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Effect of Seasonal Variation on the Nutrient Composition in Selected Fish Species in Lake Kainji-Nigeria.

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ABSTRACT: Samples of three (3) species of freshwater fishes *Citharinus citharus, Clarias anguillaris* and *Hemisynodontis membranaceus* from Lake Kainji, Nigeria were analyzed during the dry and wet seasons to study the variation in proximate composition of nutrients, mineral content and amino acid profile using standard procedures in wet weight basis. Result of the proximate composition of nutrients showed higher levels in the dry season samples than the wet season. This trend was observed also in the amino acid profile and the mineral content in all the fish samples. However, statistical analysis showed no significant difference in the samples at 0.05 level of significance. [Nature and Science. 2008;6(2):1-5]. ISSN: 1545-0740.

Keywords: Dry season, wet season, tropical fish, freshwater, mineral, amino acid.

INTRODUCTION

The developing countries, Nigeria inclusive are characterized by low food intake and poor nutritional status especially in the area of protein and energy. In Nigeria, starchy foods from root crops and cereals form majority of staple foods depending on the area of the country involved.

In recent years, research into increase production of fish as a cheap and available source of animal protein has been on with the assistant of government in various areas. Research findings have also rated fish nutrients quality very high thus making it an ideal source of vital nutrients both for nourishment and medicinal purposes.

Given that there are considerable evidences in the use of fish and fish products for solving health problems (Mumba, 2005; Onasanya, 2002; Hetzel, 1994), the need therefore arise for investigation into the nutritional composition of freshwater fishes in respect to seasonal variations.

It does appear that seasonal changes with its resultant effect on the activities of fishes may cause variations in their nutrient quality. This experiment was therefore conducted to ascertain such possibility.

MATERIALS AND METHOD

Replicate samples of three freshwater fish species, *Citharinus citharus, Clarias anguillaris* and *Hemisynodontis membranaceus* were bought from Monnai and Cover Dam fishing settlements in the Lake Kainji area, Nigeria. They were gutted, thoroughly washed and weighed for analysis in their fresh state in both dry and wet season samples. The study was conducted covering the period of wet and dry seasons from June 2005 to May 2006 in Lake Kainji, Nigeria.

Proximate composition of the following nutrients was determined using standard procedures of AOAC (2000): moisture, crude protein, lipid, crude fiber and Nitrogen free extract (NFE).

Amino acid profile of the fish samples was determined using the method of Abdullahi (2001). The minerals in the ash were brought into solution by wet digestion using concentrated HNO_3 (63%), per chloric acid (60%) and Sulphuric acid (98%) in the ratio of 4: 1: 1. Potassium and calcium was determined using flame photometer. Phosphorous was determined using spectronic 20 E, other mineral by Perkins Elmer Atomic Absorption Spectrophotometer model 2900 (U.S), (AOAC, 2000).

RESULTS AND DISCUSSION

The proximate composition of nutrients in the experimental fish for dry and wet seasons is presented in Tables 1 and 2. All the species had high moisture content in the range of (76.70% - 84.95%). High moisture contents have been similarly reported in other freshwater species (Abdullahi, 2001; Abdullahi *et al.*, 1999; Effiong, 2005). Differentiation in moisture and lipid content between dorsal and ventral portions of three farmed fish species has also been reported by Silvia et al., (2006).

The values of crude protein (18.50% - 22.87%) in dry and wet season samples of the three species indicate that they are rich source of concentrated protein to consumers. This finding is similar to that

reported by Mumba and Jose (2005). The values were higher than those reported in beef, pork, lamb, mackerel and oyster (Eyo, 2001; 1998). Eyo (1992) also reported similar result from clupeids.

Abdullahi (2001) reported that the protein content in fish might vary with species due to certain factors such as the season of the year, effect of spawning and migration, food available etc.

The ash content of dry and wet samples of *Citharinus citharus, Clarias Anguillaris* and *Hemisynodontis membranaceus* were generally low (0.40% - 1.35%); the crude fat also ranged from (2.45% - 6.85%). Abdullahi (2001) reported higher values (30.0 – 31.3/100g) from *Chrysichthys nigrodigitatus, Bagrus filamentosus* and *Auchenoglanis occidenntialis.* The lower values recorded in this experiment may be attributed to the fish species. Nuray and Ozkan (2007) reported significant differences between moisture and ash contents in *Dicentrarchus labrax* and *Sparus aurata* respectively. There were no significant differences in the proximate composition of nutrients in both species.

In the dry season samples the crude fiber and nitrogen free extract were all negligible. For the wet season sample, crude fiber had a range of 0.68% - 0.90% while nitrogen free extract was negligible. Negligible amounts of these nutrients have been reported by other authors (Oladele et al., 2005; Effiong, 2005).

The result of the amino acid analysis from the experimental fish for both dry and wet seasons is shown in Table 3. The findings are similar to those reported in other freshwater species (Eyo, 2001; Borressen, 1992; Abdullahi et al., 1999). The values were close to or above the FAO (1993) reference value implying that the species contain protein of high quality and good source of dietary essential amino acid. The amino acids of the three species were on the average higher in the dry season than in the wet season.

Generally, the nutrients content of the three species analyzed were not significantly different between the dry and wet season samples. The slight variation observed could be due to increase activity in fish species especially reproduction during the wet season. Various authors have reported the seasonal variation of protein and amino acid in fishes. Most of the authors reported higher values during dry season than the wet season. Although in most cases, there were no significant differences when analyzed statistically (Abdullahi, 2001).

Otitologbon (1997) reported that there was no significant difference in his investigation of the amino acid composition of the whole body tissues of three tropical fish species. Variations in nutrient composition have been reported in other fish species, *Sardinops sagax* (Gamex-meza et al., 1999); Pike perch, Rainbow trout and Eel (Mustafa et al., 2001).

The result of the amino acid profile showed that glutamic acid was the highest for both seasons in all the experimental fish samples. This result is in agreement with the work of other authors as shown earlier in this discussion. It does appear that glutamic acid is highest in the amino acid profile of fishes.

The mineral content of all the three experimental fish samples showed calcium as the highest present in both the dry and wet season samples (Table 4). Saadettin et al., (1999) reported that the most abundant microelements in fish were Zn and Fe followed by Cu with the remaining elements present in amounts below toxic levels. The variation in moisture and ash content in the fish species were within a narrow range according to the same authors.

However, the overall result indicated that mineral content was higher in the wet season samples than the dry season. Kriton (2007) reported differences in trace mineral content in sea bream (*Sparus aurata*) and sea brass (*Dicentrarchus labrax*), two of the most important Mediterranean fish species. He also observed higher ash content in sea brass but lower muscle fat and higher muscle moisture content in sea bream.

Eyo (2001) reported that the mineral content of fish makes fish unavoidable in the diet, as it is a source of different minerals that contribute greatly to good health.

Although there was no significant difference in the nutrient composition of the fish samples between dry and wet season samples, observed variations could be attributed to changes in activities in the fish species when related to variation in seasons. These activities include spawning and migration. The findings of other authors in relation to this study followed the same trend.

Table 1: Proximate Analysis of nutrients in Dry Season Samples

Fish species	**Moisture content**	**Ash content**	**Crude protein**	**Crude fat**	**Crude fiber**	**N.F.E**

Citharinus citharus	76.70%	0.40%	22.87%	2.45%	Neg.	Neg.
Clarias anguillaris	80.95%	0.50%	18.52%	2.65%	Neg.	Neg.
Hemisynodontis Membranaceus	79.75%	0.60%	21.00%	4.05%	Neg.	Neg.
STD	2.19	0.1	2.18	0.82		

All data are not significantly different (p>0.05)

Table 2: Proximate Analysis of nutrients in Wet season samples

Fish samples	**Moisture content**	**Ash content**	**Crude fat**	**Crude protein**	**Crude fiber**	**N F E**
Citharinus citharus	84.70%	1.35%	4.50%	21.625	0.75%	Neg.
Clarias anguillaris	80.85%	0.90%	6.85%	19.22%	0.60%	Neg.
Hemisynodontis membranaceus	84.20%	0.75%	5.00%	21.52%	0.90%	Neg.
STD	2.09	0.31	1.24	1.36	0.13	

All data are not significantly different (p>0.05)

Table 3: Amino acid values of experimental fish samples

	Citharinus citharinus		***Clarias anguillaris***		***Hemisynodnotis membranaceus***	
Amino acid	**Dry season**	**Wet season**	**Dry season**	**Wet season**	**Dry**	**Wet season**
Alanine	4.18	4.06	6.36	6.05	6.50	6.61
Arginine	4.05	3.89	6.81	6.74	5.86	5.88
Aspartic acid	9.19	10.0	11.40	9.89	12.43	12.84
Glutamic acid	15.26	15.6	15.42	15.68	15.59	15.76
Glycine	2.84	2.52	9.19	9.98	5.87	6.25
Histidine	3.26	3.07	3.18	3.49	2.52	3.24
Isoleucine	2.58	2.49	2.68	3.01	4.24	4.65
Leucine	-	-	7.29	7.68	8.52	8.10
Lysine	8.15	8.39	6.34	6.33	10.52	10.62
Methionine	3.06	3.04	2.42	2.31	-	-
Phenylalanine	2.70	2.54	4.39	4.68	3.88	4.87
Proline	2.91	2.76	6.46	5.39	4.43	3.87
Serine	2.63	2.57	5.28	5.18	4.41	4.56
Threonine	4.12	4.08	5.10	5.01	5.23	4.89
Tyrosine	3.14	3.27	3.33	2.96	3.46	2.98
Valine	4.67	4.18	4.24	4.36	5.17	5.18

Table 4: Mineral Composition of dry and wet season samples

Minerals	***Citharinus citharus***		***Clarias anguillaris***		***Hemisynodontis membranaceus***	
	Dry	**Wet**	**Dry**	**Wet**	**Dry**	**Wet**
Calcium	**2.55**	**2.77**	**2.85**	**2.91**	**2.86**	**2.89**
Potassium	**0.63**	**0.76**	**0.71**	**0.78**	**0.64**	**0.71**
Magnesium	**0.21**	**0.35**	**0.28**	**0.32**	**0.23**	**0.29**
Phosphorus	**0.023**	**0.036**	**0.021**	**0.029**	**0.02**	**0.027**

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CTT Multiplex System is a quick and inexpensive method to exclude innocent suspects during criminal inquiries

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Abstract. CTT multiplex (CSF1PO, TPOX, THO1) is a highly polymorphic STR among most populations. During criminal inquiries there is a great need for a rapid and inexpensive method to exclude innocent suspects and to zoom down further inquiries on the smallest possible number of suspects. In this article we present 5 legal cases representing different applications of CTT print. Those applications included inexpensive search for the true offender among large number of suspects, solving human identification problems in cases where tiny amounts of biological material is available such as cigarette butts or residual bone tissue resulting from sawing a corpse, establishing a crime of committing adultery on a married woman by exclusion of biological paternity of her child to her husband and quick liberation of those innocent suspects in custody if CTT print excluded their presence at the crime scene. The results presented herein suggest that CTT multiplex serves as a DNA fingerprint in cases where discordance of DNA prints are sufficient to make a decision. [Nature and Science. 2008;6(2):6-19]. ISSN: 1545-0740.

