was used in this study.

6.3.4. HSV-1 yield reduction assay

Vero cells were seeded into 96-well tissue culture plates at a concentration of 10,000 cells in 0.2 ML of minimal essential medium with Earle salts, MEM(E), supplemented with 10% FCS, and incubated at 37^{o}C in a humidified 3% CO_2 – 97% air atmosphere. After 24 hrs, plates were inverted over a waste vessel, medium was shaken out, and the plates were allowed to drain for 5 to 10 s on a sterile paper towel. Cultures were incubated with HSV-1 at a multiplicity of infection (MOI) of 5 PFU/cell in 0.2 mL of MEM (E) supplemented with 5% FCS, 100 U penicillin/mL, and 100 µg streptomycin sulfate/mL. Cultures were incubated at 37^{o}C for 2 hrs to permit virus adsorption. Virus inoculum's was replaced with 0.2 Ml of fresh medium and test compounds were added to the cultures.

The first row of 12 wells was left undisturbed and served as virus controls. Each well in the second row received an additional 0.1 mL of MEM (E) containing 5% FCS, antibiotics, and test compound at three times the desired final concentration. The contents of the 12 wells were mixed by repeated pipetting and then serially diluted 1:3 down the plate by repeated transfer and mixing of 0.1 mL of drug-containing medium. In this manner, six compounds could be tested in duplicate on a single plate with a concentrations range of nearly 1000-fold between the highest and the lowest dilutions (0.10 µM to 80 µM minuscule example). Plates were incubated at 37^{o}Cover night and then subjected to one cycle of freezing at –76^{o}C and thawing at 37^{o}C to disrupt the cells. Aliquots of 0.1 mL from each of the eight wells of a given row were transferred to the row of a fresh 96-well monolayers culture of Vero cells. Contents were mixed and serially diluted 1:3 across the remaining 11 rows of the second plate. Each row of the original primary plate was diluted across a separate plate in this manner. A culture was incubated

at 37°C for 2 h to permit virus adsorption and then the virus inoculum's was replaced with 0.2 mL of fresh medium. Cultures were incubated for 2 days, medium was removed, and the cell sheets were stained with0.1% crystal violet in 20% methanol. Plaques were counted under 20-fold magnification in the row of wells having the dilution which gave 5 to 20 plaques per well. Virus titers were calculated according to following formula:

titer (PFU/mL) = number of plaques x 5x 3^n; where n represents the n^{th} dilution of the virus used to infect the well in which plaques were counted.

6.4. Docking methodology

Docking studies were performed using Molecular Operating Environment (MOE) [41] version 2008.10 running on an Intel Core 2 Duo PC running Windows 7 as operating system. Crystal structure of herpes simplex virus type-1 thymidine kinase in complex with acyclovir (PDB code 1KI5) [43] was retrieved from the Protein Data Bank. From the PDB file of the complex, all the water molecules were removed and the missing hydrogens and partial charges were added using MOE. For ligands, 3D structures were constructed using the Builder module of MOE and optimized by energy minimization using MMFF94X force field. All docking calculations were carried out using the MOE Dock module. The parameters used were alpha triangle as placement methodology and London dG as scoring function with force field refinement. The highest scoring pose for each ligand was selected for further investigation. Ligand interactions were generated using the Ligand Interactions module in MOE.

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Analysis of medications returned to community pharmacies in Alexandria, Egypt

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Abstract: This study aimed to determine the patterns of returning unused medications to a sample of the community pharmacies affiliated to Medical Central Region in Alexandria. A cross-sectional descriptive study design was used. All drugs returned unused by all individuals attending the selected 60 pharmacies over a period of one month were documented. The randomly selected pharmacies were visited by the researcher and invited to participate in the current study. When a medicine was returned, the pharmacist interviewed the person returning it to complete a questionnaire that was especially developed for the study. This study demonstrated that an enormous amount of drugs are returned to community pharmacies in Alexandria, Egypt. "Treatment change" was the most frequent reason for the drug returns, with cardiovascular and anti-infectives are the predominant groups returned. Investment in proper patient and health-care provider education is an appropriate first step in reducing medication waste. Changing the prescription policy is needed to overcome this waste.
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1. Introduction

The problem of unused medicines is widespread throughout the world, with complex multifaceted causes and multiple effects on the cost of healthcare, public health, and environment [1]. In addition to the costs of unused drugs, their economic value includes the time needed to prescribe and dispense these medications, also poor adherence leads to increased health costs through additional hospital admission and doctor visits [2].

An unused drug is a drug which is purchased after a prescription or not, but which is not taken [3]. It is likely that a number of factors influence the quantities and types of medicines that are unused. These factors may include oversupply, changes in therapy, errors in prescribing or in supply, adverse drug reactions, poor compliance or death of the patient [1]. Other factors include expired medications, patient felt better, allergic reactions and patients didn't want to take the drugs [4].

It appears that, while there are variations between different countries, the reasons for unused medicines and even the types of medicines that are commonly unused are similar across the world [1]. More than two thirds of returned medicines mostly capsules and tablets are prescription drugs; the remainder consists of over the counter products and a few samples [5]. Most returned drug classes, however, aren't necessarily those that cost the most. Studies have found that 20% to 53% of returned medicines were unopened, with many of the remainder being almost complete [6,7].

In some Arab countries, over 50% of drug products obtained from community pharmacies are purchased either without a prescription or on the advice of the pharmacist. In addition, many medicines that require a prescription in more developed world can be purchased over the counter in these countries [8, 9] .

Unused medicines generally have only been the subject of a small number of studies worldwide and consequently the data available on these remains limited and many attempts to minimize the incidence of unused medicines have been based on anecdotal evidence and estimates [1].

In Egypt, unwanted medicines are accepted for resale by the pharmacies which are not accepted from ethical and safety point of view, because it is not possible to guarantee that they were stored under appropriate conditions. Investigating medication returns may indicate areas for targeting interventions to reduce waste. To our knowledge there is a paucity of published research examining unused medicines in Alexandria, Egypt. Therefore the aim of this study was to quantify the amount of returned unused medicines to a sample of the community pharmacies in Alexandria and identify the most contributing reasons to their return.

2. Material and Methods

Study Setting and Design

The study was carried out in a sample of the community pharmacies in Alexandria, Egypt. A cross-sectional descriptive design was used for the conduction of this study.

Sampling Design

Using MedCalc 11/5/1/0 trial version, and based on an average cost per returned item of medication of 13 Egyptian Pounds (LE.) [10], taking a 95% confidence level, with 80% power and assuming a SD double the mean with an accepted error of E3, the minimum required sample size was approximately 600 unused returns.

Based on pilot study, 60 pharmacies would be needed to reach the required sample size in one month. A two-stage random sample was used, where one district was selected randomly from among the seven districts in Alexandria. A list of all pharmacies was obtained from Central Medical Region at Ministry of Health. This region contains 600 community pharmacies and the required number of pharmacies was selected from the list randomly. A number of pharmacies were invited to participate and it was stated that participation would be voluntary. Sixty community pharmacies were randomly selected, with a response rate of 93%. All the drugs returned unused by all individuals attending theses pharmacies were documented. A total of 657 drugs were returned by 600 patients to the 60 pharmacies during the study period.

Data Collection Methods

Data were initially gathered over a 4-week period from community pharmacies during March 2011. Data were collected using interview technique. Pharmacists working at the pharmacies that agreed to participate were asked to collect the drugs that were returned voluntarily by individuals attending their pharmacies. When a medicine was returned or when a person asked the staff members to discard a medicine, the pharmacist interviewed the person returning it to complete a pre-designed questionnaire that was especially developed for the study. Pharmacists working at the selected pharmacies were trained by the researchers on filling the questionnaire.

The information recorded on the questionnaire included: pharmacy name, patient's personal data, who was returning the drug, who recommended the drug, number of returned drugs, returned medicine data (trade name, pharmaceutical form, pharmacological category, expiry date, amount to be discarded, total sales price of amount to be returned or discarded), payment method (health insurance system, patient himself) and the reason for returning or discarding the medicine.

