Thyme (*Thymus capitatus)* regulating altered hepatic mRNA expression of apoptotic genes during Nickel Chloride exposure

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Abstract: It has been reported that oxidative stress plays a vital role in nickel-induced biochemical and molecular alterations. The effects of thyme on the cytotoxicity and genotoxicity induced by nickel chloride were examined with respect to the DNA fragmentation in liver and testis, micronuclei formation, intracellular glutathione and alteration in gene expression. The obtained data from this study revealed that treatment of mice with NiCl2 (20 mg/kg) for two consecutive days, exhibited significantly ($p \leq 0.05$) bone marrow cytotoxicity and a server reduction in number of PCEs was detected, in addition a significant ($p \leq 0.05$) induction in micronucleated PCEs (Mn-PCEs) (28.2 ± 0.75) compared with control group (3.0 ± 0.48). Further more, NiCl2 significantly depleted intracellular glutathione (GSH) levels in hepatic and testicular tissues by 27 & 31.5 % below that of control group and led to apoptotic changes in both tissues as evidenced by DNA fragmentation (21.5% compared to 8.5% in liver & 24.6% compared to 5.0% in testis) as measured by diphenylamine assay. However, pretreatment with thyme oil, significantly ($P \leq 0.05$) increased the number of PCEs and decreased the frequencies of Mn-PCEs (10 ± 0.48).The GSH levels were significantly increased in group pretreated with thyme in hepatic and testicular tissues (34.8% and 29.75 % above control values). Moreover, DNA fragmentations were significantly reduced reached to 62.7% in testis and 65.2% in liver when mice pretreated with thyme. Hepatic mRNA levels for Fas, the apoptosis-promoting gene Bax, and TNFά, were up regulated following NiCl2 exposures in mice as compared to vehicle controls. In summary, the results suggest that the chemo preventive role of thyme against nickel chloride-induced testicular and liver genotoxicity may be due to its intrinsic antioxidant property and it exhibits a hepatoprotective effect, the mechanism of which may involve thyme anti-inflammatory and antiapoptotic effects.
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1. Introduction:

Apoptosis is the phenomenon of programmed cell death; it can be triggered by developmental or environmental stimuli that activate diverse cellular events that eventually culminate in cellular death. Apoptosis is characterized by DNA fragmentation, chromatin condensation, membrane blabbing, cell shrinkage and disassembly into membrane-enclosed vesicles **(**Searle *et al.*, 1982; Wyllie et al., 1980 and Shi 2002). Apoptosis has been reported for various hepatotoxicants, including carbon tetrachloride (Shi *et al.,* 1998), cadmium (Habeebu *et al.*, 1998) and cyclohexamide (Ledda-Columbano *et al.*, 1992), yet the molecular mechanism(s) of xenobiotic-induced toxicity remain poorly understood. Apoptosis is a binary event and is characterized by the presence of DNA laddering **(**Ioannou and Chen, 1996). DNA is broken down into segments and DNA fragmentation is an important feature that occurs very early in the apoptotic process, usually appears several hours before cell viability starts to decrease (Rangamani and Sirovich 2007., Claudia et al., 2010).

Exposure to toxic metals has become an increasingly recognized source **of** illness worldwide. Modern industrialization has introduced harmful metals into the environment by redistributing them from immobilized ores and minerals, thereby exposing humans and animals to more metal salts. Among the myriad environmental pollutants, Nickel, a heavy metal, merits special consideration as a potential toxic element (Das and Dasgupta, 2002). Animal data indicate that nickel is a reproductive toxicant in animals, but it is not known whether occupational or environmental exposure to nickel could result in reproductive effects in humans (Hassan and Barakat 2008). In mammalian cells, induction of DNA single-strand breaks, DNA protein cross links, sister chromatid exchanges and chromosomal aberrations has been demonstrated with various Nickel salts (Kawanishi et al., 1989; Torreilles and Gurein, 1990 and Kasprzak, 1991). Growing evidence suggest that the nickel (III) / nickel (II) redox couple facilitates oxygen free radical reactions, which may represent one of the molecular mechanisms for genotoxicity of nickel compounds Doreswamy et al. (2004). The in vitro and in vivo genotoxicity data indicate that nickel is genotoxic. Nickel has been

reported to interact with DNA, resulting in cross links and strand breaks Das and Dasgupta 2000.