Keywords: CTT Multiplex; Exclusion; Forensic; Inexpensive; Suspects

INTRODUCTION

DNA fingerprinting is a very important tool in the search for justice as it provides prosecutors with a way to pinpoint suspects with a high degree of certainty, and on the other side can exonerate others without the expense and suffering caused by a trial (Clayton *et al*., 2004).The amplification of short tandem repeat (STR) loci using the polymerase chain reaction (PCR) is currently the method of choice in all forensic investigations. Sprecher *et al*., 1996 having several advantages over conventional Southern blotting method of the larger variable number of tandem repeats (VNTRs) (Kirby and T. Lorne,1990). producing reliable and highly discriminating results with 1ng or less of sample material where casework samples (ex: blood stains, hair roots, cigarette buts, seminal stains,…etc) may contain limited amounts or partially degraded DNA as the only evidence that may link a suspect to a crime (Edwards, 1991**)**. Discrete alleles from STR systems may be obtained due to their smaller size, which puts them in the size range where DNA fragments differing by a single tri or tetra-nucleotide repeat in size may be differentiated. Determination of discrete alleles allows results to be compared easily between laboratories without binning. The inclusion of allelic ladders with each STR multiplex system provides a rapid and accurate method of allele determination that is easy to present to a judge without necessitating a detailed explanation of the underlying scientific principles (Egyed *et al*.,2006)

CTT marker (table1) is one of the most polymorphic multiplex systems characterized by high heterozygosity, distinguishable alleles, regular repeat unit and robust amplification

Table (1): Locus-Specific Information (Edwards, 1991).

STR locus	Chromosomal location	Locus definition	Repeat sequence 5' - 3'
THO1	**11**p15.5	Intron 1 of human tyrosine hydroxylase gene	$AATG^2$
TPOX	**2**p25.1-pter	Intron 10 of human thyroid peroxidase gene	$AATG^2$
CSF1PO	**5**q33.3-34	Human c-fms proto-oncogene for CFS-1 receptor gene	$AGAT^2$

In police investigations CTT marker serves as a rapid DNA profiling test which provides a powerful scientific tool that helps detecting the criminal who usually confesses before being presented to court. Hanson and Ballantyne, 2005. However in those cases where several persons are suspected and police investigators need to free those innocent suspects whose DNA prints are not related to biological remains left at crime scene, a quick inexpensive test is required for rapid decision making. In this manuscript, the significance of using the CTT multiplex to exclude innocent suspects is clarified in various forensic applications (murder, robbery, rape disputed paternity, and identification of dead body remains). Five legal cases are presented where different biological impacts were left at crime scenes (ex: dried blood stains, hairs, cigarette buts, muscle tissues, seminal fluid and vaginal secretion).

MATERIALS AND METHODS

DNA Extraction:

Extraction of total human genomic DNA was performed on biological samples left at crime scenes using 5 % Chelex 100 as a medium for extraction according to the following protocols:

1. a. Blood samples: 150 µl of whole blood, or 1 cm^2 portion of blood stained material was placed into a sterile 1.5 ml microcentrifuge tube containing 0.4 ml of sterile deionized water, then mixed gently and incubated at room temperature for 15 minutes, Centrifuged at 13,000 rpm for 5 minutes, then Carefully all but 20-30 µl of the supernatant was removed from each sample and discarded leaving the remaining pellet containing DNA.
   b. Hair samples: Samples were placed into a 1.5 ml mirocentrifuge tube containing sterile deionized water and shaken vigorously to remove surface dirt and to reduce contamination.
   c. Cigarrate buts: samples were prepared by cutting a 0.5 cm wide strip from the filter end of each cigarette butt, then the strip was cut into smaller pieces and placed into a sterile 1.5 ml microcentrifuge tube.
   d. Seminal stains, vaginal stains and buccal swabs: 1 cm^2 portion from the sample was placed into a sterile 1.5 ml microcentrifuge tube containing 0.4 ml of sterile deionized water, then mixed gently and incubated at room temperature for 15 minutes, centrifuged at 13,000 rpm for 5 minutes, then carefully all but 20-30 µl of the supernatant was removed from each sample and discarded leaving the remaining pellet containing
2. (30-100) µl of 5 % Chelex 100 were added according to the type and volume of the sample.
3. (3-10) µl of Proteinase K (10 mg / ml) were added according to the type and volume of the sample .
4. 10 µl of 1M Dithiothreitol (DTT) were added. (On seminal, vaginal, and buccal swab samples only).
5. Samples were incubated at 56 °C for (30min.-overnight) according to the type of the sample then vortexed on high speed for 5-10 seconds.
6. Samples were incubated at 100 °C for 8 minutes, vortexed on high speed for 5-10 seconds, and then centrifuged at 13,000 rpm for 5 minutes at room temperature. Finally the DNA, which is in the supernatant, is ready for amplification.

DNA Amplification:

For each sample, DNA amplification was performed in a sterile laminar flow hood. The reaction mix contained the following components

PCR Master Mix Component	Volume Per Sample (µl)
Sterile deionized water	14.8
STR10x Buffer	2.5
CTT Multiplex 10x Primer Pair Mix	2.5
Taq DNA polymerase (at 5 u / µl)	0.2
Extracted DNA sample	5
Total volume	25

Amplification was performed according to the following thermal cycling protocol

Initial incubation	Cycling for first 10 cycles	Cycling for last 20 cycles	Hold step
96 °C for 2 minutes	94 °C, 1 minute 64 °C, 1 minute 70 °C, 1.5 minutes	90 °C, 1 minute 64 °C, 1 minute 70 °C, 1.5 minutes	4 °C

A positive control was included by adding 5 µl of K562 DNA instead of sample DNA to 20 µl of PCR master mix. A negative control was included by substituting sample DNA with 5 µl of sterile deionized water in a tube containing 20 µl of PCR master mix. At the end of thermal cycling samples were stored at -20 °C until performing polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis

Amplified DNA samples were prepared by mixing 1 to 1 (V/V) with 2x STR loading solution containing (10mM NaOH, 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol FF) followed by heating at 95°C for 3 minutes then chilling immediately in ice. The polyacrylamide gel was pre-run at 40 watts for at least 30 minutes. Denatured samples were resolved on 4% polyacrylamide gel in 0.5 X TBE buffer using a sequencing gel apparatus (30 cm width x 40 cm length). Samples were allowed to resolve at 40 watts for at least 75 minutes.

Silver Nitrate staining

After electrophoresis, plates were separated carefully using a plastic wedge. The gel (attached to one plate) was placed in a shallow plastic tray, and then subjected to treatment with silver stain according to the following steps:

Step	Solution	Times
a	Fix / stop solution	20 minutes
b	Deionized water	2 minutes
c	Repeat step b, twice	2*2 minutes
d	Staining solution	30 minutes
e	Deionized water	10 seconds
f	Developer solution	Up to 5 minutes (until alleles and ladders are visible)
g	Fix / stop solution	5 minutes
h	Deionized water	2 minutes

The gel (on a plate) was positioned upright and allowed to dry then photographed for documentation.

RESULTS

CASE NO 1

Crime scenario

A master felon committed more than 15 crimes including robbery and setting fire, and never leaves behind him any materialistic evidence that indicates his presence in any of the places where he committed his crimes. There was only one evidence that indicates his responsibility for the crime; a paper he usually writes while planning for each crime. He leaves this paper for the investigators at the crime scene as a kind of belittling the policemen abilities in solving the mystery of the crime as well as threatening them to commit other similar crimes. One night, this felon decided to rob a safe in the office of the undersecretary of the ministry of health and population located in the training center building in Asyout (a major city in Upper Egypt). At 2:00 am, the felon sneaked into the office, turned over the safe, and started to saw certain part of it using some metal tools that he had brought. While he was sawing the safe, his forefinger was

injured and a drop of blood fell on the metal body of the safe front from the inside. The criminal lab expert removed the blood stain using a piece of gauze wetted with de-ionized water and sent it to the central criminal lab for analysis. Results were documented for future comparisons. Because of the high proficiency that was noticed in all those crimes, it was decided to inspect CTT print in all felons having similar criminal records. Besides, the CTT print of the blood stain was compared with prints stored for police staff databases. Surprisingly an ex-sergeant who left the service after several years working with the criminal inquiry staff had a similar CTT print to that found at crime scene.

DNA Analysis

A sample DNA profiling gel showing CTT multiplex of 4 suspects plus the offender and the blood stain CTT multiplex.

Analysis was carried out using polyacrylamide gel electrophoresis followed by silver stain detection and the results were shown in fig (1).

The lanes contain the following samples:

- Lane 1: CTT allelic ladder.
- Lane 2: -ve control.
- Lane 3: +ve control.
- Lane 4: Blood stain at the crime scene (over the safe).
- Lane 5: Blood sample taken from the ex-police staff.
- Lane 6: Blood sample taken from felon 1
- Lane 7: Blood sample taken from felon 2.
- Lane 8: CTT allelic ladder.
- Lane 9: Blood sample taken from felon 3.

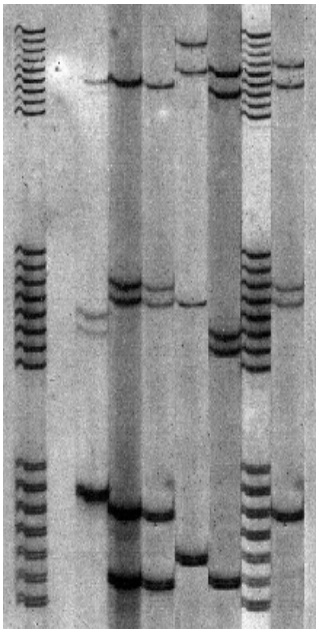

Fig (1): Case no 1

Interpretation of results

CTT profile of the sample in lane no 4 is similar to the CTT profile of the sample in lane no 5 as both have the profile 6-9, 10-11, 10-10 for the genetic loci: THO1, TPOX and CSF1PO respectively. None of the other suspects had similar CTT print to that of the blood stain.

Trial

Investigation authorities decide that the second, third, and fourth suspects are innocent and immediately set them free. Both ex-sergeant's blood and blood stain were further analyzed by CODIS STR loci to create a compelling evidence on the true offender before sending him to court.

Case no 2

Crime scenario

A criminal decided to kill his rival who swindled him out of a great sum of money. He visited him in his apartment where they had supper and smoked cigarettes then the felon volunteered to prepare tea. He went to the kitchen, brought a big knife, sneaked to his victim, and stabbed him in his back. The victim tried to defend himself, holding the felon's head but in vain, the criminal stabbed him a number of times in the chest. After making sure of his victim's death, the criminal took the knife and escaped.

When the investigators entered the apartment they found the corpse of the victim in the midst of a blood pool in the hall. Entertainment impacts and two cigarette buts were found on the table beside the corpse. The criminal lab expert took a sample of blood from the knife wound in the chest of the corpse. While examining the corpse, he noticed that the victim's right hand fingers are contracted gripping some locks of hair (snatched from the head as they have their roots). He took these snatched locks and the two cigarette buts found on the table beside the corpse for analysis. A week later, the criminal inquiry resulted in restricting the suspicion to four persons, as there was a great hostility between these persons and the victim on materialistic affairs.

DNA Analysis

Whole blood samples were taken from those four suspects and analyzed in comparison to the samples taken from the crime scene.