The drugs were counted and classified according to the British National Formulary classification of active ingredients [11]. Costs were determined by multiplying cost per pill by the estimated number of pills remaining in the container according to the list of drug prices provided by the Egyptian Ministry of Health. Cost was calculated in Egyptian currency (1 USD equals 5.93 LE.).

Statistical analysis

The collected data were entered into a data base and a descriptive statistical analysis was performed using Statistical package for Social Sciences (SPSS) Version 11.5 (SPSS Inc., Chicago IL, USA).

3. Results

In 60 community pharmacies, 657 returned drugs were collected during one month from 600 patients with an average number of drugs returned per patient of 1.09. Males constituted the higher percentage of the participants (56.7%). Elderly having 60 years or above constituted the highest proportion of the sample (28.3%), while the lowest percentage (4.0%) was within the age group "10 to less than 20". Concerning the sample's occupations, the highest percentages of the patients pertained to the employee category (29.8%) followed by not working category (28.3%).

Of the 657 returned drugs, the predominant groups were cardiovascular system (19.4%) and anti-infectives (19.2%), as shown in Table 1.

Figures 1& 2 showed that in more than half of the returned drugs the patients in person (57.2%) returned their drugs. Relatives returned a considerable proportion of drugs (32.9%). The majority of drugs had been prescribed by physician (53.7%), or pharmacist (24.9%). Friends recommended 11.8% of the returned drugs and 9.4% were chosen by the patient himself.

The reasons for the drugs being returned were listed in Table 2. The most frequent reason stated was treatment change (35.2%) followed by ineffective drug (12.0%). On the other hand, error in dispensing was the reason with the lowest frequency (0.8%).

Regarding the cost of the returned medicines, data from Table 3 illustrated that the average drug cost per pharmacy per month was 825.1 LE (140 $). The estimated total cost was 49507.2 LE (8348.5 $), with the mean drug cost of 75.5 LE. "Cardiovascular" drugs was the therapeutic category of the highest cost percentage (44.6%) total cost of 22.115 LE., while "ear, nose, throat" was the therapeutic category of lowest cost percentage (1.12%) with a total cost of 556.2 LE. Concerning who had initially paid for retuned drugs, 68.6% of the cost had been borne by patient himself, with the remaining percent (31.4%) had been borne by health insurance system (data not shown).

4. Discussion

Improper drug use has major ramifications not only in the therapeutic and economic fields, but also from the environmental perspective [4, 7] . Moreover, the number of times a drug is returned gives an indication of frequency of prescribing and the level of medication noncompliance [12].

Table 1 Therapeutic classifications of drugs returned to the selected community pharmacies during the study period.

Drug category	Number	%
Anti-infectives	126	19.2
Cardiovascular system	127	19.4
Endocrine system	49	7.5
Ear-Nose-Throat	7	1.1
Gastrointestinal system	66	10.9
Genitourinary system	7	1.1
Musculo-skeletal system	2	0.3
Nutrition and Blood	69	10.6
Nervous system	61	9.3
Non-steroidal anti-inflammatory	64	9.8
Respiratory system	58	8.9
Skin care	19	2.9
Total	**657**	**100**
Average number of drug returned per patient	**1.09**	

Table 2 Reasons for returning drugs to the selected community pharmacies during the study period.

Reason	Number (N=657)	%
Treatment change	178	35.2
Ineffective drug	79	12.0
Get back money	74	11.3
Oversupply	71	10.8
Patient feels better	69	10.5
Passed expiry date	68	10.4
Inconvenience to use	59	9.0
Patient non compliance	36	5.5
Patient died	31	4.7
Side effects	26	3.9
Manufacture problem	20	3.0
Error in dispensing	17	2.6

Table 3 Cost of drugs returned to the selected community pharmacies during the study period.

Drug Category	Cost (LE.)	Cost percent
Anti-infectives	5801	11.71
Cardiovascular system	22115.75	44.6
Endocrine system	3621.2	7.3
Ear-Nose-Throat	556.2	1.12
Gastrointestinal system	2999.3	6.05
Genitourinary system	648	1.3
Musculo-skeletal system	194.5	0.39
Nutrition and Blood	3786.2	7.6
Nervous system	5117.95	10.3
Non-Steroidal anti-inflammatory	1241.1	2.5
Respiratory system	2200	4.44
Skin Care	1225	2.44
Total	**49507.5=8251 $**	**100**
Mean drug cost ± SD 75.5 L.E.		
Average drug cost per pharmacy per month	**4950.6 E.P (825.1 $)**	

LE stands for Livre Egyptian (the Egyptian currency).

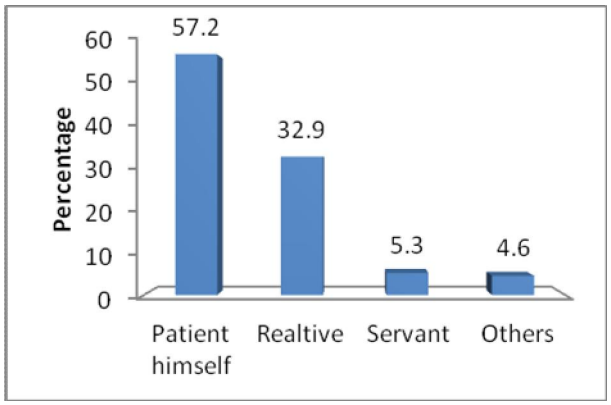

Figure 1: Person who returned the unused drugs to the selected community pharmacies during the study period (N=657).

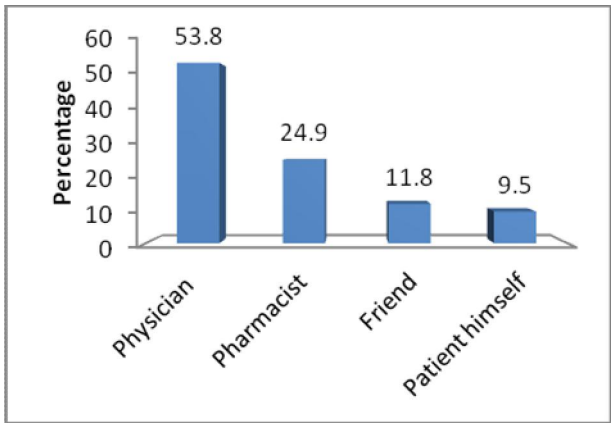

Figure 2: Person who recommended the drugs returned to the selected community pharmacies during the study period (N=657).

No drugs were returned during the study period in only 10 out of 60 pharmacies that participated in the present study. This is considered within the normal variability among pharmacies, their locations and number of patients they are serving.

Patients aged 50 years or over made half of the return events (51.5%). This may not necessarily indicate that this age group uses less of their prescribed medications. However, there is some evidence that inappropriate prescription may decrease adherence in elderly patients [13, 14].This aligns with other studies which showed increased spending on prescribed medications with increasing age [14- 16].

The two main reasons indicated for the medications not being used were 'treatment changes' (35.2%) and 'ineffective drug' (12%). Other frequent reasons declared by the present respondents were 'oversupply', 'patient feels better' and 'sell to get back money'. These reasons were somewhat consistent with that of other published studies [6, 12, 14].

Change of treatment as a frequent reason for returning drugs is important as the most likely time for changing the prescribed medications for a patient is during the early phases of treatment, so it may be prudent not to dispense the whole of the prescription quantity when treatment is being initiated. This may also allow the prescriber to more closely monitor the effectiveness of the chosen treatment. The huge percentage of patients returned their medications due to treatment change in the present study indicates an urgent need to change the current dispensing practices. Trial prescriptions have been implemented in Canada to overcome this problem and they lead to reducing the direct cost of medication wastage [17].

Medication wastage resulting from oversupply can be reduced through reducing the dispensed package size or by adapting the pack to the most frequent dosages, or by synchronization of prescription quantities and the deletion of inappropriate items from the repeat order forms or even by dispensing through individualized dosing system depending on each prescription.

'Patient feels better' was one of the frequent reasons for returning drugs in the current study (10.5%). This might give an indication to the important role that should be played by comprehensive doctor/patient counseling on appropriate drug use especially in chronic conditions where long term treatment course should be followed.

Considerable proportions of the current participants returned unused drugs to get their money back, and this might be due to the finding that the highest percentage of payment for drugs was by the patient himself.