Fresh and dried aromatic plants as well as their processed products have been widely used as flavorings since ancient times, however, during last few decades they also have become a subject for a search of natural antioxidants and antibacterial agents (Evans & Reyhout, 1992; Pokorny, 1991; Madsen & Bertelsen, 1995; Venskutonis, 2004). The genus Thymus comprises 215 species with *Thymus capitatus b*eing one of the most important and thoroughly investigated aromatic plant. It is a perennial plant commonly used as a spicy herb and locally known under the common name "Zaatar". Chemistry, processing and application of Thymus species were previously investigated. Thymus species as well as many other aromatic plants biosynthesize remarkable amount of volatile compounds referred as the essential oil; therefore chemical classification of such plants is based on the main essential oil components. Chemical polymorphism is characteristic to the species of Thymus; numerous chemotypes have been defined, such as carvacrol and thymol, a-terpineol, thujone, geraniol, linalool and others (Thompson, et al., 1998). Essential oils containing high amount of thymol and carvacrol were reported to possess the highest antioxidant activity (Aeschbach et al., 1994; Dapkevicˇius, et al., 1998**;** Farag et al., 1989). In addition, these compounds exhibit other bioactivities, e.g. thymol is an antiseptic, while carvacrol possesses antifungal properties (Menphini, et al., 1993). Non-volatile antioxidants, such as flavonoids and vitamin E were also found in the extracts of T. vulgaris (Dapkevicius et al., 2002; Guille´n & Manzanos, 1998). Therefore essential oils of thyme can be used as natural preservative ingredients in the food industry **(**Banias, et al., 1992; Conner & Beuchat, 1984; Curtis et al., 1996; Economou et al., 1991; Karapmar & Aktug, 1987; Shapiro et al., 1994). Thymus vulgaris has anti-apoptic and anti-inflammatory properties that protect tissues and enhance the regeneration of tubular epithelial.

The aim of the current study was to evaluate the protective effects of thyme oil extract against nickel chloride-induced oxidative stress, DNA damage and alteration in gene expressions in liver of mice.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals:

Thyme oil was purchased from El-Captain Company (CAP PHARMA), 6th October City, Egypt. Nickel chloride was purchased from El-Gomhouria Co., Cairo, Egypt. All other chemicals were of analytical grade.

2.1.2. Animals: Forty Swiss albino adult male mice, 10 – 12 Weeks old and weighing 20 – 25 g. were used from the department of animal house in Science Faculty,

El Malk Abd –El Aziz University, Gada, Saudi. Animals were randomized and housed under ambient room-temperature and relative-humidity conditions, a commercial diet and water were provided ad libitum. All mice were acclimatized for at least one week prior to dosing.

2.2. Methods

2.2.1.Experimental design:

Experiments were carried out to evaluate the antioxidant effects of Thyme against genotoxicity induced by Nickel Chloride using different cytogenetic and molecular tests. Animals were divided into four equal groups (10 mice/group) and caged separately.

Group I (control) mice were maintained on standard diet,

Group II male mice were orally administered thyme oil at 0.1 ml /mouse/day for 10 days.

GroupIII were initially administered (intra-peritoneally) nickel chloride (dissolved in distilled water) at 20 mg/kg for two consecutive days,

Group IV in which mice orally administered thyme oil at 0.1 ml /mouse/day for 10 days and then administered (intraperitoneally) nickel chloride at 20 mg/kg for two consecutive days. After 24 h from the last dose animals were scarified and different samples were collected.