DNA profiling test was carried out on the crime scene samples and the samples taken from the four accused persons simultaneously using the CTT multiplex. Analysis was carried out using polyacrylamide gel electrophoresis followed by silver staining and the results are shown in figure (2).
The lanes contained the following samples:

- Lane 1: blood sample taken from a knife wound from the chest of the corpse.
- Lane 2: CTT allelic ladder.
- Lane 3: blood sample taken from the blood pool surrounding the corpse.
- Lane 4: one cigarette butt found on the table beside the corpse.
- Lane 5: another cigarette but found on the table beside the corpse.
- Lane 6: hair lock found in between the right hand fingers of the victim's corpse.
- Lane 7: blood sample taken from suspect 1.
- Lane 8: blood sample taken from suspect 2.
- Lane 9: blood sample taken from suspect 3.
- Lane 10: CTT allelic ladder.
- Lane 11: blood sample taken from suspect 4

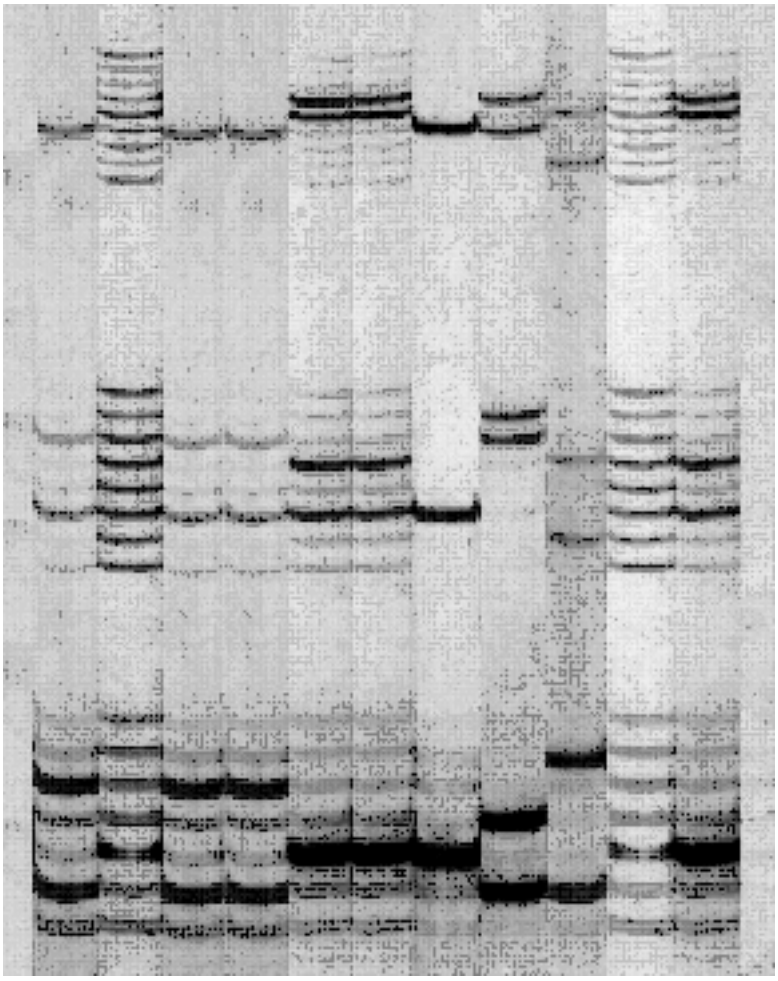

Fig (2): Case no 2

Interpretation of results

- DNA profiles of samples in lanes; 1, 3, and 4 have the typing: 6-9, 8-11 and 10-10 for the genetic loci: THO1, TPOX, & CSF1PO respectively. This indicates that the blood samples taken from the knife wound, the blood pool and from one cigarette butt all belong to the victim.
- DNA profiles of samples in lanes; 5, 6, & 11 have the typing: 7-7, 8-10, 11-12 for the genetic loci: THO1, TPOX, & CSF1PO respectively. Therefore; the second cigarette butt found on the table and the hair lock found in the right hand fingers of the victim's corpse belong to suspect number 4.
- DNA profiles in lanes no 7, 8 and 9 have the typing: 7-7, 8-8, 10-10, 6-8, 11-12, 10-12 and 6-9.3, 7-10, 8-11 which belong to suspects 1, 2 and 3 respectively. None of these DNA fingerprints were detected in the crime scene.

Investigation authorities concluded that suspects 1,2 and 3 were innocent and immediately liberated them. Police investigators faced suspect 4 with the DNA typing results and advised him to confess to improve his legal situation in the case or otherwise greater number of genetic loci will be utilized to provide perfect evidence to the court. At this point the suspect confessed of committing the crime which has been taken in consideration later on by the judge.

CASE NO 3

Crime scenario

A corpse of a strangled naked woman was found laid on the bed in her apartment where she lives alone. There were traces of bloody injuries in the chest, on the face and in the neck showing that she was strangled. Also signs of sexual assault that happened shortly before the death were noticed. Blood samples from a deep wound in the victim's neck and a vaginal swab were taken for examination. Criminal inquiry pointed to two delivery men of either the grocery or the pharmacy.

DNA Analysis

A sample of blood was taken from each of those two accused persons and transformed to the criminal lab to be examined and analyzed in comparison to the samples taken from the crime scene.
DNA profiling test was carried out on the two samples taken from the corpse of the victim and those taken from the two accused persons simultaneously using the CTT multiplex. Results are shown in figure (3).

The lanes contained the following samples:

- Lane 1: CTT allelic ladder
- Lane 2: blood sample taken from the grocery delivery servant.
- Lane 3: blood sample taken from the pharmacy delivery servant.
- Lane 4: blood sample taken from a wound in the neck of the corpse.
- Lane 5: vaginal swab taken from the corpse.
- Lane 6: CTT allelic ladder**.**

Interpretation of results

- DNA profile of the sample lane 5 is 7-8-9, 8-8, 10-11-12 for genetic loci: THO1, TPOX, & CSF1PO respectively, which represents that it contains more than one human nuclear genome to more than one individual.

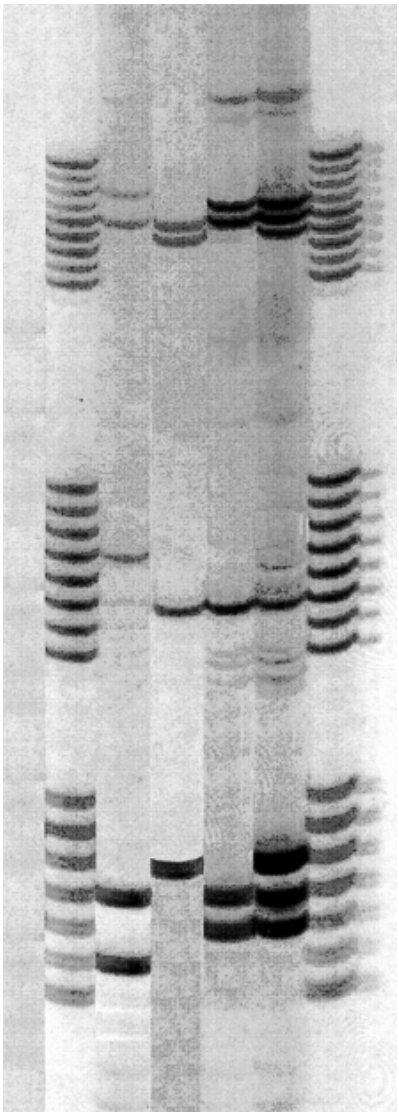

Fig (3): Case no 3

- DNA profile of the sample in lane 4 is 7-8, 8-8, 11-12 for genetic loci: THO1, TPOX, & CSF1PO respectively, which identifies the victim's profile.
- DNA profile of the sample in lane 3 is 9-9, 8-8, 10-11 for genetic loci: THO1, TPOX, & CSF1PO respectively. which identifies the CTT of suspect 1(pharmacy delivery man)
- DNA profile of the sample in lane 2 is 6-8, 10-10, 11-13 for genetic loci: THO1, TPOX, & CSF1PO respectively. which identifies the CTT of suspect 2 (grocery delivery man).
- Therefore; by excluding the typing result of the sample in lane 4 taken from a wound in the victim from that in lane 5 taken as a vaginal swab from the corpse, it was conclude that typing of the criminal's DNA profile found in his seminal fluid excluded suspect 2 who was immediately freed, while suspect 1 was arrested.

The accused felon did not take much time to confess that he raped the woman and killed her being convinced that he could improve his legal situation in the case and reduce the verdict emitted by the court.

Case no 4

Crime scenario

A criminal decided to kill his wife. He slaughtered her neck , cut her corpse into parts and put each part inside a plastic bag then discarded the bags in the desert. After he carefully mopped all traces left at crime scene. Few days later the victim's father reported his daughter's absence to police and accusing her husband. When investigating the conjugal home the criminal lab men found no signs of violence except for a tiny piece of bone that was found hanged at the cesspool.

DNA Analysis

Blood samples were taken from the victim's parents and CTT profiling was carried out simultaneously on parents` samples together with the bone sample found in the bathroom. Results of CTT print are shown in figure (4).

The lanes contain the following samples:

- Lane 1: CTT allelic ladder.
- Lane 2: blood sample taken from the mother of the victim.
- Lane 3: bone sample found at the crime scene.
- Lane 4: blood sample taken from the father of the victim.
- Lane 5: CTT allelic ladder.

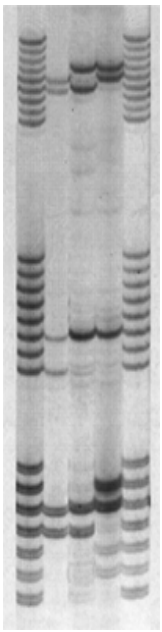

Fig (4): Case no 4

Interpretation of results

- CTT print in lane 2 is 8-9, 6-8, 10-11 for loci: THO1, TPOX, & CSF1PO respectively.
- CTT print in lane 3 is 8-9, 8-8, 10-12 for loci: THO1, TPOX, & CSF1PO respectively.
- CTT print in lane 4 is 9-10, 8-8, 11-12 for loci: THO1, TPOX, & CSF1PO respectively.
- This indicates that the tissue sample found at crime scene shares a common allele with the father and the mother of victim thus suggesting that the such tiny piece of tissue may belong to their missing daughter.

Police investigators confronted the husband with the DNA typing results asking him to explain how his wife's bone was hanged at the cesspool of bathroom.. They advised him to confess or other wise scientific evidences will be completed and presented to court besides the accusation report provided by the victim's father. The accused husband reported a complete confession during the investigation and narrated how and why he committed the crime.

Case 5

Scenario

A married man accused his wife of mistakenly ascribing her new born to him. He proved that he was abroad when pregnancy occurred and accused his wife's cousin for being the true father of the child. When investigators
arraigned the wife and her cousin for in questing both denied the impeachment of the husband. Buccal swap samples were taken from the child as well as from the husband, his wife and her cousin and simultaneously analyzed for CTT print.

DNA Analysis

Results of CTT profiling test are shown in figure (5).