Drug reaching expiry date was among frequent the reasons for returning unused drugs. However, no information about the date of dispensing could be recorded in order to evaluate how long people tend to keep drugs they have stopped using. The information on the reason for returning each drug together with a note stating the age of the returned drug would help to provide a quantitative estimate of the relative importance of the reasons for drug being returned unused.

The therapeutic groups responsible for the most present returns were 'cardiovascular system', 'anti-infectives' and 'gastrointestinal' (Table 1). This finding is somewhat consistent with that of another Egyptian study [12]. Studies from different countries showed variability in the returned drugs by therapeutic groups, with cardiovascular and nervous systems among the highly ranked groups [14, 18, 19].

Generally, the majority of patients taking medications for cardiovascular system are chronic patients. Patient adherence to prescribed therapy (especially with chronic illness) is often not ideal, which may explain why a large number of returned drugs belonged to the cardiovascular system. Patients' non adherence to treatment could be the root cause that stands behind many other cited reasons [20]. Unfortunately, over-the-counter antibiotic use is common in Egypt, with evidence on over use of many antibiotics [21, 22]. This mostly explains why anti-infective ranked the first on the list of restored drugs in the present study.

When comparing based upon the cost of the returns, in the current study the therapeutic categories that showed the highest percentages of occurrence were also responsible for the highest cost, where the 'cardiovascular' group represents 19.4% of returns by number, and 44.6% of the cost. In a study conducted in New Zealand, although cardiovascular category showed the highest percentage of return, respiratory system category showed the highest cost [14].

In relation to the cost of the returned drugs in the present study, the average drug cost per pharmacy per month was 825.1 LE (140 $). This value is higher than that was reported by another Egyptian study (average drug cost per pharmacy per month of 549.4 LE (103.5 $) [12]. This confirms the substantial economic value of the unused drugs. Unfortunately, when considering the total number of pharmacies in Alexandria Governorate (3443), the average drug cost per month would be expected to reach 1.891.584 LE (318.986 $).

The present data also indicated that patients themselves paid for almost 70% of the returns. In contrast, a study conducted in Spain revealed that the public insurance paid for more than 50% of the returns [18]. Payment systems for drugs vary between countries due to differences in the health care systems.

We acknowledge that there are limitations to the present study. The questionnaire on the returned medicines was answered by the patient, relative or others, which highlights the subjectivity of the answers. In some instances, respondents may not have known exactly why the medicines had not been used, especially when the patient was deceased.

Also the return of unused medicines may be subjected to seasonal variations, so data better to be collected over a longer period (1 year) before a reasonable annual figure can be estimated. This study had not attempt to quantify the other routes of disposal or to estimate quantities of unused medicines in patient's homes, therefore, it is likely that it has substantially underestimated the extent of unused medicines in the community.

Conclusion

In this study, more than one third of the return events made were attributable to changes in prescribed therapy. Additionally, cardiovascular and anti-invectives drugs were the most frequent therapeutic categories returned. This emphasizes the need for changing the drug dispensing policy, especially in chronic patients, and for anti-infectives, policy revision would have the highest priority. Based on the present findings, it is necessary to consider new measures to reduce the size and the cost of unused drugs in Egypt that entails more efficient prescription and dispensing systems. An investment in proper patient -and health provider education is an appropriate first step in reducing medication waste.

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Conflicts of interest

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The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Efficiency of Some Disinfectants on Bacterial Wound Pathogens

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Abstract: Disinfectant are chemical agent used on inanimate object but can also be employed as antiseptic at a very low concentration. It is therefore imperative to determine the efficiency of some commonly used disinfectants on the frequently encountered bacterial wound pathogens. The antibacterial effects of these chemical agents were carried out using standard microbiological techniques. Results showed that the investigated disinfectants at 50% and 100% concentration cause 100% bacterial cell reduction. The Minimum inhibitory concentration of the investigated disinfectant ranged from 0.78 – 6.25% while the MBC ranged from 3.13 – 12.5%. The MBC to MIC ratio also ranged from 1 – 4, asserting the bactericidal power of the tested disinfectants. It can therefore be concluded that professionals involved in the care of wounds should consider the use of these agents for washing the surfaces of infected wounds in order to minimize the possible spread of multi-drug resistant bacterial pathogens from wound to other sources.

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KEYWORDS: Disinfectants, bacteria, wound, pathogens

1. Introduction

Wound infection has been defined as the presence of pus in a lesion, as well as other general or local features of sepsis including pyrexia, pain and indurations (Shija, 1973). Wound may be encountered in clinical practice either post operatively, following trauma, in association with haemoglobinopathy or could primarily be of infective origin (Sule *et al*., 2002). All wounds, regardless of their origin may be contaminated by microorganisms or foreign bodies or both and all are likely to contain a significant amount of devitalized or necrotic tissue (Bell Chan *et al*., 1999). Wound infections represent an important cause of mobidity and account for 70 – 80% mortality (Wilson *et al*; 2004). The development of such infections represent delayed healing causing anxiety and discomfort for patient, longer stays in hospitals and add to cost of health care services significantly (Mohantay *et al*, 2004). If infection is deep seated or becomes generalized, appropriate systemic treatment must be administered (Murtlay *et al*., 1998). However, the management of infected wound is a challenge (Sule *et al*., 2002 but it is important that, the entry site be cleansed daily and treated with appropriate antiseptic (Kiernan, 1998). The present study was therefore designed to determine the efficiency of some commonly used disinfectants on the frequently encountered bacterial wound pathogens.

2. Materials and Methods

2.1 Disinfectants

Three commonly used disinfectants were selected for this study and they included; Methylated spirit, Dettol and Lysol. The table below presents the common names, scientific name and the commercial concentration of the selected disinfectants.

2.2 Test Organisms

Staphylococcus aureus, Enterococous faecalis, Pseudomonas aeruginosa, Escherichia coli, Klebsiella species, staphylococcus epidermidis and Proteus mirabilis were obtained from the Department of Medical Microbiology of the Olabisi Onabanjo University Teaching Hospital, Ogun State, Nigeria. Isolates were from clinical wound samples. The isolates identities were further confirmed in our laboratory using standard biochemical procedures (Barrow and Feltham, 1993). The isolates were maintained on Trytone soy agar (TSA) (Oxoid) at 4^{O}C before use for this work (Efuntoye *et al*., 2010).

Table 1. Types and Commercial Concentration of Disinfectants used in the study

Disinfectants	Scientific Name	Commercial Concentration (Percentage (%))
Dettol	Chloroxylenol	4.8 w/v oleum pini
Methylated Spirit	Idoptrophy Alcohol	95% Alcohol (v/v)
Lysol	Saponated cresol	5% cresol

2.3 Antibacterial Activity of the Disinfectants

The antibacterial activity of the selected disinfectant on the frequently encountered bacterial wound pathogens was evaluated using time kill test as describes by Ogunledun (2008). The minimum inhibitory concentration and the Minimum bactericidal concentration were carried out as described by NCCLS (2002). The minimum bactericidal concentration was defined as the lowest concentration of the disinfectants that produced negative subcultures. The MBC to MIC ratio was also determined and interpreted as described by Hazen (1998).

3. RESULTS

The results of the effect of the disinfectants on the frequently encountered bacterial wound pathogens as summarized in table 2 and 3 showed that the disinfectants were very effective at both 50% and 100% concentrations. These agents causes 100% reduction in the bacterial growth examined. Results of the minimum inhibitory concentration of the tested disinfectants showed that the disinfectants demonstrated inhibitory activities against the test organisms to varying degrees. The minimum bactericidal concentrations of all the disinfectants ranged from 3.13 – 12.5%. The minimum bactericidal concentration to the minimum inhibitory concentration (MBC/MIC ratio) were found to be between 1- 4%.