2.2.2. **Micronucleustest**

The micronucleus test was performed according to Adler (1984). Femurs were removed from the animals and a drop of bone marrow was placed on another drop of fetal calf serum on one end of a clean dry glass slide. Cells were spread with the aid of another slide and left to dry. Two slides were obtained per animal and stained with May-Gruenwald-Giemsa before microscopic examination. A total of 2000 polychromatic erythrocytes (PCEs) were analyzed per mouse, and the frequency of micro nucleated PCEs (MnPCEs) were determined. The ratio of PCEs to normo-chromatic erythrocytes (NCEs) was calculated to determine the cytotoxicity in bone marrow**.**

2.2.3. DNA Fragmentation Assays for Apoptosis Protocol

A distinctive feature of apoptosis at the biochemical level is DNA fragmentation. This method was used as a semi-quantitative method for measuring apoptosis.

Apoptotic changes in testis and liver were evaluated calorimetrically by DNA fragmentation and by agarose gel electrophoresis according to the procedure of (Perandones *et al.,* 1993). Testis and liver samples were homogenized in 7oo µl hypotonic lysis buffer and centrifuged for 15 min at 11,000 rpm. The supernatants (SN) containing small DNA fragments were separated; one-half the volume was used for gel electrophoresis and the other half, together with the pellet containing large pieces of DNA were used for quantification of fragmented DNA by the Diphenyl amine (DPA) assay. The samples were treated with equal volumes of absolute isopropyl alcohol and 0.5 M NaCl to precipitate DNA. The samples were then kept at -20° C overnight and centrifuged at 11,000 rpm for 15 min. The pellets were then washed with 500 µ l of 70% ethanol and allowed to dry at room temperature. Extracted DNA was reconstituted in 12 µl of Tris-EDTA buffer and 3 µl loading buffer. The samples were incubated at 37° C for 20 min, then electrophoresed on 1% agarose gels containing 0.71 µg/ml ethidium bromide. At the end of the runs, gels were examined using UV trans-illumination. The Diphenyl amine (DPA) assay reaction was modified by (Perandones *et al*. 1993) from (Burton 1956). Briefly, perchloric acid (0.5 M) was added to the pellets containing native DNA (reconstituted in 400 µl of the hypotonic lysis buffer) and to the supernatants containing fragmented DNA followed by the addition of 2 volumes of DPA solution. The samples were kept at 4° C for 48 h. The coloremetric reaction was then measured spectrophotometrically at 575 nm. The percentage of DNA fragmentation was calculated in both tissues (liver and testis).

2.2.4. Measurement of intracellular level of reduced glutathione

Liver and testis tissues (about 0.2 g) were homogenized in 600 µl hypotonic lysis buffer and centrifuged for 15 min at 11,000 rpm. The levels of reduced glutathione (GSH) in SN were measured according to the method of Silber *et al*., (1992). 5,59-dithiobis-2 nitrobenzoic acid (DTNB) oxidises the sulfhydryl groups in GSH to GSSG with stochiometric formation of 5-thio-2 nitrobenzoic acid (TNB) that has a yellow color. The concentration of GSH was quantified spectro-photometrically at 412 nm using a GSH standard curve.

2.2.5. RNA isolation

Liver tissue cells were ground in liquid nitrogen and total RNA was extracted from the five groups of the experiment (five samples from each group). The extraction of total RNA was performed using Biozol reagent according to the manufacturer's procedures. The concentration and purity of RNA was measured at 260/280 nm using ultraviolet spectrophotometer (ratios fell between 1.75 and 1.9, indicating very pure RNA in all cases). Equal amounts of RNA isolated from individual mice of each group were prepared for the semi-quantitative RT-PCR (Marone et al., 2000).