The lanes contain the following samples:

- Lane 1: CTT allelic ladder
- Lane 2: CTT print of the wife.
- Lane 3: CTT print of the disputed child.
- Lane 4: CTT print of the alleged father.
- Lane 5: CTT print of the accused father.
- Lane 6: CTT allelic ladder**.**

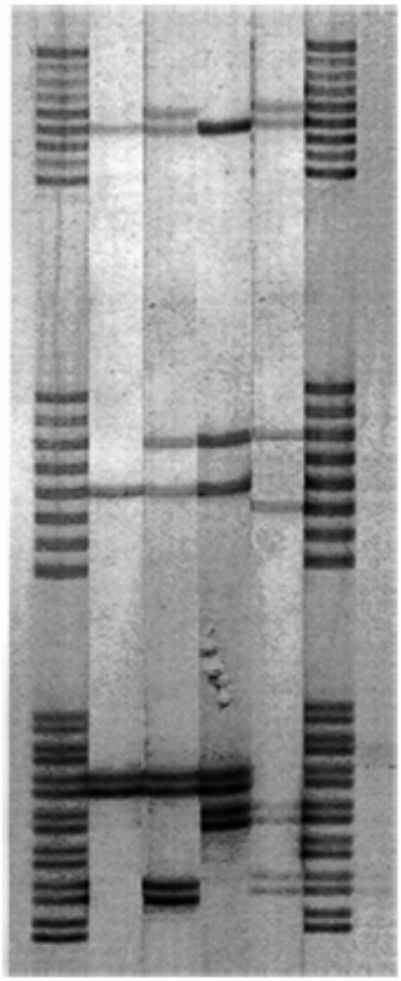

Fig (5): Paternity case

Interpretation of results

- CTT profile in lane 2 is 9-9, 9-9, 10-10 for the genetic loci: THO1, TPOX, & CSF1PO respectively.
- CTT profile in lane 3 is 6-9, 9-11, 10-11 for the genetic loci: THO1, TPOX, & CSF1PO respectively.
- This indicates that the child shares one allele in each genetic locus with that of the wife thus concluding that the biological father must contain the allele (**6**) in the **THO1** locus, the allele (**11**) in the **TPOX** locus and the allele (**11**) in the **CSF1PO** locus.
- However, CTT profile in lane 4 is 8-9, 9-11, 10-10 for the genetic loci: THO1, TPOX, & CSF1PO respectively.
- Therefore; the alleged father (husband) cannot be the biological father of the child, **thus establishing an obvious adultery case.**
- On the other hand, CTT profile in lane 5 is 6-8, 8-11, 10-11 for the genetic loci: THO1, TPOX, & CSF1PO respectively.
- **Therefore; the wife's cousin may be the biological father of the child an assumption that had been confirmed later on by comparing 16 different STR loci.**

Investigators confronted the wife and her cousin with the typing results which proved that the husband can not be the biological father of the child and advised the cousin to assign the child to his name to avoid being sentenced by the court. Upon writing his official confession the case was closed without having a trial.

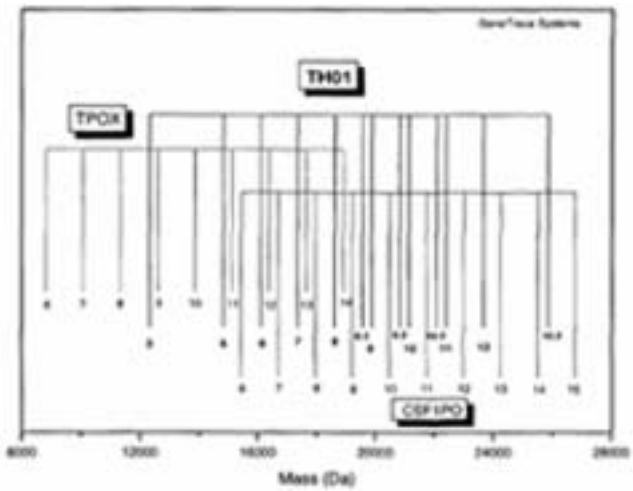

Fig (6): Schematic of expected allele masses for a CSF1PO-TPOX-TH01 (CTT) multiplex involving overlapping allele size ranges. All known alleles are fully distinguishable by mass with this interleaving approach (Butler *et al*., 1997).

DISCUSSION

There are literally hundreds of STR systems which have been mapped throughout the human genome. Several dozens of systems have been investigated for application in forensic purposes. The CTT print is one of the most polymorphic STR multiplex systems included in the 13 core STR loci designated by the Federal Bureau of Investigations (FBI) in the USA. The expected masses for the CTT multiplex including the STR loci CSF1PO, TPOX, and THO1 are schematically displayed in fig (6) (Hammond *et al.* , 1994). .All known alleles for those loci are fully resolvable and are far enough apart to be accurately determined. For example THO1 alleles 9.3 and 10 fall between CSF1PO alleles 10 and 11. For all three STRs in the CTT multiplex, the AATG repeat represents the building unit, which means that alleles within the same STR system differ by at least 1260 Daltons (Denise,1996). This helps in presenting a clear DNA typing result to the court, without explaining the scientific molecular techniques used in evaluating the result. An important reason for utilizing CTT print is the relatively great genetic diversity of the three loci in Egyptians as CSF1PO locus has 8 alleles with frequency range of 0.003 – 0.335, while TPOX locus contains 7 alleles with frequency varying from 0.004 – 0.492 and THO1 locus has 6 alleles with a frequency range of 0.028-0.330 (Refaat,2005). In those forensic application where more than a single person are suspected of committing the crime the investigators need to obtain a rapid and in expensive evidence to exclude innocent suspects and push true offenders to confession, the master of evidences. In this article, the significance of using the CTT multiplex in various applications is clarified through the presentation of five different legal cases (forensics and paternity) in which different biological sources of DNA were involved.

The first case was very mysterious and difficult to solve since the offender seemed to be experienced in this type of crimes. Therefore police investigators decided to focus their CTT screening on felons with criminal records of committing similar robberies and on police staff members for whom CTT data bases were already available. To do rapid inexpensive screening on huge number of suspects CTT print was sufficient to exclude subjects with no relation with the blood stain left at the crime scene. Such typing indicated that none of the felons with criminal records of robbing safes had the same CTT. Then it was decided to launch massive screening of a large number of inhabitants in the village using CTT print to reduce costs

and time when compared with the CODIS system. The results showed no similarities with the CTT of blood on the safe. Finally, when the later CTT was compared with the police staff data base the investigators were able to pinpoint the true offender who gained huge experience in criminal inquiry secrets through his work as a policeman. Needless to say CODIS analysis was required to present a successful case to court when the offender denied the charge.

In the second case it was necessary to free those suspects who have no materialistic linkage to the crime. The availability of diverse sources of DNA at crime scene has facilitated the process of cornering the offender with multiple evidences simultaneously since CTT multiplex results showed identical prints between the offender's DNA and those left in the victim's hands as hair lock and on the table as cigarette butts

In the third case analysis of the victim's vaginal swab was the key to solve the case through which the CTT print of the offender was concluded by subtracting the victim's CTT print (taken from the corpse) from the mixed CTT prints in the vaginal swab and, therefore, having no evidence to keep the other suspect under custody. Therefore, CTT Multiplex is thought to provide a powerful and unique materialistic evidence through the analysis of a post coital mixed stain providing a decision that the grocery man is not guilty and represents a scientific pressure tool which obligated the true offender to confess or otherwise further analysis would be carried out on the extracted DNA samples using larger number of STR loci reaching the assertion state that proves that he is the true offender.

In the fourth case the missing corpse (the major materialistic evidence of a murder crime) could complicate the case because the offender was very careful to hide all traces of violence and the subsequent failure of police investigators to prove the crime of murder. The very tiny (even microscopic) piece of human hard tissue that was recovered from the crime scene could belong to the husband himself or even could be a non human tissue. The later assumption was denied via the biological investigations which had been carried out on this sample and indicated that it is from a human origin. The CTT profiling test could easily exclude that it belongs to the husband's genome. Again a rapid comparative CTT profiling of both the victim's parents and the piece of bone directed the police investigators` attention towards a strong possibility that a case of murder had happened in the conjugal apartment and the husband's confession was a natural result of evidential confrontation from criminal inquiry men.

In the paternity case, CTT print of the disputed child and the alleged father (husband) was sufficient to exclude paternal relation between them thus establishing an obvious adultery case for which the wife should be sentenced according to the Egyptian law. On the other hand the concordance of CTT print of the wife's cousin (accused father) with the child's CTT print opened the gate for friendly dialog between police investigators and him to confess and assign the child for his name or otherwise will be subjected to CODIS analysis and face the verdict emitted by the court.

CONCLUSION

We conclude that CSF1PO, TPOX, TH01 genetic loci are genetically heterogeneous so that they are useful in certain forensic applications. The (CTT) multiplex can act as a scientific pressure on accused persons to confess and simultaneously can exclude other innocent persons who may face injustice being arrested for long time during criminal investigations.

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Anthelmintic Effect of *Solanum lycocarpum* in Mice Infected with *Aspiculuris tetraptera*

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Abstract: This approach intends to add new data on the helminthes parasites of laboratory mice. It has been investigated the anthelmintic activity of *Solanum lycocarpum* (*Solanaceae*) extracts against *Aspiculuris tetraptera* in mice naturally infected. The extracts were applied for oral saw (intragastric), into the volume of 0.04 mL g^{-1} , with the employing of a dead and bend probe during three consecutive days. The fecal material, collected 24 h after each application, performing a total of four fecal collection, have been softened previously, transferred about to sieve of network of 125 μm and tested under microscope stereoscope, with the objective of behave the identification and counting from the worms eliminated of the second to the fifth day of the experimental. Tukey-Kramer Multiple Comparisons Test was applied to compare the results. According to the analysis of the results it was observed and an extremely significant difference between TM and C (from 5.64±3.16 to 1.56±3.16). It was published that medicinal plants which were reported as useful in the treatment of diabetes the *S. lycocarpum* was the sixth most frequently mentioned. According to the results obtained in the present study, we can speculate that the anthelmintic effect of *Solanum lycocarpum* was noticed due to the concentration of steroidal alkaloid oligoglycosides and short-chain fatty acids.

Key words: *Solanum lycocarpum*, *Aspiculuris tetraptera*, anthelmintic, mice, extracts, medicinal plants

INTRODUCTION

Solanum lycocarpum was collected in the City of Três Marias, State of Minas Gerais and in the City of Seropédica, State of Rio de Janeiro. The botanical identification was carried through in the Department of Botany of the Rural Federal University of Rio de Janeiro, having been the exsiccates deposited under numbers RBR 28010 and RBR 14072.

S. lycocarpum is a plant which is shrubs ranging in height from 1.2 to 3 m. The fruit is yellow in color and resembles a medium sized tomato. Parts of the plant are poisonous if it gets in your system. When it is in bloom, it is medium blue. It blooms in the late winter, early spring, late fall, early winter and mid winter. It is velvety or fuzzy. It needs water regularly. It is found in the Brazilian savannah but has been said to grow in San Antonio, Texas. *S. lycocarpum* is commonly used in Brazilian folk medicine. The Brazilian flora is one the world richest sources of bioactive material due to its biodiversity. Several plants are currently used in Brazilian traditional medicine to treat diabetes. *Solanum lycocarpum* St. Hill., *Solanaceae* has been widely used and commercialized as a hypoglycemic agent in Brazil. It was described by Vieira *et al*. (2003) the anti-inflammatory effects of the crude ethanol extract and its alkaloid fraction from *S. lycocarpum* fruits. Recently, it was carried out a chemical analysis of the starch and tried to correlate its supposed hypoglycemic activity with the polysaccharide content. However, these investigators did not conduct any experimental test to directly demonstrate the hypoglycemic effect attributed to the starch. Solanaceae or Lobeira is a plant used as a hypoglycemic agent. A study reported that the extract reduces glycemia in alloxan induced diabetic rats. It was reported that the potential of *S. lycocarpum* as antioxidant was capable reduce in 27%

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nitrate generation in diabetic animals. In literaturature has been demonstrated that *S. lycocarpum* is not ulcerogenic and restored haemoglobin and haematocrit to normal values in diabetic animals (Perez *et al*., 2006).