Table 2: Effect of Some Disinfectants on bacterial wound pathogens at 100% concentration

Organisms	Dettol				Lysol				Methylated Spirit			
	Bactericidal growth (%)											
	30S	60S	90S	120S	30S	60S	90S	120S	30S	60S	90S	120S
SA	O	O	O	O	O	O	O	O	O	O	O	O
EF	O	O	O	O	O	O	O	O	O	O	O	O
PA	O	O	O	O	O	O	O	O	O	O	O	O
EC	O	O	O	O	O	O	O	O	O	O	O	O
KS	O	O	O	O	O	O	O	O	O	O	O	O
SE	O	O	O	O	O	O	O	O	O	O	O	O
PM	O	O	O	O	O	O	O	O	O	O	O	O

Table 3: Effect of some disinfectants on bacterial wound pathogens at 50% concentration

Organisms	Dettol				Lysol				Methylated Spirit			
	Bactericidal growth (%)											
	30S	60S	90S	120S	30	60	90	120	30	60	90	120
SA	O	O	O	O	O	O	O	O	O	O	O	O
EF	O	O	O	O	O	O	O	O	O	O	O	O
PA	O	O	O	O	O	O	O	O	O	O	O	O
EC	O	O	O	O	O	O	O	O	O	O	O	O
KS	O	O	O	O	O	O	O	O	O	O	O	O
SE	O	O	O	O	O	O	O	O	O	O	O	O
PM	O	O	O	O	O	O	O	O	O	O	O	O

Table 4: Minimum Inhibitory Concentrations of the selected Disinfectants on Bacterial Wound Pathogens

Organisms	Disinfectants (%)		
	Dettol	Lysol	Methylated Spirit
SA	3.13	6.25	6.25
EF	3.13	6.25	6.25
PA	3.13	3.13	6.25
EC	1.56	3.13	3.13
KS	1.56	3.13	3.13
SE	3.13	6.25	3.13
PM	0.78	1.56	1.56

Table 5: Minimum Bactericidal Concentrations of Some Selected Disinfectants on Bacterial Wound Pathogens

	Disinfectants (%)		
Organisms	Dettol	Lysol	Methylated Spirit
SA	6.25	12.5	12.5
EF	6.25	12.5	12.5
PA	6.25	6.25	12.5
EC	3.13	6.25	12.5
KS	3.13	6.25	12.5
SE	3.13	12.5	12.5
PM	3.13	6.25	6.25

Table 6: Minimum bactericidal concentration and Minimum inhibitory concentration of the tested disinfectants.

	MBC/MIC ratio for the investigated disinfectants		
Organisms	Dettol	Lysol	Methylated Spirit
SA	2	2	2
EF	2	2	2
PA	2	2	2
EC	2	2	4
KS	2	2	4
SE	1	2	4
PM	4	4	4

5. Discussion

Over the years, disinfectants have played important roles in the control of infections (Rutala, 1996). All the tested disinfectants were very active against the wound pathogens. This finding is contrary to the findings of Ihsan and Thuraya (2011) who reported some commonly used disinfectants in Iraq which were not effective against bacterial wound pathogens at 100% and 50% concentration. The difference observed in our study could be due to difference in the species or strains of the organisms used. The minimum inhibitory concentrations for all the disinfectants were found ranging from 0.78 – 6.25%. This observation corroborates that of Frohm *et al*. (1996) who also asserted that bacteria will continue to be killed even at a surface level if they come in contact with a disinfectant regardless of the concentration of such disinfectants. The MIC to MBC ratio ranged from 1 – 4 and incidentally falls within the range reported by Hazen (1998) to be cidal for any agents. This observation further stressed that these agents are effective bactericidal agents. It can therefore be concluded that these agents should be used for cleansing the inanimate objects that could serve as formites for wound pathogens and also, at a very low concentration, should be considered good cleansers for infected wounds.

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Reduction of Toxic Cr^{6+} Ions in Presence of Non Toxic Acid

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Abstract: Chromium VI is a toxic and carcinogenic element; it could be detected in waste water getting out from many industries as tannery, textile and electroplating of metals. Oxidation reduction process is the most economic and simple way of removing Cr^{6+} by conversion into Cr^{3+} (non hazard). The rate of reduction reaction of Cr^{6+} to Cr^{3+} in $K_2Cr_2O_7$ has been studied in absence of nontoxic acids and it is found to be first order reaction. Different acids as acetic acid, formic, glycinecitric acids were also tested at different temperatures and reactions. The rate of the reaction in each case was measured. Thermodynamic parameters ΔG^*, ΔH^* and ΔS^* are estimated from the experimental measurements.
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1. Introduction

Environmental preservation is nowadays matter of deep concern; that is why much work has been done on this subject in the last years. The word "pollution" has no many presences in our lives, due to the great development in the industrial field. Pollution can be identified as very contaminants that could change the nature balance due to human levels [(1)]. Water is essential for life, since it is required for various purposes in community life such as cocking, drinking, washing, warring gardens, etc. but as the community developed and with concentration of pollution in cities, the demand of water increases. Also, with the rapid industerlization-take place, a vast amount of water is required to run industries. Subsequently, amount of industrial wastes discharged into water resources increased, and the demand for industrial water treatment, particularly containing heavy metals, in necessary.

Heavy metals release in waste waters is one of the most worrying pollution causes. As it affects on life may particularly serious, referring to plants and animals (mainly fishes), or to man, reached indirectly by the toxics through food chain.

The removal of heavy metals from waste is a topic with great industrial importance, as there are a great number of large industries, which must spend millions of dollars each year to deal with this issue. The most common heavy metals to be removed are: cadmium, chromium, cobalt, copper, lead, manganeses, mercury, vanadium and zinc.

There are many industries where process operations results in large quantities of the pollutants in the effluents being discharged, e.g., refineries, gas, gas undertakings, alloys, electroplating rinse solution, metal finishing industries and reach and effluent solutions from the manufacture of chemicals.

Processes of metal removal from waste water effluents must be selected to remove the metal either in this existing form, or converted to a form suitable for the removal processes. In general, metals must either be precipitated or else attached to an insoluble species, e.g. through adsorption or ion exchange [(2)].

Chromium:

Chromium is steel-grey, lustrous, hard, metallic, and takes a high polish. Its compounds are toxic. It is found as chromites ore. Siberian red lead (crocoites $PbCrO_4$) is a chromium ore prized as a red pigment for oil paints [(3)].

Chromium exists in several valence states, ranging from chromium (III) to chromium (VI). However, the three major forms of chromium that commonly exist in the environment are chromium (0), chromium (III) and chromium (VI). Only trivalent and hexavalent are biologically significant [(4)]. Hexavalent chromium compound appear to be 10 to 100 times more toxic than their Cr^{3+} counterparts when both are administers the oral route [(5)]. Metallic chromium (0) is relatively non toxic.

1.3 Hexavalent chromium

Hexavalent chromium compounds are chromium trioxide, chromium anhydride, chromium acid and dichromate salts.

Cr^{6+}, a know carcinogen, forms relatively soluble precipitates, and dose not absorb readily. Cr^{6+} is carcinogenic because it is highly reactive interacting with the body's chemistry and functions [(6-9)].

Health effects

Chromium III is required for health, and all ordinary exposures are considered to be safe. Chromium VI can produce liver and kidney damage, internal hemorrhage, dermatitis, respiratory damages and lung cancer.

Environmental effects

Chromium has been associated with soil infertility only in a few places because of high concentration. Chromium in the form of chromate

chemicals is toxic to plant. Chromium VI is toxic to aquatic life. Chromium water concentration should not exceed 10 ppm for protection of aquatic species.

Cementation is one of the oldest and simplest hydrometallurgical processes, which has been used as a means of extracting metals from solution. Only in the past 20 years, considerable attention has been paid to two main industrial applications of cementation. The first involves the recovery of metals from leach solution [10-12] and the second is concerned with the purification of electrolyte solutions to remove metals which are more electropositive than the metal to be deposited, e.g. Cu, Co, Ni, Cd from $ZnSO_4$ electrolyte [13-15].

Many applications have been reported in industry [7-11] for the recovery of metals and purification of electrolyte solution. Almost all the authors have reported that electrochemistry of the reaction at room temperature is diffusion-controlled [20].

The most important source of chromium pollution is dusterial. Hexavalent chromium compounds causes dermatitis [21], perforation of nasal septum and inflammation of larynx and liver. Skin lesions and kidney damage could be produced as a result of occupational exposure to hexavalent chromium compound is probably a carcinogen and the lung is principle site of action [22].