2.2.6. **Reverse transcription-polymerase chain reaction (RT-PCR).**

RT was performed in a 20-µl volume that contained 5 µM oligo dT_{12-18}, 2 µg total liver RNA, 200 U Superscript™ II reverse transcriptase (Life Technologies) at 42°C for 10 min followed by 42°C for 1 h. In a total volume of 20 µl, the PCR mixture contained 150 µM dNTPs, 1 µM antisense and sense primers for Fas or TNFα, 1 µl reverse-transcribed cDNA, and 2 U Taq polymerase (PE Applied Biosystems, Foster City, CA). The sequences of oligonucleotide primers were: **5`-Fas, CGC CTA TGG TTG TTG ACC; 3`-Fas, CTC CAG ACA TTG TCC TTC; 5`-TNFα, ACA GAA AGC ATG ATC CGC; 3`-TNFα, GTA GAC CTG CCC GGA CTC.,** Amplification conditions were (94°C 15 s, 54°C 1 min, 72°C for 30 s) for 15–35 cycles. The expected amplicon lengths were 477 bp for Fas and 692 bp for TNFα. An aliquot of the RT-PCR reactants (10 µl) was separated on a 1.2% agarose gel containing ethidium bromide, visualized under UV light, and analyzed using NIH Image software.

2.2.7. Statistical analysis

Data are reported as means ±standard deviations (SD). Comparisons between groups were performed using one-way analysis of variance with a *post-hoc* Bonferroni test correction (SPSS 11.0 for Windows; SPSS Inc., Chicago, IL, USA). Differences between groups were deemed to be statistically significant at $p<0.05$.

3. RESULTS

3.1. Micronucleus test

During the preliminary study, slides from live animals that had received nickel chloride at (20 mg/kg) exhibited significantly ($p\leq0.05$) bone marrow cytotoxicity and a server reduction in number of PCEs was detected, (0.57 ±0.45), indicating severe bone marrow toxicity. Treatment with thyme oil at 0.1 ml/mice /day did not induced evident bone marrow cytotoxicity in comparison to the control group. While pretreatment with thyme oil, significantly ($P\leq0.05$) increased the number of PCEs and the ratio of PCEs to NCEs were enhanced to reach the same manner to the control group. In addition, nickel chloride-treated mice group showed a highly significant ($p\leq0.01$) induction in micronucleated PCEs (Mn-PCEs) (28.2 ±

0.75) compared with control group (3.0 ±0.48), while the frequencies of Mn-PCEs observed in control group and thyme oil-treated group were found to be in the same range as illustrated in Table (1). Pretreatment with thyme oil significant decreased (P≤0.05) the frequencies of Mn-PCEs (10 ± 0.48), in spit of this reduction the frequencies of Mn-PCEs in this group was still higher than control group.

Table (1): Inhibitory effect of Thyme oil on bone marrow genotoxicity induced by Nickel chloride

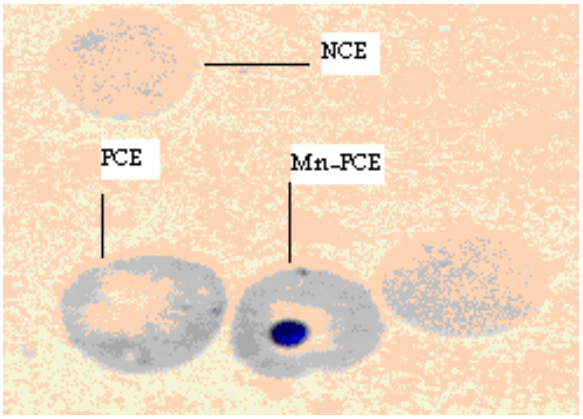

Fig (1): showing normal erythrocyte (NCE), polychromatic erythrocyte (PCE) and micronucleated PCE (Mn-PCE).

3.2. DNA fragmentation

NiCl2 induced DNA damage in liver and testis was evaluated by measuring the level of fragmented DNA colorimetrically using Diphenylamine (DPA) and by detecting DNA ladders on agarose gel electrophoresis. The results showed that NiCl2 caused marked DNA fragmentation in testis and liver (24.6% and 21.5%) compared to control untreated cells (5% and 8.5%) as indicated by DPA assay. Simultaneous treatment with thyme oil and NiCl2 significantly decreased NiCl2-induced DNA fragmentation to 12.3% in testis and to 13.0% in liver (Table 2). DNA fragmentation in response to NiCl2 treatment was also detected by gel electrophoresis as DNA ladder representing a series of fragments that is multiples of 180–200 bp. (Figure 2). Treatment with thyme led to significant protection against NiCl2-induced DNA fragmentation reached to 62.7% in testis and 65.2% in liver (Figure 3). The results indicated that, thyme oil could counteract NiCl2-induced apoptosis in mice.