It this plant contains steroidal glycoalkaloids that can be transformed into an intermediate for steroidal drug production. In this way, it is very possible that these glycoalkaloids and its aglycone, once in the body by ingestion of *S. lycocarpum* fruits, may act by disrupting the endocrine system. Because its fruits may be consumed by pregnant animals in the fields, various studies determined the possible toxic effects of exposure to *S. lycocarpum* fruit from gestation. The unripe fruits contained 0.6% of solamargine and 0.9% of solasonine. It was related that *S. lycocarpum*, during gestation and the beginning of lactation reduces intrauterine growth. It is known that during adulthood, female offspring showed impaired sexual behavior and male offspring showed prominent degeneration of testis germinative cells, characterized by a reduced number of germ cells and vacuolation. It has been documented that the exposed offspring showed reduced hypothalamic norepinephrine (NOR), vanillylmandelic acid (VMA), 3-methoxy-4-hydrophenylglycol (MHPG) and homovanillic acid (HVA) levels and reduced striatum NOR, HVA, VMA, MHPG, dopamine (DA), dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindolacetic acid (5-HIAA) levels. It is suggest that the fruit may act as an estrogen, with a long-term effect, impairing the receptive lordosis behavior of female offspring and promoting testis abnormalities in male offspring at adulthood. It appears to disrupt brain organization since important central monoamine level alterations were also related (Schwarz *et al*., 2005a).

Rodents, as mice and rats are the most common laboratory animals used in research and testing. They are seldom investigated for autochthonous ecto and endoparasites prior their utilization in the experiments. Pinworms commonly infecting laboratory rodents include mainly the mice pinworms *Aspiculuris tetraptera* (Perec-Matysiak *et al*., 2006; Gilioli *et al*., 2000).

Some plant extract may act differently due to its action against the parasite. In a study the anthelmintic activity of the extracts obtained from *Luxemburgia octandra* was evaluated naturally infected mice with *Aspiculuris tetraptera* and *Vampirolepis nana*. The leaves extracts were given to the animals during three days. The ethanolic and ethyl acetate extracts presented significant increase of the *V. nana* elimination, but did not present the nematicide effect against *A. tetraptera* (Silva *et al*., 2005).

In the present study we evaluated the anthelmintic activity of *Solanum lycocarpum* extracts in a concentration of 5% against *Aspiculuris tetraptera* in mice in naturally infected.

MATERIALS AND METHODS

Vegetal extracts: Dried leaves of units of had been used in the anthelmintic tests *Solanum lycocarpum* had been gotten by infusion (tea), submitted to the filtration in nylon and the express concentrations in g/100 mL (p/v).

Animals and anthelmintic tests: For anthelmintc test have been used lots of albinos mice, male and females weighted in media of 25 g and naturally infecting for *Aspiculuris tetraptera*, originated from Oswaldo Cruz Foundation-FIOCRUZ and held into the Institute of Biology from Rural Federal University from Rio de Janeiro. The animals have been held into bird cages individual of polypropylene (30×20×13 cm), it has at the bottom road of screen stark and stiff (network of 7×7 mm) upon a sheet of absorbent paper with the aim to facilitate the collection diary of excrement (Amorim *et al*., 1987; Amorim and Borba, 1990). The referred study was conclude in the Laboratory of Activity Anthelmintic of Plants from Rural Federal University from Rio de Janeiro. The extracts were applied for oral saw (intragastric), into the volume of 0.04 mL g^{-1}, with the employing of a dead and bend probe during three consecutive days. The excrement, collected 24 h after each application, performing a total of four fecal collection, have been softened previously, transferred about to tames of network of 125 μm and evaluated under microscope stereoscope, with the objective of behave the identification from the worm eliminated of the second to the fifth day of the experimental. Into the fifth and last days from the tests, the mice have been sacrificing for inhalation of vapors of ether ethyl, examining humid weight of the contents of the small intestine, in order to access the number of the proglotes collected of *A. tetraptera* remnants (Amorim *et al*., 1999). On the tests have been used the extracts of *S. lycocarpum* (leaves dried from Três Marias in the concentration of 10%) and (leaves dried from UFRRJ in the concentration of 10%). Additional lots of mice have been used with standard, they receiving doses of 20 mg kg^{-1} day^{-1} of mebendazol and 100 mg kg^{-1} day^{-1} of nitroscanato and they were submitted to the identical assessment anthelmintic description about to the animals treated with the plant extracts. A batch control, without a treatment served about to appraise the elimination spontaneous from the

helminthes studied. The outcome antinematode also was denominated in terms percentile average of roundworm eliminated, considering the number of roundworm eliminated in the excrement in relation to the total number. Statistical analysis were performed and Tukey-Kramer Multiple Comparisons Test was applied to compare the results.

RESULTS AND DISCUSSION

According to the analysis of the results it was observed that there were no differences ($p>0.05$) in the % of elimination between TM and UR (from 5.64±3.33 to 3.15±3.16), UR and C (from 3.15±3.16 to 1.56±3.16) and an extremely significant difference between TM and C (from 5.64±3.16 to 1.56±3.16) (Table 1).

The extracts were applied for oral saw (intragastric), into the volume of 0.04 mL g^{-1}, with the employing of a dead and bend probe during three consecutive days. The excrements, collected 24 h after each application, performing a total of four fecal collection, have been softened previously, transferred about to tames of network of 125 µm and evaluated under microscope stereoscope, with the objective of behave the identification of the worm eliminated of the second to the fifth day of the experimental. Tukey-Kramer Multiple Comparisons Test was applied to compare the results.

Animal models have been exhaustively investigated regarding aspects related to their suitability for the development of experimental protocols under laboratory conditions. Nevertheless, in most of the adopted procedures, the prior detection of their ecto and endo parasites are generally overlooked related to the really effects of natural extracts in their biological cycle.

In the Brazilian cerate, a preparation obtained from the fruits of *Solanum lycocarpum* St.-Hill. (Solanaceae), popularly known as fruta-de-lobo (wolf-fruit), have been widely employed for diabetes management, obesity and to decrease cholesterol levels. The medicinal preparation consists of the green fruits which are ground in aqueous solution and filtered. The white gum deposited is decanted and slowly dried providing a powder which is commercialized in capsules with the name of polvilho-de-lobeira. Through phytochemical analysis of this phytomedicine and the fruit of *S. lycocarpum* were found polysaccharides as the main component. Some polysaccharides slow gastric emptying and act on the endocrinous system affecting the liberation of gastrointestinal hormones, lowering blood glucose levels. According to Schwarz *et al.* (2005b) it is well known that this plant contain steroidal glycoalkaloids that can be transformed into an intermediate for steroidal drugs production, like oral contraceptives. In this way, it is very possible that these glycoalkaloids and its aglycone, once in the body by ingestion of *S. lycocarpum*, may act disrupting to the endocrine system as well as it may probably affect the reproductive system of helminthes. The hypocholesterolemic activity could be due to the increased fecal bile acid excretion as well as to the action of the short-chain fatty acids, coming from fermentation, on the synthesis of delta-aminolevulinate and by the increase of the cholesterol 7-alpha-hydroxylase and 3-hydroxy-3-methylglutaryl CoA reductase synthesis (Dall'Agnol and Von Poser, 2000).

Due to the effect related it may be possible that these fatty acids could act as an anthelmintic, although in he present study there was not observed differences between TM and UR extracts related to % of elimination in comparison one to another, although in comparison to the control group was evident a significative difference due to the TM group. Related to the obtained results due to the action of the TM extract it may be explained by their concentration as well as originated region which may explain the effect due to the biochemistry compounds in the equivalents proportions in spite of different conditions as soil composition, light and water availability.

The effect of TM extract may be support by possible modifications in ribosomal DNA spacer region suggesting that it could result in genetic and geographical variability as well as different bioactivity which may not be effective depend on the concentration of the extract (Arruda *et al.*, 2003).

Table 1: Anthelmintic activity of the extracts obtained of *Solanum lycocarpum* in the elimination of *Aspiculuris tetraptera* in mice naturally infected

Used parts	Administration form (%)	No. of animals	No. of Helminthes		Elimination (%)
			Fecal exam	Necropsy	
Leaves dried from três marias (TM)	10	10	61	1082	5.64±3.33
Leaves died from UFRRJ (UR)	10	12	54	1717	3.15±3.16
Nitroscanato (NIT)		12	499	282	64.00±0.00
Mebendazol (MEB)		10	324	0.0	100.00±0.00
Control (C)		10	45	2836	1.56±3.16

We can speculate that the other effect would be related to the low concentration of steroidal alkaloid oligoglycosides which in a optimal concentration may suppress the transfer of sucrose from the stomach to the small intestine which could diminish the support of glucose to helminthes together with its antioxidant effect which is capable of reducing the nitrate generation which can be used in the protein synthesis.

CONCLUSION

Based on the results we can suggested that the anthelmintic effect of *Solanum lycocarpum*, TM extract, was observed related to the possible concentration of steroidal alkaloid oligoglycosides as well as the short-chain fatty acids presents in the extract. The similar action of the extracts may be explained by adaptation mechanisms related to the genetic and geographical variability.

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Selective Antimicrobial properties of *Phyllanthus acidus leaf* extract against *Candida albicans, Escherichia coli* and *Staphylococcus aureus* using Stokes Disc diffusion, Well diffusion, Streak plate and a dilution method

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ABSTRACT:

The antibacterial and antifungal activities of Phyllanthus acidus was investigated against S.aureus (gram+ve), E.coli (gram-ve) and C.albicans using the Stokes disc diffusion, the Pour plate, Well diffusion and Streak plate methods. The solvent type extracts were obtained by three extractions with hexane, CH_2Cl_2, EtOAc and CH_3CH_2OH respectively. Solvents were removed in vacuo to yield viscous oils and paste which were made up to a concentration of 0.035g in 0.01L(10 mL)of the respective solvents. These were tested in varying volumes of 0.2-0.6 ml/plate. The solvents were used as control whereas ampicillin and nystatin were used as references for bacteria and fungal species respectively. The solvents had no effect on the microorganisms whereas ampicillin and nystatin inhibited microbial growth. Phyllanthus acidus showed antimicrobial inhibitory activity at 0.18mg/10mL plate of medium with activity most prominent with the ethanol extracts and negligible with the hexane. This study suggests that the ethanol extracts of Phyllanthus acidus ,can be used as herbal medicines in the control of E.coli and S.aureus following clinical trials. [Nature and Science. 2008;6(2):24-38]. ISSN: 1545-0740.

Keywords: Antimicrobial; *Phyllanthus acidus; S.aureus; E.Coli*; *C.albicans*; Stokes Disc diffusion; Well diffusion; Streak plate; dilution method; herbal medicines.

1.0. Introduction:

This paper discusses the antimicrobiological (antibacterial and antifungal) activity of leaves of *Phyllanthus acidus* also known as *gooseberry* from the coastal plain of the Guyana flora and its possible use as an herbal cream/herbal medicine. Its antimicrobial properties were investigated against *S.aureus* (gram+ve), *E.coli* (gram-ve) and *C.albicans* strains using the Stokes disc diffusion sensitivity technique, Well diffusion, Streak plate and a dilution method. An antimicrobial is a compound that kills or inhibits the growth of microbes such as bacteria (antibacterial activity), fungi (antifungal activity), viruses (antiviral activity) or parasites (antiparasitic activity).