Aim of work

The objective of the present work is devoted to study the kinetics of the reduction of Cr^{+6} to Cr^{3+} on rotating iron cylinder in presence of some non toxic organic acids at different concentrations and different rpm to remove as much as possible the Cr^{+6} from waste water.

2. Experimental work

Figure (1) is a block diagram of the apparatus used in recovery of (Cr^{+6}) from the solution which permits the rotation of an immersed iron cylinder in a 600ml glass beaker containing 499ml of experimental solution. The iron cylinder used in each run is of 7cm length and 1.4cm diameter, only the peripheral surface of pure iron was exposed to the solution. The cylinder was rotated in experimental solution with variable speed motor. The frequency of rotation recorded as revolution per second was counted by an optical tachometer.

Kinetic measurement

Analar potassium dichromate and redistilled water containing 1.5 mol l^{-1} sulfuric acid (98% w/w) were used in the preparation of blank solution (0.05 mol l^{-1}) as well as in the presence of five different concentrations of acid. The rate of reaction was determined at different temperature 25, 30, 35 and 40° C as well as at different rotations 650, 500, 375, 250 and 125 r.p.m. Analar 4 non toxic acids were used.

Through the proceeding reaction, at different time intervals 10, 20, 30 minutes, 1ml sample was taken from reaction solution and diluted to 10ml by redistilled water. The determination of hexavalent (Cr^{+6}) concentration was carried out at 365 nm using UV-160A spectrophotometer through the equation (7).

3. Results and Discussion

Chromium occurs in liquid wastes in two forms, trivalent and hexavalent. Hexavalent chromium is toxic and known to be carcinogenic substance. It is responsible for lung cancer, chrome ulcer, perforation of nasal septum and kidney damage. According to IS: 2490 and IS: 2296 (environmental protection agency 1985) the threshold-limiting value for hexavalent chromium is 0.1 mg/l. the limit of liquid wastes discharged into the sea is 1 ppm. For trivalent chromium, it is a practice to keep the concentration below 4 ppm.

Chromic acid and its salts are used in tanning industries and as oxidizing agents in the manufacture of organic chemicals. They are also widely used in electroplating industries for the deposition of chromium metal [16].

In fertilizer-industry waste, hexavalent chromium is between 20 and 30 ppm and must be removed before biological treatment. It finds its way into the liquid effluent through the blow-down of the cooling tower. Chromates and dichromates are present in the cooling water as scale and corrosion inhibitors. Blow-down is done when the total amount of dissolved solids in the water increases to around 700 ppm from an initial value of 100 ppm.

Reduction precipitation

The reduction precipitation [7, 9] method finds wide applications in the treatment of chromium. It is economical and the removal efficiency is high (98-99 per cent). However, there are three steps involved in this methods:

1. pH adjustement
2. Reduction
3. Precipitation.

pH adjustement is achieved with the use of sulphuric acid whereby the pH is reduced to 2-3. At this level, the reduction of Cr^{+6} to Cr^{+3} can be achieved very efficiently. The equalization technique where acidic waste from some other plant is mixed with the liquid waste can also be used, thereby reducing the cost of treatment.

3.3 The order of the reaction

In our work, oxidation-reduction reaction between potassium dichromate and iron takes place to

produce trivalent chromium ions (Cr^{+3}). The kinetics of this reaction have been studied extensively [23-25] where, the rate controlling step was found to be the diffusion of hexavalent chromium ions (Cr^{+6}) to iron surface. In such case, the rate of change of hexavalent chromium ions (Cr^{+6}) concentration in the solution is followed as in equation (1)

$$\frac{dc}{dt} = -KA * \frac{C}{V} \qquad (1)$$

Assuming that (Cr^{+6}) concentration is negligible at iron solution interface, C is (Cr^{+6}) concentration represented in (mol l^{-1}) at time t (sec), K is the mass transfer coefficient which depends upon fluid flow and temperature conditions (cm/sec), A* is the exposed area of the iron cylinder (cm^2) and V ' represents the volume of the solution (cm^3).

Accordingly, the rate of decreasing the hexavalent chromium ions (Cr^{+6}) concentration is proportional to its concentration and the exposed iron area, the mechanism of the reaction seems to be electrochemical in nature i.e. it takes place through a galvanic cell where Fe act as the cell anode where Cr^{6+} reduction take place at cathode site as follows

Reaction at the anode (a) $3Fe \longrightarrow Fe^{+2} + 6e^-$ (dissolution of metal in acidic medium)

Reaction at the cathode (b) $Cr^{+6}_{bulk} \longrightarrow Cr^{+6}_{interface}$ (mass transfer step)

(c) $Cr^{+6} + 6e^- \longrightarrow Cr^{+3}$ (charge transfer step)

This reaction is diffusion controlled whose rate in a batch reactor can be represented by integration equation (2)

$$\text{Ln } C_o/C = KA^*/V' \qquad (2)$$

This equation may be represented as a straight line where, C_o is (Cr^{+6}) concentrations at zero time and the other symbols have the definition mentioned above. Figure (2) shows the relation between log C_0/C against time for the blank solution while figure (3) demonstrate the relation between log C_0/C against time at different concentrations of acetic acid at 250 rpm. Table (1) and figures (2,3) show that, the reaction is a first order reaction in according to equation (1) and (2).

The rate constant of reaction for different acetic acid derivatives composition were calculated from the slopes of $\ln C_0/C$ Vs time lines. Table (1) summarizes the obtained results at different temperatures. It is found that Cr^{+6} reduction process is inhibited by addition of acids. The percentage of inhibition for Cr^{+6} reduction is calculated from the following relation:

$$\% \text{ inhibition} = \frac{k - \bar{k}}{k} 100 \qquad (4)$$

where k is reaction rate constant in blank solution at [250 rpm].

$\bar{k}$ = rate constant of the reaction in presence of organic acid. Table (3) gives the relation between the percentages of inhibition of the rate of Cr^{+6} reduction and organic acid concentration at 25° C. It was found that the % inhibition depending on the types of organic acid and its concentration. The order of Cr^{+6} reduction inhibition is

citric >glycine> Acetic acid > formic

The decrease in the rate of Cr^{+6} reduction in the presence of acid may be attributed to:

Organic acid may form a thin adsorbed film on the iron metal which leads to decrease the rate of reduction reaction, also adsorption of organic acid on the surface depends mainly on the structure. Citric, glycine have more than one functional group which are adsorbed on the surface of the metal more than acetic acid which has one functional group [25, 26].

(i) The decrease in the diffusion coefficient (D); of Cr^{+3} in solutions containing acids is due to the increase in the interfacial viscosity η in accordance to Stokes-Einstein equation [9, 10]

$$\eta \frac{D}{T} = \text{constant} \qquad (5)$$

Where T is the absolute temperature. The increase in the interfacial viscosity is caused by the adsorption of acids molecules on the iron surface.

Trichloro acetic acid is more inhibitors than other because it has more than one functional group. From table (3) it is obvious that the order of decreasing the rate of reduction as follows:

Formic <Acetic <glycine< Citric

3.4 Effect of stirring on the reaction

Figures (4,5) give the variation of log C_0/C with time at different speed of rotation (rpm) of iron cylinder at 25^{0}C for acidified 0.05 mol l^{-1} potassium dichromate solution as a blank solution as well as in the presence of formic acid. The effect of rotational speed on the mass transfer coefficient (K) can also be used to determine whether the reaction is diffusion or

chemically controlled. If the mass transfer coefficient increases with increasing stirring speed, then the reaction is diffusion controlled. If the mass transfer coefficient (K) is independent on stirring speed, then the reaction is chemically controlled. The data shown in table (3) represent that the reaction is diffusion controlled [16 and 17].

Adsorption isotherm

The degree of surface coverage θ was calculated by the relation

$$\theta = \frac{K - \overline{K}}{K} \qquad (6)$$

$\overline{K}$ = rate of the reaction for blank solution

K = rate of the reaction in presence of additive

The adsorption of surfactant on Zn electrode is found to obey Langmuir isotherm equation (7)

$$\frac{\theta}{1-\theta} = Ac\left[\exp\frac{\Delta G}{RT}\right] \qquad (7)$$

where A = constant

C = concentration of organic substances

ΔG = energy of adsorption

R = Gas constant, [8.314 $J.mol^{-1}.l^{-1}$]

Fig. (4) and table (4) represents a typical plot of log (θ/1-θ) vs. log c which would give straight line. The slopes of the curves are nearly equal to unity and are reasonably linear in accordance with Langmuir isotherm equation (7).