Table 2: Effect of NiCl2 and thyme on DNA fragmentation in testis and live

Treatment	MnPCEs/1000 Mean ± SE	PCEs % Mean ± SE	NCEs % Mean ± SE	Ratio of PCEs : NCEs
Control	3.0 ± 0.48	47.1 ± 0.43	52.9 ± 0.50	0.89 ± 0.07
Thyme	4.1 ± 0.5	51.0 ± 0.86	49.0 ± 0.48	1.04 ±0.03
Nickel Chloride	28.2 ± 0.75**	36.5 ± 0.59*	63.5 ± 0.40*	0.57 ± 0.45**
Thyme + Nickel Chloride	10.3 ± o.48*	44.8 ± 0.45*	55.2 ± 0.53	0.81 ± 0.06

Treatment	Percent of DNA fragmentation		Change	
	Testis	Liver	Testis	Liver
Control	5.0 %	8.5 %		
Thyme	4.3 %	7.2 %	- 0.7	- 1.3
Nickel Chloride	24.6 %**	21.5 %**	+ 19.6	+ 13.0
Thyme + Nickel Chloride	12.3 %*	13.0 %*	+ 7.3	+ 4.5

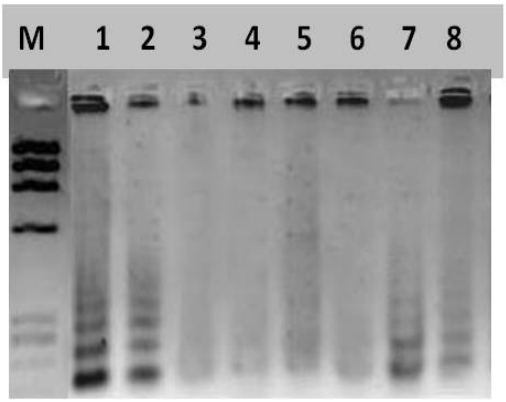

Figure 2: Agarose gel electrophoreses showing DNA fragmentation in mouse testes and liver cells induced by NiCl2: Lane M DNA molecular weight marker Lanes 1 & 2 NiCl2 treated groups (liver & testes), Lanes 3 & 4 control liver & testes, Lanes 5 & 6 thyme treated liver and testes while Lanes 7 & 8 combined treatments (thyme + NiCl2) in liver and testes

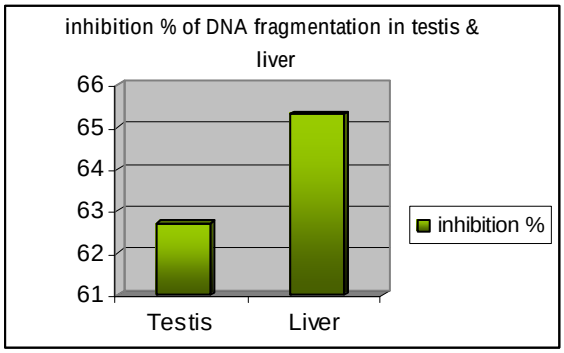

Figure 3: Histogram showing the inhibition percent of DNA fragmentation in testis and liver cells of mice

3.3.Measurement of intracellular level of reduced glutathione

The results of glutathione (GSH) in liver and testis are presented in Table (3). It is clear that nickel chloride treatment resulted in a significant decreased in GSH reached to 38.6 and 43.7 % in both liver and testis respectively. While thyme oil treatment succeeded to increase GSH in liver by 34.8 % and 29.75 % in testis. Animals treated with thyme oil and received nickel chloride showed a significant improvement in GSH and normalized it in both tissues.