Guyana has a rich biodiversified flora whose crude extracts, both organic and aqueous can be investigated for their antimicrobial activity. In addition, their role as global CO_2 sinks (in the context of global warming) is noted. Also, the extracts of the specified plants parts of the same species, fractionated for natural products whose antimicrobial activity can also be correlated with that of crude extracts. Following this, clinical trials can lead to the formulation of an herbal plant cream or herbal medicine. A few herbal medicine shops have now been established in Guyana. Plants extracts and fractionated plant extracts have been used for their antimicrobial properties[1-15]. Besides used as an herbal cream, following clinical trials, crude plant extracts can be chromatographed leading to the isolation and purification of new and known bioactive natural products/phytochemicals, whose medicinal activity can also be investigated.

For example, two new *ent*-trachylobanes (1) and (2) were isolated from *Xylopia langsdorffiana* and their *in vitro* cytotoxicity assay investigated against a permanent lung fibroblast cell line derived from Chinese hamsters (V79) and rat hepatocytes using MTT[15-16] method and gave IC_{50} values of 224 and 231 uM respectively.

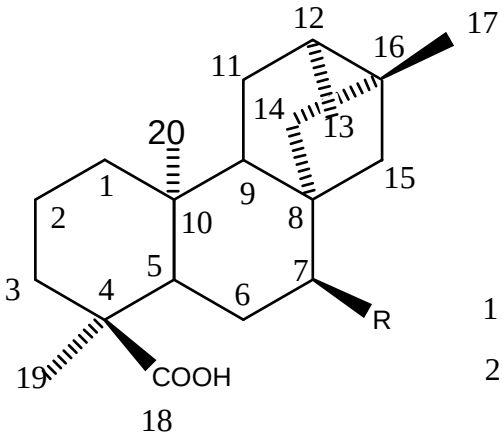

Fig. 1.0. Structure of *en*trachylobanes (1) and (2).

There is an urgent need to revolutionalsed and intensify research in herbal medicine and isolated drug discovery, considering the threat to mankind of incurable diseases as HIV AIDS and other new emerging disease such as SARS, bird flu (H5N1) etc. Plants are a good source of herbal medicine and natural products/ phytochemicals[1-17] . Thus, research in herbal medicine needs to be intensified. Many synthetic drugs owe their discovery and potency as a result of a mimic of structures from isolated natural products from plants rather than to the creativity and imagination of contemporary organic chemists. For example, the drug taxol, paclitaxel, one of the most powerful anticancer drug known , first isolated from the bark of the yew tree *Taxus brevifolia* has yielded two approved drugs for breast and ovarian cancer[6 14.] Paclitaxel is a mitotic inhibitor used in cancer chemotherapy.

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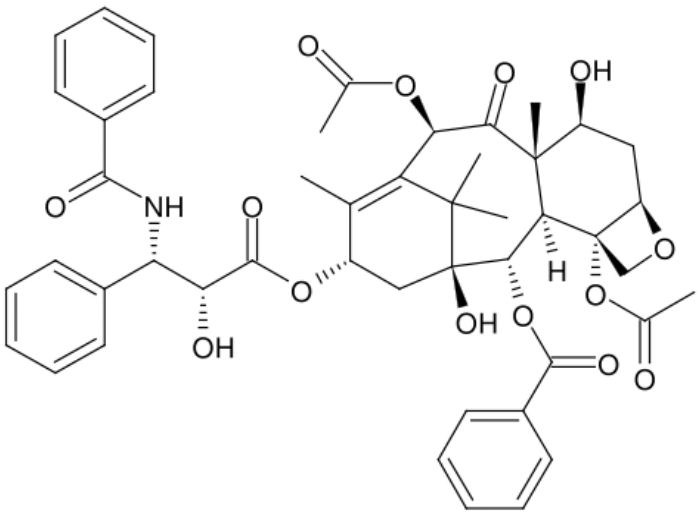


Fig 2.0. Paclitaxel, “Taxol” (3)

In Guyana, there are many medicinal folklore practises but most are without scientific research. Its our scientific endeavour, to correlate antimicrobial activity of *Phyllanthus acidus* with its folklore practices. In Guyana's traditional medicine, an infusion of the herb is taken for the relief of dysentery and also as a blood purifier (bitter tonic to reduce blood sugar level). Also, an infusion or tea for women who are dieting and wish to remain slim. However, little is known of the antimicrobial properties of *Phyllanthus acidus.* As part of a project to investigate extracts and chromatographic fractions from plants of the Guyana's flora[7-15, 17] for antimicrobial activity, we report here, the antimicrobial properties of *Phyllanthus acidus*.

Phyllanthus is the largest genus in the family *Phyllantheae*. *Phyllanthus* has a remarkable diversity of growth forms including annual and perennial herbaceous, arborescent, climbing, floating aquatic, pachycaulous, and phyllocladous. *Phyllanthus acidus* is an annual erect little branched herb, 10-50 cm high [18-20]. Its completely green including the flowers. Leaves are simple, oblong, acute or obtuse, slightly oblique to 14 mm long and 6 mm broad and bear the inconspicuous flowers in pairs in their axils. Each pair of flowers comprises one male and one female. The capsule is a flattened globose about 2 mm in diameter. The classification of the plant is given in Table 1.0:

Table 1.0 Classification of *Phyllanthus acidus*.

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Rosidae
Order	Euphorbiales
Family	Euphorbiaceae
Genus	*Phyllanthus*
Species	*Phyllanthus acidus*

The microbes studied are *Eschericia coli, Staphylococcus aureus and Candida albicans. Escherica. coli* can cause several intestinal and extra intestinal infections such as urinary tract infections, meningitis, peritonitis, mastitis, septicemia and gram-negative pneumonia[21]. *Staphylococcus aureus* can cause furuncles (boils), carbuncles (a collection of furuncles)[22]. In infants, *Staphylococcus aureus* can cause a severe disease Staphylococcal scalded skin syndrome (SSSS). Staphylococcal *endocarditis* (infection of the heart valves) and pneumonia may be fatal. *Candida Albicans* is a diploid fungus (a form of yeast) and is a casual agent of opportunistic oral and genital infections in humans[23-24].

2.0. Procedure:

2.1: **Collection of Plant materials**: The leaves of the above plant was collected from the University of Guyana. The detached plant leaves were subjected to aerial drying for three weeks, removed and placed in separate conical flasks. It was then extracted with the required solvents.

2.2 Extraction: Using selective solvent extraction**,** the leaves were first extracted thrice in hexane over a period of five days[1-13].Water was removed from the accumulated extract by stirring over anhydrous Na_2SO_4 and extract filtered. Solvents were removed in *vacuo* using a rotor vapor. The extracts was placed in sample vials and allow to evaporate. Further drying was done in a dessicator to remove residual solvents. Extracts were stored in capped vials and were weighed. The above procedure was repeated with the same leaves but with different solvents of increasing polarity: CH_2Cl_2, EtOAc, and then CH_3CH_2OH. At the end of drying process, plant extract was either viscous oils, solid or paste.

2.3. Antimicrobial activity tests

2.3.1. Making up extract solution

Approximately 0.035g of dried crude extract of *Phyllanthus acidus* was weighed and transferred to a 10 ml volumetric flask. The respective solvent was then added to make up the 10 ml solution (0.035g in 0.01L).

2.3.2. Microorganisms:

Micro organisms: *Staphylococcus aureus, Escherichia coli and Candida albicans* were obtained from the Georgetown Public Hospital (GPH) microbiology laboratory and was stored in a refrigerator at the Food and Drug microbiology lab.

2.3.3. Agar Preparation:

Two types of agar were used, nutrient agar to make up the medium for bacteria and PDA (Potato Dextrose Agar) to make up the medium for fungi.

2.3.4. Potato dextrose agar (PDA) [25]

The potato *was peeled* and 100g was measured, finely chopped and boiled to a mash in distilled water.The dextrose was measured (12.5g) and placed in a 1L measuring cylinder. Agar was measured (12.5g) and added to the measuring cylinder (with the dextrose).The potato mash was stirred and strained into the cylinder. Hot distilled water was added to make up 500mL.The contents was continuously poured and stirred until consistency was achieved. The content was then poured into a conical flask, plugged with cotton wool, over which aluminium foil was tightly wrapped. The flask was then autoclaved at 121 ^{0}C for 24hrs. The pH range was between 6.5-7.0.

2.3. 5. Reference and Control:

The references were antibiotic in nature. *Ampicillin* and *Nyastatin*. *Ampicillin* was choosen as the reference for all bacterial species used: *E.Coli* and *S.aureus*. *Nyastatin* was used as the reference for the fungus, *Candida.albicans.* The Control experiment consists of a plate of solidifying agar onto which was inoculated pure solvent with microorganism mixed in a 1:1 portion [25].

2.3.6. Aseptic conditions:

The aseptic chamber which consists of a wooden box (1m x 1m x 0.5m) with a door, was cleaned with 70% ethanol and irradiated with short wave UV light (from a lamp).

2.3.7. Mother plates:

These were made by culturing *C.albicans* on PDA. A sterilized 6 mm cork borer was used to cut agar discs in the plate.

2.3. 8. Nutrient Agar:

Nutrient agar was purchased from the International Pharmacy Association in Guyana. 14g of nutrient agar was suspended in 500ml of distilled water in a 1L flask, stirred, boiled to dissolve and then autoclaved for 15 minutes at 121^{0}C. The pH range was between 7.0-8.0. The plates were poured in a sterile environment and allowed to cool for 2 hours. Under aseptic conditions, the micro organisms were streaked onto separate

plates and the discs were applied with a forceps.They were labeled and placed in an incubator at 37 ^{0}C for 24 and 48 hours for bacteria and fungi respectively.

2.3.9. Disc diffusion: Stokes Disc diffusion sensitivity technique [25].

Using Stokes Disc diffusion sensitivity testing technique[25], an inoculum containing bacterial or yeast cells was applied onto nutrient agar plates. On each plate, a reference antibiotic was also applied.The reference antibiotic disc contained 200mg of antibiotic/ml. The discs were made by cutting discs (5-6mm) from a filter paper with a perforator, placing 5 of these discs in a vial and adding 0.2mL of each extract solution. These were left to dry. Discs were also made for the controls: ampicillin for the bacteria and nystatin for the fungus. Each disc was impregnated with the anticipated antimicrobial plant extract at appropriate concentration of 200 mg/ml using a microlitre syringe. This was then placed on a plate of sensitivity testing nutrient agar which was then incubated with the test organism:Bacteria/fungi. Incubation was done at 37ºC for 24 hr and 48 hr for the bacteria and *Candida albicans* species respectively. The antimicrobial compound diffuses from the disc into the medium. Following overnight incubation, the culture was examined for areas of no growth around the disc (zone of inhibition). The radius of the inhibition zone was measured from the edge of the disc to the edge of the zone.The end point of inhibition is where growth starts. Larger the inhibition zone diameter, greater is the antimicrobial activities. It is anticipated through the antimicrobial activity of plant extract, no area of growth will be induced around the disc. Bacteria or fungal strains sensitive to the antimicrobial are inhibited at a distance from the disc whereas resistant strains grow up to the edge of the disc. Discs applied to the plates already streaked with bacteria and the fungus.