It has been postulated on the derivation of Langmuir isotherm that adsorbed molecules do not interact with each other [15]. Interaction of adsorbed species by mutual repulsion or attraction would make the slope of the plot deviate from unity.

Thermodynamic treatment of the reaction

From the integrated form of Arrhenius equation

$$\ln K = \frac{-E}{RT} + \ln(A) \qquad (2)$$

where R is the gas constant, E is the activation energy and A is the frequency factor. The values of E are given in table (6).

The values for enthalpy of activation, ΔH*, entropy of activation ΔS*, and free energy of activation ΔG*, can be obtained by using the following equations:

$$\Delta H^* = E - RT \qquad (7)$$

$$\frac{\Delta S^*}{R} = \ln(A) - \ln\left(\frac{\alpha T e}{h}\right) \qquad (8)$$

$$\Delta G^* = \Delta H^* - T\Delta S^* \qquad (9)$$

where α is the Boltzman constant, e is 2.7183 and h is Plank's constant.

Although the change in the free energy of activation, ΔG*, with the acids concentration for all used acids is only small, (Tables 6), and variations occur in the enthalpy of activation ΔH* and the entropy of activation ΔS*, with acids concentration where in all these cases ΔH* and ΔS* compensate each other to produce little changes in ΔG*.

It is noticed that all values of ΔS* are highly negative values, indicating a more ordered system and non-random distribution of the acids on the electrode. These values are found to be independent of the type of acids and the number of the substituent present in each acid, where these acids compounds affect the process by dissolution of copper metal.

In general, it is found that the values of E and ΔH* decreases as the acid concentration decreases as shown in Table (6), which may be attributed to that; the acids increase the local solution viscosity at the Fe surface with a consequent decrease in the diffusivity of Cr^{6+} ion, and , also , the acids molecules accelerate the natural convection flow arising from the density difference between the bulk solution and the solution at the electrode surface due to the repulsion force between the Fe and the COOH group of the acid, leading to decrease in the rate of oxidation.

Table (1) The values of rate constants ($k.10^3$) for solution of different concentration of acids at 25°C and 250 rpm.

Acid	Conc. 10^4 mol l^{-1}					
	0.0	0.5	1	5	10	50
	k. 10^3 sec^{-1}					
Acetic	7.6	5.7	4.4	3.3	2.7	2.0
Monochloro Acetic	7.6	5.00	3.85	3.1	2.65	1.90
Dichloro Acetic	7.6	4.6	3.53	2.90	2.60	1.82
Trichloro Acetic	7.6	4.4	3.40	2.81	2.51	1.71

The values of the rate constants k. 10^3 at different temperatures for all acids and at 250 rpm c = 1x 10^{-4} mol. l^{-1}

Table (2a) The values of rate constant for blank solution at different rpm and 25˚C.

Acid	Temperature			
	25	30	35	40
Acetic Acid	4.3	5.14	6.12	7.18
Formic Acid	3.85	4.55	5.55	6.53
Glyceric Acid	3.53	4.28	5.23	6.34
Citric Acid	3.4	4.2	5.01	6.10

Table (2b) The values of rate constant for solutions in presence of $5x10^{-5}$ mol l^{-1} CH_3COOH.

rpm	k. 10^3
125	2.13
250	3.11
375	4.70
500	7.60

rpm	k. 10^3
125	1.22
250	1.75
375	3.10
500	4.7

Table (3) :The values of percentage inhibition for all acids at 25˚C and 250 rpm.

Acids	Conc. 10^4				
	0.5	1	5	10	50
Formic Acid	25	39.3	56.57	64.5	73.7
Acetic Acid	34.2	49.3	59.21	65.5	75.0
Glyceric Acid	39.47	53.55	61.85	66	76.1
Citric Acid	42.1	55.26	63.0	67	77.5

Table (4):The values of the rate constants k.10^3 for solution in presence of different concentration of acids at different temperatures and 250 rpm.

C.10^4 mol l^{-1} / T˚C	0.0	0.5	1.0	2.0	3.0	4.0	5.0
a) Formic acid							
25	7.7	5.7	4.4	3.3	2.66	2.2	2.0
30	8.5	6.6	5.2	3.7	3.2	2.63	2.37
35	9.86	7.91	6.15	4.75	3.7	3.3	3.1
40	11.11	9.1	7.3	5.61	4.7	4.0	3.7
b) Acetic acid							
25	7.6	5.70	3.85	2.95	2.30	2.0	1.63
30	8.5	6.0	4.6	3.55	2.70	4.33	2.1
35	9.86	7.20	5.65	4.30	3.43	3.2	2.7
40	11.11	8.50	6.8	5.25	4.21	3.8	3.3
C)glycineacid							
25	7.6	4.6	3.6	2.6	2.1	1.7	1.3
30	8.5	5.6	4.3	3.15	2.15	2.15	1.7
35	9.86	6.8	5.3	3.8	3.1	2.7	2.10
40	11.11	8.10	6.4	4.7	3.8	3.31	2.7
c) Citric acid							
25	7.6	4.4	3.4	3.01	2.9	2.7	2.6
30	8.5	5.2	4.0	3.3	3.2	3.1	2.5
35	9.86	6.3	5.4	5.2	4.8	4.5	4.2
40	11.11	8.0	6.2	5.4	5.1	4.8	4.7

Table (5): The relation between the degree of coverage and concentration of acid

Acids	$C.10^4$ Mol. l^{-1}	θ	1-θ	$\frac{\theta}{1-\theta}$	$\log \frac{\theta}{1-\theta}$	log C
Acetic Acid	0.5	0.342	0.658	0.519	-1.715	-5.30
	1	0.493	0.507	0.972	-1.9878	-5.0
	5	0.592	0.408	1.45	0.1616	-4.30
	10	0.655	0.345	1.898	0.278	-4.00
	50	0.75	0.25	3.0	0.477	-3.20
Formic Acid	0.5	0.25	0.75	0.333	-0.477	-5.30
	1	0.343	0.607	0.647	-0.188	-5.0
	5	0.566	0.454	1.246	0.096	-4.30
	10	0.645	0.455	1.417	0.1513	-4.00
	50	0.737	0.263	2.802	0.448	-3.20
Glyceric Acid	0.5	0.395	0.605	0.6528	-0.185	-5.30
	1	0.536	0.464	1.155	0.063	-5.0
	5	0.619	0.38	1.628	0.212	-4.30
	10	0.66	0.34	1.941	0.288	-4.00
	50	0.755	0.25	3.00	0.477	-3.20
Citric Acid	0.5	0.421	0.589	0.721	-0.14	-5.30
	1	0.553	0.447	1.237	0.092	-5.0
	5	0.63	0.37	1.70	0.230	-4.30
	10	0.67	0.33	2.030	0.31	-4.00
	50	0.775	0.225	3.444	0.537	-3.20

Table (6): Thermodynamic parameters for all acid used

Acid	Parameter	C x 10^4 (mol / l^{-1})						
		0	**0.5**	**1.0**	**2.0**	**3.0**	**4.0**	**5.0**
Acetic Acid	E	19.986	26.20	29.683	29.817	31.518	34.4	36.9
	ΔH	17.507	23.70	27.207	27.338	29.04	32.11	34.3
	-ΔS	226.8	210.5	199.95	201.7	198.11	188	183
	ΔG	85.13	86.00	86.819	87.500	88.1	88.6	88.9
Formic Acid	E	19.986	24.603	26.188	28.568	28.71	31.36	32.891
	ΔH	17.507	22.125	23.709	26.09	26.262	28.881	30.30
	-ΔS	226.8	213.7	210.5	205	206.2	194.0	195
	ΔG	85.13	85.84	86.479	87.25	87.742	88.215	85.461
Glyceric Acid	E	19.986	29.382	21.35	30.56	33.21	34.6	37.3
	ΔH	17.507	26.90	18.875	28.00	30.73	32.1	34.9
	-ΔS	226.8	199.0	228	200.5	193	190	183
	ΔG	85.13	85.357	86.895	87.79	88.9	88.8	89.50
Citric Acid	E	19.986	19.04	32.655	34.31	32.6	32.62	33.8
	ΔH	17.507	16.67	30.1	31.833	30.13	30.1	31.3
	-ΔS	226.8	234.1	191	186.6	192	193	189.4
	ΔG	85.13	86.4	87.14	87.50	87.57	87.5	87.9