Table 3: Effect of Nickle and Thyme on glutathione concentration in liver and testis of mice

Treatmen	Glutathione concentration ir Liver µmo l / gm tissue		Glutathione concentration in testi µmo l / gm tissues	
	Mean ±	Change	Mean ± S	Change %
Control	8.63 ± 0.	----	8.0 ± 0.2	----
Thyme	11.62 ± 0	+ 34.8	10.38 ± 0.	+ 29.75
NiCl2	6.30 ± 0.	- 27	5.48 ± 0.1	- 31.5
Thyme+Ni	9.68 ± 0.	+ 81.1	7.28 ± 0.1	+ 62.5

3.4. Gene expression

The results of gene expression evaluated as the ratio between maximum optical density (max OD) for each band of the target amplification product and the corresponding max OD of β-actin. These results indicated that treatment with NiCl2 resulted in up regulating in mRNA Fas and TNF genes expression in hepatic tissue compared to the other groups. Treatment with thyme induced insignificant change in the expression of both genes compared to the control group. However, the combined treatment with thyme plus NiCl2 significantly regulated and normalized the expression of mRNA (Fas & TNF) compared to the control (Fig. 4 & 5).

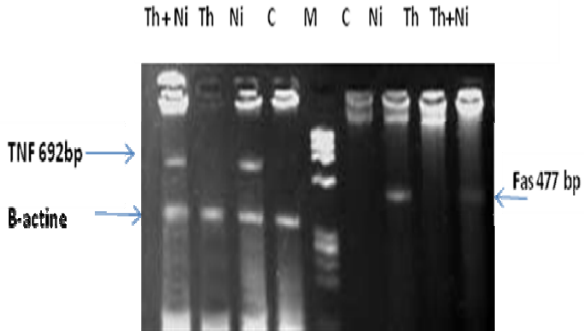

Fig 4:
Hepatic mRNA levels of Fas and TNFα in mice. Liver RNA was isolated from control (C), mice, mice treated with NiCl2 (Ni), or treated with thyme (Th) and combined treatment (Th+Ni). Fas and TNFα expression were normalized to the housekeeping gene β-actine.

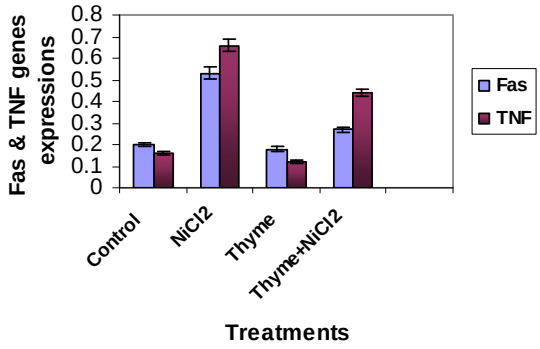

Fig (5). **FAS and TNF mRNA expression in liver of mice determined by semiquantitive RT-PCR analysis The FAS and TNF genes expressions were greatly higher expression in NiCl2 treated group, compared to the other groups.**

4. DISCCUSION

The present investigation was carried out to explore the possible antioxidant and ameliorative role of Thyme herb extract on Nickel chloride-induced genotoxic and cytotoxic effects of hepatic and testicular tissues (DNA fragmentation and glutathione content), micronucleus inductions in bone marrow as

well as up-regulating of apoptotic genes in liver of mice.

Pro-oxidant and antioxidant balance is vital for normal biological functioning of the cells and tissues (Fujii *et al*., 2003). The antioxidant system comprises enzymatic antioxidants such as SOD, GSH-Px and GSH are major enzymes that scavenge harmful ROS in organs (Fujii *et al*., 2003). GSH repairs oxidized and damaged molecules and play a role in regulating a variety of cellular functions. In the present study GSH levels were significantly increased in mice pretreated with Thyme in hepatic and testicular tissues. Thyme extract succeed to protect testis and liver against GSH depletion induced by Nickel chloride. The ability of thyme to maintain hepatic and testicular GSH homeostasis might be due to activation of the glutathione reductase enzyme as well as their anti-oxidant and free radical scavenging effects (Lee *et al*., 2005, Javanmardi *et al*., 2003, Madsen *et al*., 1996, and Madsen and Bertelsen, 1995). The antioxidant activities of thyme have been investigated using various model systems and assays (Lozien *et al*., 2007, and Lee *et al*., 2005).