2.3.10. Well Diffusion Plate Method Diffusion plate (Well diffusion)[25]:

A fungus (*Candida albicans*) was inoculated into a test tube containing three ml of distilled water (medium), using a flamed loop. Drops of fungus/water culture was mixed with the warm, melted, autoclaved PDA and poured into separate plates under aseptic conditions.The plates were covered and allowed to cool. As soon as the agar was partly solidified, the plates were inverted and left for 2h. When cooled, a well was made at the centre of the plate. The well was made by using a 6 mm cork borer or puncher that was sterilized with alcohol and flame. Plant extracts dissolve in solvent at final concentration of 0.035g/0.01L was pipette into the different wells in a sterilized environment at different volumes (0.2-0.4-0.6ml) in separate plates, using a micro liter syringe. The four solvents (hexane, CH_2Cl_2, EtOAc and CH_3CH_2OH) at different volumes were used as control whereas nystatin dissolved in dichloromethane at same concentration with plant extract (0.035g/0.01L) at different volumes (0.2-0.4-0.6ml) was used as the reference.The plates were labelled, covered, inverted and placed in a fume hood (no incubator was available) for 48h.

2.3.11. Streak Plate Method:

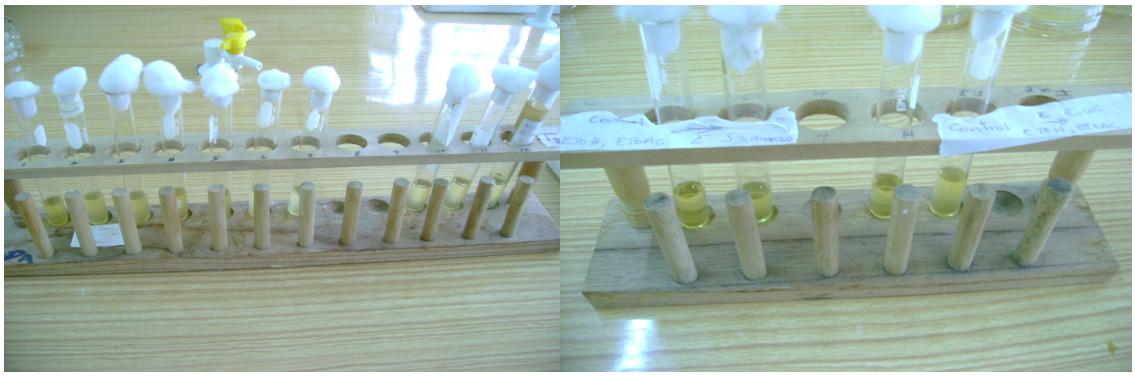
Nutrient agar was prepared as described above and 10 ml was poured into plates. Plant extracts dissolved in solvent at a final concentration of 0.035g/0.01L were pipette into three sterilized plates under asceptic conditions at different volumes (0.2-0.4-0.6 ml), using a micropipette. The plates were allowed to cool and then the bacteria were streaked onto the surface of the solidified agar/plant extract medium. A flame loop was used to inoculate the bacteria from their cultures These plates were left for 24 hours in a dessicator.The plates with inhibition were used in further experiments. A reference experiment was setup using an antibiotic (ampicillin capsule) at the same concentration as plant extracts (0.035g/0.01L) at different volumes (0.2-0.4-0.6ml). Controls were also setup using solvents: hexane, CH_2Cl_2 and EtOAc at the different volumes.

2.3.12. LB (*Luria-Bertani)* broth :

Luria –Bertani broth (LB broth) is a rich medium used to culture bacteria such as *E.Coli and S.aureus.* To make it, tryptone (10g), yeast extract (5g) and sodium chloride (10g) were measured and placed in a 1L cylinder. Distilled water was added to make up the 1L solution and the mixture was poured and re-poured until the contents were dissolved. The pH of the solution was adjusted to 7.4 using sodium hydroxide. 3mL each of LB broth was placed in 56 test tubes. The tubes were plugged with cotton wool foil and wrapped over each top. The tubes were placed into a beaker and autoclaved at 121 ^{0}C for 2h. These tubes were used in the dilutions experiments.

2.3.13. Dilution Method:

This method was used to test the plant extracts for antimicrobial activities against bacteria by investigating whether there was turbity or not. Turbidity represents microbial growth, while no turbidity represents inhibition of microbes. One set of tubes containing LB (*Louria Bertinieia*) broth was inoculated with *Staphylococcus aureus* and the second set was inoculated with *Escherichia coli* using a loop, flame and alcohol. Under aseptic conditions, the plant extracts (dissolved in solvent at concentration 0.035g/0.01L) and which showed inhibition in the streak plate were added to the one set of test tubes containing *E. Coli* and the other set, *S.aureus* with LB broth(medium) in differing volumes (0.2-0.4-0.6ml). Two sets of four tubes each were treated with the four solvents (hexane, CH_2Cl_2, EtOAc and CH_3CH_2OH). One set was inoculated with *S.aureus* and the other with *E.coli,* Fig 3.0. Cotton wool was used to plug test tubes. The tubes were observed after 24 hrs.



(a) Actual experiment (b) Control Experiment

Fig. 3.0. Dilution tubes after 24 h: (a) *Phyllanthus acidus* extract with *S.aureus & E.coli in* LB broth *(b) Control solvent added to LB broth.*

2.3.14. Retention Factor: R_f = Distance moved by sample

Distance moved by solvent front.

In general, the most polar compound has the lowest R_f value.

2.3.15. Thin Layer Chromatography (TLC):

A baseline was drawn on the TLC plate. A spot of the plant extract was placed on the baseline with use of the pipette and allowed to dry. The plate was placed in the developing jar with the solvent. When taken out of the jar, the solvent front was drawn. The plates were then held in the iodine jar for a few seconds, shaken and taken out. They were examined under the UV/Vis lamp and the spots were circled with a pencil. The plate was further examined under UV lamp and any new spots were marked. The spots were labelled and their distances from the baseline were measured. The distance between the baseline and the solvent front was also measured. The R_f values were calculated.

2.3.16. Results:

Mass of dried leaves used for *Phyllanthus acidus species was* 8.55g respectively. These extracts were in the concentration of 0.035g in 10ml of solvent except for *Phyllanthus acidus* with ethanol which was 0.5g in 25ml. This works out to 0.0003mg/uL and 0.02mg/uL of crude extract respectively. The ampicillin and nystatin controls were in concentration of 250mg in 10ml.

Disc diffusion:

Table 2.0. Antimicrobial activity of Plant extract as shown by the inhibition zone diameter**.**

Area of inhibition. (mm^2) using *E.Coli*	**Area of inhibition. (mm^2) using** *S.aureus*	**Area of inhibition. (mm^2) using** *Candida albicans*	**Plant Extracts** *Phyllanthus acidus*	**Reference compound (Ampicillin) (mm^2)**	**Control Experiment**
					No zone of inhibition
< 5	**< 5**	**< 5**	Hexane extract	**27**	No zone of inhibition
< 5	**< 5**	**< 5**	CH_2Cl_2 extract	**28**	No zone of inhibition
20	**15**	**18**	EtOAc extract	**28**	No zone of inhibition
22	**21**	**20**	CH_3CH_2OH extract	**30**	No zone of inhibition

Table 3.0. Results of Well diffusion method for plant extracts *Phyllanthus acidus* against *C.albicans.*

Extract	**Volume of Extract (mL)**		**Diameter of Zone of Inhibition (mm^2)**
Phyllanthus acidus with Hexane	0.2	No zones of inhibition visible, scattered colonies.	**-**
	0.4	" "	**-**
	0.6	" "	**-**
Phyllanthus acidus with CH_2Cl_2	0.2	Zones of inhibition visible.	**67**
	0.4	" "	**79**
	0.6	" "	**79**
Phyllanthus acidus with EtOAc	0.2	Zones of inhibition visible.	**-**
	0.4	" "	**69.5**
	0.6		**79**
Phyllanthus acidus with CH_3CH_2OH	0.2		**75**
	0.4		**80**
	0.6		**84**
Reference(Nystatin)	0.2	Zones of Inhibition visible	**50**
	0.4	" "	**51**
	0.6	" "	**51**

Controls (CH_2Cl_2, EtOAc, CH_3CH_2OH)	0.2	No zone of inhibition	-
	0.4	No zone of inhibition	-
	0.6	No zone of inhibition	-

Streak Plate Method:

Table 4.0. Results obtained from Streak plate method for the bacteria's *Escheria coli* and *Staphylococcus aureus* against different volumes of dissolved plant extracts at a final concentration of 0.035g/0.01 L and controls. Inhibition or no growth of microbes were represented by a positive sign (+), while the negative sign (-) represents no inhibition or growth of microbes**.**

Plant extract dissolved in solvent	Volume of dissolved plant extract used in (ml) at concentration 0.035g/0.01L	Inhibition or no growth of microbe, *Escherichia coli*	Inhibition or no growth of microbe *Staphylococus aureus*
Phyllanthus acidus dissolved inn hexane	**0.2**	-	-
	0.4	-	-
	0.6	-	-
Phyllanthus acidus dissolved in CH_2Cl_2	**0.2**	-	+
	0.4	-	+
	0.6	-	+
Phyllanthus acidus dissolved in Et(OAc)	**0.2**	+	-
	0.4	+	-
	0.6	+	-
Phyllanthus acidus dissolved in CH_3CH_2OH	**0.2**	+	+
	0.4	+	+
	0.6	+	+
Reference(Ampicillin with same concentration as dissolved plant extracts(0.035g/0.01L).			
	0.2	+	+
	0.4	+	+
	0.6	+	+
Hexane	**0.2**	-	-
	0.4	-	-
	0.6	-	-
CH_2Cl_2	**0.2**	-	-
	0.4	-	-
	0.6	-	-
EtOAc	**0.2**	-	-
	0.4	-	-
	0.6	-	-

Dilution Method:

T_0 = No Turbidity = Inhibition
T_1 = Lightly Turbid = Moderately Inhibited
T_2 = Moderately Turbid = Lightly Inhibited
T_3 = Very Turbid = No Inhibition

Table 5.0. Table showing degree of turbidity of dissolved *Phyllanthus acidus* extracts at a concentration of 0.035g/0.01L at different volumes against *Escheria coli* microbe.

Plant extract dissolved in solvents at concentration of 0.035g/0.01L	Volume of dissolved plant extract (ml) 0.2 ml	Volume of dissolved plant extract (ml) 0.4ml	Volume of dissolved plant extract (ml) 0.6 ml
Phyllanthus acidus with hexane	**T_3**	**T_3**	**T_3**
Phyllanthus acidus with CH_2Cl_2	**T_3**	**T_3**	**T_3**
Phyllanthus acidus with EtOAc	**T_2**	**T_1**	**T_o**
Phyllanthus acidus with CH_3CH_2OH	**T_0**	**T_0**	**T_0**

Table 6.0. Table showing the degree of turbity of dissolved plant extract at concentration 0.035g/0.1 L at different volumes against *Staphylococcus aureus* microbe.

Plant extract dissolved in solvents at concentration of 0.035g/0.01L	Volume of dissolved plant extract (ml) 0.2 ml	Volume of dissolved plant extract (ml) 0.4ml	Volume of dissolved plant extract (ml) 0.6 ml
Phyllanthus acidus with hexane	**T_3**	**T_3**	**T_3**
Phyllanthus acidus with CH_2Cl_2	**T_0**	**T_0**	**T_0**
Phyllanthus acidus with EtOAc	**T_0**	**T_0**	**T_0**
Phyllanthus acidus with CH_3CH_2OH	**T_0**	**T_0**	**T_0**

Table 7.0**.** Table showing the degree of turbity of dissolved plant extract at concentration 0.035g/0.1 L at different volumes against reference ampicillin and control.