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Plasma Levels of 25-Hydroxyvitamin D and Dress Style in a Sample of Egyptian Female University Students

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Abstract: Background: Sunlight exposure is the most important source of vitamin D. Nevertheless, there are indications of a high prevalence of vitamin D deficiency in a number of sunny countries. Concealed clothing, as it blocks the absorbance of UV light, is hypothesized to be the cause of impairment of vitamin D production.The objectives of the present study were to determine the prevalence of vitamin D deficiency and whether vitamin D status is related to the dress style among apparently healthy female university students in a prototype of Egyptian governorates *which enjoy* a good deal of *sunny* weather. **Methods:** A random sample of 120(90 females; 30 males) apparently healthy undergraduate students, Zagazig University, Sharkia, Egypt, were enrolled in a cross sectional study. Females were divided according to their dress style into three groups, Western, Hegab and Nekab dress style group. Vitamin D status was determined in terms of plasma 25(OH) D levels using electrochemiluminescence immunoassay (ECLIA). **Results:** Mean serum 25(OH)D level was 23.7 ±12.681 ng/mL. Levels did not differ between males and females or between females grouped by dress style. Using a cutoff point of 30 ng/mL, 74.6% of the sample (61.7% and 79.2% of males and females, respectively) had low vitamin D status. **Conclusions:** This study demonstrates a high prevalence of low vitamin D status among university students in Zagazig, Egypt. Results, however, are not in support of the hypothesis that concealing clothing is the cause of the vitamin D insufficiency. Thus, other factors must be sought to explain the low vitamin D levels despite the sufficient solar source of this vitamin.
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1. Introduction

The steroid hormone vitamin D, also known as the "sunshine vitamin," plays a major role as a precursor in calcium homeostasis of the human body. By far, the most important source of vitamin D is sunlight exposure. Hence, limited skin exposure to sunlight is presumed to be the cause of vitamin D deficiency(**Holick, 2005**).The liver and other tissues metabolize vitamin D, whether from the skin or oral ingestion, to 25-hydroxyvitamin D (25(OH)D) which is the principal circulating form of vitamin D. Further metabolism of 25(OH)D to the active metabolite 1,25-dihydroxyvitamin D occurs mainly in the kidneys (**Henry, 2011**).Although 1,25(OH)D is the principal hormonal form of vitamin D, it is not the ideal measure for vitamin D status. This is mainly because the plasma half-life of circulating 1,25(OH)D is only 4-6 hours and the 1,25(OH)D circulating levels are thousand fold less than those of 25(OH)D (**Anderson *et al.,* 2003**). By contrast, 25(OH)D, the principal circulating form of vitamin D has a half-life of approximately 2-3 weeks. Therefore, measurement of 25(OH)D is widely considered a robust 'gold standard' indicator of vitamin D status (**Hollis, 2005; Hollis and Horst**, **2007; Springbett *et al.,* 2010**).

Low levels of vitamin D metabolites are associated with malabsorption of calcium, which results in bone loss and variety of many other conditions including cancers of prostate, colon and breast, hypertension, lack of proper immune modulation and diabetes (**Khazai *et al.,* 2008**).This is especially true for people more prone to vitamin D deficiency, including people of darker skin color photo proteced by melanin (**Brok *et al.,* 2011**). Because of recent recognition of its broad pathophysiological importance, vitamin D has become a hot topic in medical research during the last decade (**Grant, 2006; Bacchetta *et al.,* 2010;**).

There appears to be an unrecognized epidemic of vitamin D deficiency in many parts of the world (**Holick and Chen, 2008**). However, there are wide regional differences in the 25(OH)D levels with an observed latitude effect, which has already been addressed in several countries by implementing dairy fortifications policies (**Rucker *et al.,* 2002, Kull *et al.,* 2009;**). Most of the studies concerning vitamin D status have focused on Western countries situated at temperate latitudes, though studies in the East have shown similar observations. Thus, a greater prevalence of the disease has been reported in the northern regions of China where the intensity of summer ultraviolet (UV) light is less than in the south (**Strand *et al.,* 2009**).This suggests that vitamin D deficiency is simply the consequence of inadequate exposure of the skin to the sun. Given the significant role of sunlight in vitamin D synthesis, one should expect low prevalence of vitamin D deficiency in tropical countries. Contrary to expectation, however, a number of studies

conducted in sunny countries such as Tunisia (**Meddeb *et al.*, 2005**), Morocco (**Allali *et al.*, 2009**) Lebanon (**Gannagé-Yared *et al.*, 2000**), Saudi Arabia (**Al Faraj and Al Mutairi, 2003**) United Arab Emirates (**Al Anouti *et al.*, 2011**) and Iran (**Hovsepian *et al.*, 2011; Kaykhaei *et al.*, 2011**) have reported a high prevalence of vitamin D deficiency. One plausible presumption of the high prevalence of vitamin D deficiency in the Middle Eastern countries, despite the abundant sunlight almost throughout the year, has been related to clothing style. Concealed clothing, such as veiling worn by women, which is the cultural norm in most of the Middle Eastern countries, is hypothesized to be the cause of impairment of vitamin D production as it blocks the absorbance of UV light.

Egypt, like other Middle Eastern countries, *enjoys* a good deal of *sunny* weather, and likewise, the available few studies are hinting that Egyptian females may be, nevertheless, at high risk of vitamin D insufficiency (**El Badawy *et al.*, 2009**). Also, it is not yet ascertained whether vitamin D insufficiency is because of insufficient sunlight exposure as a result of concealing clothes. Egyptian women are dressed in different styles ranging from, as locally called,"Nekab" (totally covering the whole body, including the hands and face) and "Hegab" (covering the whole body but excluding the hands and face) to the western dress styles. Laboratory *in vitro* and *in vivo* studies have shown that clothing inhibits the production of previtamin D and serum vitamin D respectively (**Matsuoka *et al.*, 1992; Salih, 2004**).

The objectives of the present study were to determine the prevalence of vitamin D deficiency by measuring plasma levels of 25(OH)D, and to find out whether vitamin D status is related to the dress among apparently healthy female university students in an Egyptian governorate.

2. Subjects and Methods

Subjects

A random sample of 130 (96 females; 34 males) apparently healthy undergraduate students, Zagazig University, Sharkia, Egypt, were enrolled in a cross sectional study. Female subjects were classified by their dress style into three groups: the "Nekab" dress group where subjects have their whole body totally covered but eyes, the "Hegab" dress group where subjects have their body covered except hands and face, and the Western dressgroup.

Exclusion criteria were chronic illnesses, chronic use of medications (including vitamin D supplementation) or failure to give written informed consent. The study was approved by the ethics committee of Faculty of Medicine, Zagazig University, Zagazig, Egypt.

Methods

All subjects were studied within the same period of one month (April, 2011) to minimize seasonal variations in the levels of vitamin D. A study questionnaire was completed for each participant to collect socio-demographic data. Dress style (for women), skin color, and milk product daily consumption were also recorded. A full clinical examination and clinical history were taken from all subjects.

Anthropometrical measurements

Height and weight were measured with the patient standing in light clothes and without shoes. Body mass index (BMI) was calculated as body weight divided by height squared (kg/m^2).

Determination of plasma 25(OH)D levels

Plasma samples were obtained in EDTA tubes, mixed and centrifuged for 15 minutes at 2500 rpm. Plasma samples were stored at -20°C until time of assay. Plasma levels of 25(OH)D were assayed in duplicate,with quality control samples,using electrochemiluminescence immunoassay (ECLIA) performed on the Cobas e601 immunoassay analyzer (Roche Diagnostics GmbH, D-68298 Mannheim, Germany),(detectable range: 3.00-70.0 ng/ml.). Values below the limit of detection are reported as < 3.00 ng/ml. Values above the measuring range are reported as > 70.0 ng/ml. A value of 30 ng/ml is defined as vitamin D sufficiency. A value of < 20 ng/ml is defined as vitamin D deficiency and values between 20 and30 ng/ml are defined as vitamin D insufficiency.