In the present study we were found that Thyme treatments significantly protected the hepatic and testicular DNA damage by enhancing the concentration of glutathione in both tissues and reducing the DNA fragmentation percent induced by NiCl2. This protective effect of thyme could be the result of direct free radical scavenger properties induced by its phenolic compound (Lozien *et al*., 2007, Lee *et al*., 2005, and Javanmardi *et al*., 2003). Phenolic compound, which are a powerful antioxidants, found in these herb could also react with membrane phospholipid bilayers to break the chain reaction initiated by ROS (Lee and Shibamoto, 2001). In particular, eugenol, thymol, carvacrol and 4-allylphenol, found in Thyme, exhibited potent antioxidant activity, comparable to the known antioxidants, BHT and a-tocopherol (Lee and Shibamoto, 2002). Considering the abundance of these aroma chemicals in natural plants, the total activity may be comparable, to those of known antioxidants. Furthermore, ingestion of these aroma compounds may help to prevent *in vivo* oxidative damage, such as lipid peroxidation and GSH depletion which is associated with cancer, premature aging, atherosclerosis, and diabetes (Lee *et al*., 2005, and Verma and Nair, 2001). Further more Loziene *et al*. (2007) clearly demonstrate anti-mutagenic potential of thyme derivatives *in vitro* while Dasgupta *et al*. (2004) investigation has demonstrated clearly that thyme and basil leafs can be used as a potential cancer chemo-preventive agent by virtue of its efficacy in inducing drug detoxification enzymes such as GST and DTD, as well as in blocking carcinogen-activating phase I enzymes (Henderson *et al*. (2000). Hassan and Barakat (2008) demonstrated that, Thyme pretreatment was shown more effective than Basil in reducing the genotoxicity induced by NiCl2. Thyme was shown to have the highest amount of phenolics whereas Basil had three times lower concentration of phenolics compounds (Kruma *et al*., 2008).

Oxidative stress induced inflammation is mediated by the activation of NF-kB and AP-1. It affects a wide variety of cellular signaling processes leading to generation of inflammatory mediators and chromatin remodeling (Rahman et al., 2006). The latter allows expression of pro-inflammatory genes such as interleukin-1beta (IL-1β), IL-8, tumor necrotic factor alpha (TNF-a), and inducible nitric oxide synthase (iNOS). The undesired effects of oxidative stress have been found to be controlled by the antioxidant and/or anti-inflammatory effects of dietary polyphenols *in vivo* and *in vitro* (Shakibaei, et al., 2007, Rahman et al., 2006 and Han et al., 2007). These result confirmed our study which indicated that, thyme succeed to inhibit up regulating expression of apoptotic genes Fas and inhibition of inhibitory κB (IκB), thus inhibiting NF-κB transactivation, as well as restoring transrepressive pathways through the activation of histone deacetylases in RAW 264.7 cells (Rahman et al., 2006).

In conclusion, the current results revealed that Nickel chloride induced a stressful effects on the liver and testis functions including, depletion in glutathione content, increased in DNA fragmentation and induced micronucleated polychromatic erythrocyted in bone marrow and up-regulated the expression of apoptotic genes in liver. Thyme oil proved to have a protective effect in all the tested parameters. Generally the protective role of thyme can be attributed to the antioxidant effect of herb by acting as free radical scavenger. The improvement of the activities of antioxidant systems might be one of the results of the free radical scavenging effect of antioxidant herb. The detailed mechanisms are worthy of further investigation.

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