Statistical Analysis

Continuous data were expressed as mean (±standard deviation) and were compared by use of Student's t-test or one-way analysis of variance (ANOVA). Categorical data were expressed as frequencies and percentages, and were analyzed with the two-tailed chi-square test. Data were analyzed using SPSS version 11.0.1. Software (SPSS for Windows, 2001). Two-tailed *p* values <0.05 were considered statistically significant.

3. Results

Mean age of participants was 21.1 ±2.17 and 20.8 ±1.96 years for male and female students, respectively. A comparison between the characteristics of these subjects by gender is shown in Table 1. A significant difference was found between males and females as regard BMI and daily consumption of milk products.

Mean plasma 25(OH)D concentration was 23.7 ±12.681 ng/ml. As shown in Table 2, mean plasma 25(OH)D level was not significantly different between males (26.6±12.19) and females (22.8±12.75). Also, no differences were noted when categorical comparisons

were made by stratifying plasma 25(OH)D levels into deficiency, insufficiency and sufficiency levels. Using a cut-point of 30 ng/ml, 74.6% of this sample (61.7% and 79.2% of males and females, respectively) had low vitamin D status.

Characteristics of female subjects, classified by their dress style, are shown in Table 3. Students dressed in Western style clothing were the youngest. Otherwise there were no significant differences between the three female groups along parameters studied. Table 4 shows that plasma 25(OH)D levels were not different between these groups. Seventy five percent,81.4% and 82.3% of females with Western, Hegab and Nekab dress style, respectively, had plasma 25(OH)D concentrations indicative of deficiency or insufficiency levels.

Table 1. Characteristics of the study sample.

Parameter	Male (N= 34) Mean (±SD)	Female (N= 96) Mean (±SD)	Total (N= 130) Mean (±SD)	Analysis
Age (yr)	21.1 (±2.17)	20.8 (±1.96)	20.9 (±2.01)	t=0.638; *p*=0.525
BMI[(1)]	26.1 (±6.53)	30.3 (±6.15)	29.2 (±6.49)	t=3.313; *p*=0.001*
	N (%)	N (%)	N (%)	
Skin type				
Fair	5 (14.7)	22 (22.9)	27 (20.8)	
Intermediate or dark	29 (85.3)	74 (77.1)	103 (79.2)	χ^2=1.029; *p*=0.310
Milk product daily consumption				
Yes	12 (35.3)	16 (16.7)	28 (21.5)	
No	22 (64.7)	80 (83.3)	102 (78.5)	χ^2=5.155; *p*=0.023*

*Significant (1) BMI= Body mass index

Table 2. Distribution of 25-hydroxyvitamin Dlevels in plasma,by gender.

25-OHD*	Male (N= 34)	Female (N= 96)	Total (N= 130)	Analysis
Mean (±SD) ng/ml	26.6(±12.19)	22.8(±12.75)	23.7 (±12.681)	t=1.512; *p*=0.133
	N (%)	N (%)	N (%)	
<20 ng/ml	6 (17.6)	31 (32.3)	37 (28.5)	
<30 ng/ml	15 (44.1)	45 (46.9)	60 (46.1)	
≥ 30 ng/ml	13 (38.3)	20(20.8)	33 (25.4)	χ^2=4.929; *p*=0.085

*OHD= Hydroxyvitamin D.

Table 3. Characteristics of the female subjects, by dress style.

Parameter	Western (N= 36) Mean (±SD)	Hegab (N= 43) Mean (±SD)	Nekab (N= 17) Mean (±SD)	Analysis
Age (yr)	19.7 (±1.72)	21.1 (±1.87)	20.9 (±2.04)	F=3.476; *p*=0.035*
BMI[(1)]	29.7 (±6.63)	30.6 (±6.23)	30.8 (±5.03)	F=0.287; *p*=0.751
	N (%)	N (%)	N (%)	
Skin type				
Fair	8 (22.2)	8 (18.6)	6 (35.3)	
Intermediate or dark	28 (77.8)	35 (81.4)	11 (64.7)	χ^2=1.937; *p*=0.380
Milk product daily use				
Yes	5 (13.9)	8 (18.6)	3 (17.6)	
No	31 (86.1)	35 (81.4)	14 (82.4)	χ^2=0.328; *p*=0.849

*Significant (1) BMI= Body mass index

Table 4.Distribution of 25-hydroxyvitamin Dlevels among females,by dress style.

25-OHD*	Western (N= 36)	Hegab (N= 43)	Nekab (N= 17)	Analysis
Mean (±SD) ng/ml	24,5(±13.86)	21.4(±12.03)	22.5(±12.76)	F=0.586; *p*=0.558
	N (%)	N (%)	N (%)	
<20 ng/ml	11 (30.6)	15 (34.9)	5 (29.4)	
<30 ng/ml	16 (44.4)	20 (46.5)	9 (52.9)	
≥ 30 ng/ml	9 (25.0)	8 (18.6)	3 (17.7)	χ^2=0.832; *p*=0.934

*OHD= Hydroxyvitamin D.

4. Discussion

Below latitude of approximately 35° North, UVB radiation is sufficient for vitamin D3 synthesis all year round (**Pierrot-Deseilligny and Souberbielle, 2010; Tsiaras and Weinstock, 2011**).The latitude of Zagazig, Egypt, where this study was carried out, is 30°N.There should be, therefore, no problem with the solar source of vitamin D. However, this study, in keeping with a number of previous studies in other countries sharing Egypt a sunny climate (**Mishal, 2001; Elsammak *et al.*, 2011**) demonstrates a high prevalence of low vitamin D status (61.7% and 79.2% of males and females, respectively) among university students in Zagazig, Egypt. Yet, in contrast to other studies which claim that covered dressing style causes vitamin D insufficiency (**Guzel *et al.*, 2001; Budak *et al.*, 2004; Allali *et al.*, 2009**) we found no differences in plasma 25(OH)D levels between groups, classified by dress style. Thus, our findings do not support the hypothesis that concealing clothing is the mechanism by which vitamin D production is impaired in this sunny country. By contrast, it could be that excess sun exposure itself is the factor responsible for the decrease in vitamin D status. As a regulatory process of vitamin D synthesis in face of continued skin exposure to UVB radiation, pre-vitamin D_3 is broken-down and vitamin D_3 is converted to inactive photoproducts and hence, an increase in vitamin D status is controlled (**Tsiaras and Weinstock, 2011; Webb and Engelsen, 2008**).

Moreover, it should be noted that in the present study, vitamin D levels did not differ between males and females or between females with Western dress style and those with more concealing dress styles, i.e., those wearing Hegab or Nekab. More than 60% of males and 75% of females with Western dress style had low plasma 25(OH)Dlevels. Thus, several reasons other than sun exposure could be involved in such reduction of vitamin D levels among Egyptian university students. Further studies are required to search for these reasons.

Our study had a number of limitations. First, we examined the plasma vitamin D in students of Zagazig University only. Thus, we cannot extrapolate our results to students in other universities or to other populations. Second, we did not measure parathyroid hormone, Ca and PO4levels to exclude any cases of secondary hyperparathyroidism. Yet, many studies have indicated that such serum biomarkers may not reflect the true status of an individual's vitamin D status. Third, while we measured baseline plasma 25(OH)D, we did not have details for sun exposure data of the participants, including holidays in the sun. Evaluation of the impact of dress style on vitamin D status might have been limited by the possibility that many students were spending most of their time indoors. Finally, a larger sample would have been preferred.

Conclusions

This study, in Zagazig, Egypt, in support of those in other Middle East countries, demonstrates a high prevalence of low vitamin D status among university students. Yet, in contrast to other studies we failed to confirm that concealing clothing is the cause of the vitamin D insufficiency. Thus, despite having sufficient solar source of vitamin D, other factors do play a role in these low levels. Further research is needed in this area of interest. While importance of vitamin D supplementation, perhaps not only for conservatively dressed females, is obvious, the most efficacious strategy to improve vitamin D levels remains a subject for further study.

Conflict of interests: None.

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