Reference (Ampicillin at same concentration as dissolved plant extract 0.035g/0.01L	Volume of Reference or control (ml) 0.2 ml	Volume of Reference or control (ml) 0.4ml	Volume of Reference or control (ml) 0.6 ml
	T_2	T_1	T_0
Control Experiment			

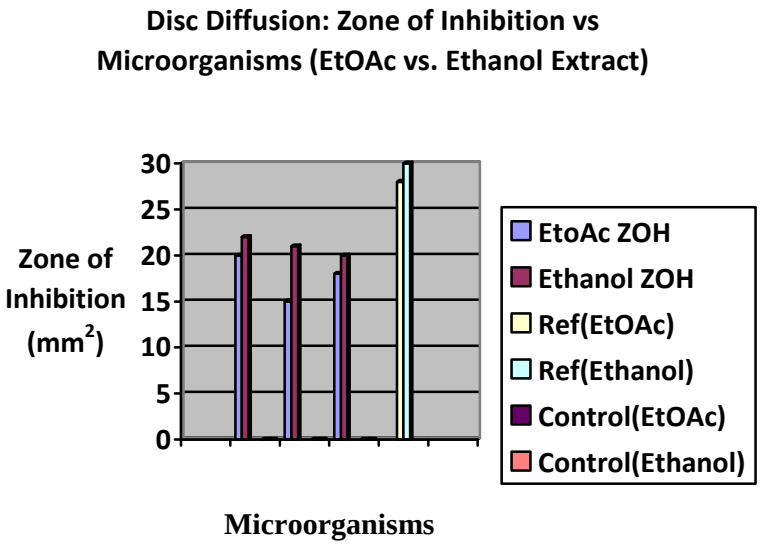
Hexane	T_3	T_3	T_3
CH_2Cl_2	T_3	T_3	T_3
EtOAc	T_3	T_3	T_3
CH_3CH_2OH	T_3	T_3	T_3

Table 8.0. showing the results of TLC for all the extracts.

Solvents	**Plants**	**No of spots visible by UV**	**R_f value**
Hexane	*Phyllanthus acidus (Hexane Extract)*	4	0.022 0.087 0.739 0.978
CH_2Cl_2	*Phyllanthus acidus* (CH_2Cl_2 Extract)	2	0.026 0.051
EtOAc/CH_2Cl_2, 90: 10, v/v)	*Phyllanthus acidus* (EtOAc Extract)	3	0.048 0.333 0.414

Graphs: Bar graphs are shown in Fig. 4.0 (a) and 4.0 (b) whereas corresponding line graph for Fig. 4.0 (a) and 4.0 (b) are shown in Fig. 5.0 (a) and Fig. 5.0 (b).

(a)



(b)

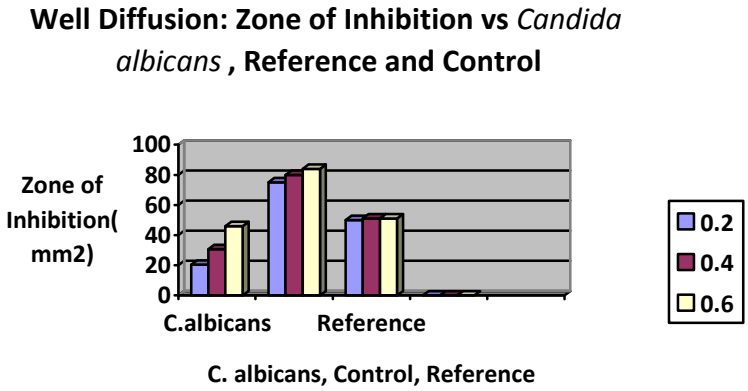
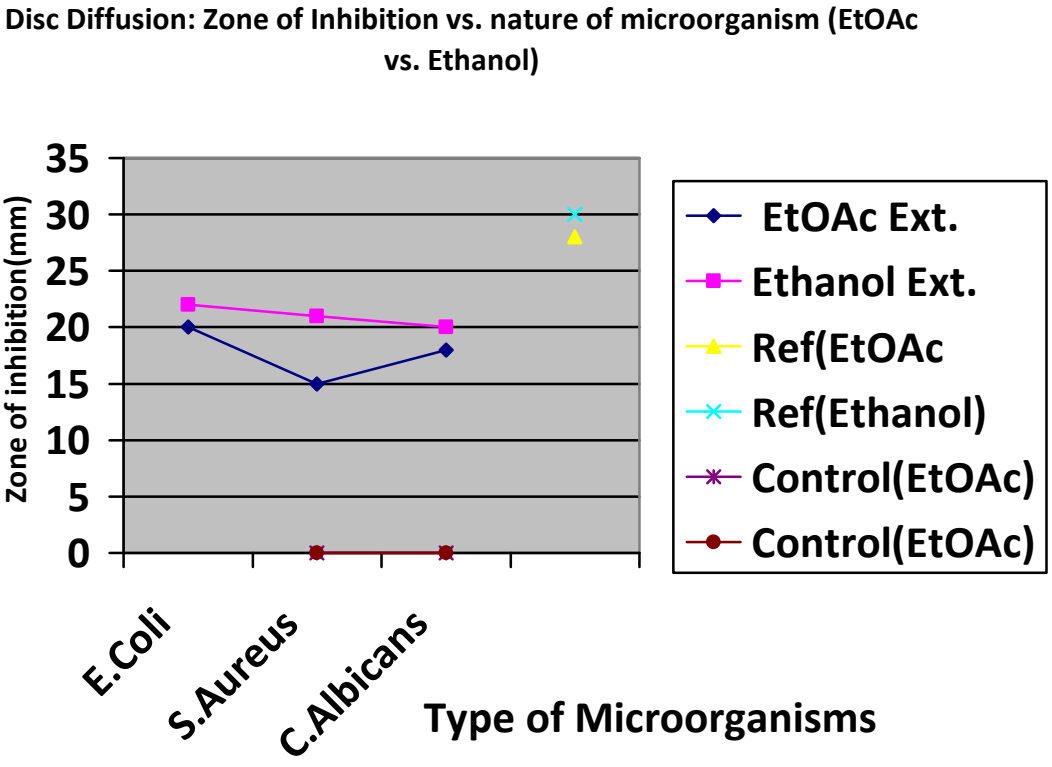


Fig. 4.0 (a) and 4.0 (b).



(b)

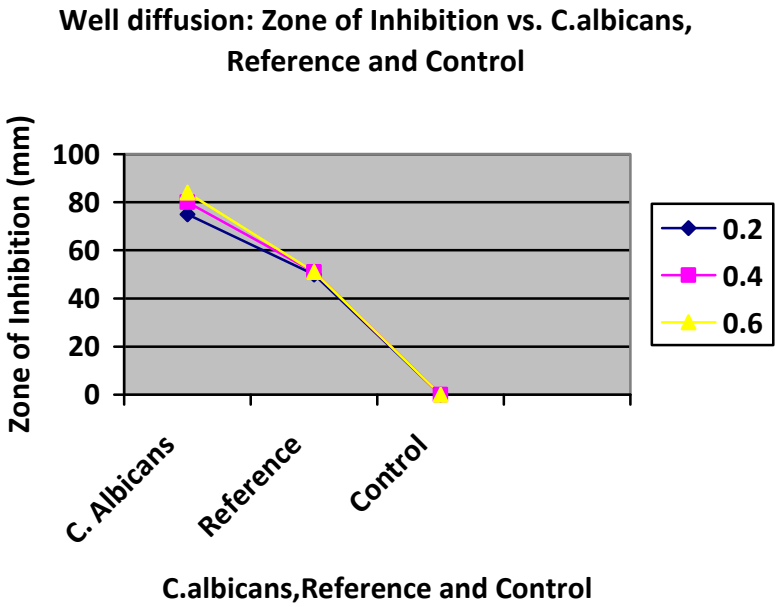


Fig. 5.0 (a) and 5.0 (b)

2.3.17. Discussion: All four methods: Stokes disc diffusion, Well diffusion, Streak plate and a dilution method were successful in determining *Phyllanthus acidus* antimicrobial activities. Several trends are noted. Antimicrobial activity follow the sequence: CH_3CH_2OH extract > EtOAc extract > CH_2Cl_2 extract > hexane extract. For example, for the disc diffusion method, zone of inhibition of 22 mm^2, 21 mm^2 and 20 mm^2 were obtained for *E.Coli*, *S.aureus* and *C.albicans* for the CH_3CH_2OH extract, whereas for the corresponding Hexane extract, zone of inhibition of 5 mm^2 were obtained for all three microbes. In another method, the Well diffusion, zone of inhibition of 84 mm^2 was obtained when the well was filled with 0.6 ml compare to the hexane extract for which the zone of inhibition was <5 mm^2. These results strongly suggest that *Phyllanthus acidus* antimicrobial active constituents are localized in the EtOAc and CH_3CH_2OH extracts. For all methods used, the control experiments which necessitate the use of pure distilled solvent alone, rather than pure plant extract induced negative result i.e no zone of inhibition or in the case of the dilution method, turbidity in test tubes containing LB broth with bacterial microbes. As anticipated, our reference antibiotic compound, ampicillin for bacteria and Nyastatin for fungi displayed positive results. For the disc diffusion method, a larger zone of inhibition was observed for the reference compound in comparison to the plant extract. The opposite was noted for the Well diffusion method . For example, for the disc diffusion method, ampicillin induced zone of inhibition of 30 mm^2 for the CH_3CH_2OH extract whereas for the turbidity experiment no inhibition was seen. For the Well diffusion method, nyastatin induced zone of inhibition of 51 mm^2 at 0.6 ml. All these results suggest that *Phyllanthus acidus* antimicrobial properties are due to the plant active constituent rather than to the distilled solvents. It should be noted that for each solvent extracts, extracts were added in increasing volume (0.2-0.4-0.6) ml to the microbial medium.

The salient feature for each method can be discussed. Disc diffusion indicates that the plant extract induced a larger zone of inhibition against *E.Coli* as compared against *S.aureus*. For example, for the EtOAc extract, zone of inhibition of 20 mm^2 and 15 mm^2 were obtained for *E.Coli* and *S.aureus* respectively. For the streak plate method, a similar trend was noted: *E.Coli* showed inhibition whereas *S.aureus* showed negligible inhibition (< 5mm^2).

The Well diffusion method was used primarily against *C. albicans* microbe. The Well diffusion indicated that a larger zone of inhibition was observed compared with the Stokes Disc diffusion method. For example, for the EtOAc extract, a zone of inhibition of 79 mm^2 was observed at a volume of 0.6 ml.

Compared with the Stokes Disc Diffusion method, zone of inhibition of 18 mm^2 were observed. This suggest that the Well diffusion method is a much more sensitive method than the Disc diffusion.

The streak plate method indicated both the hexane and CH_2Cl_2 extract induce no inhibition against *E.Coli* and *S.aureus*. However, selective inhibition was observed for the EtOAc extract. Positive inhibition or no growth of microbe was observed against *E.Coli* at volume of 0.2 to 0.6 ml whereas negative inhibition was observed against *S. aureus* at the same volume range.

The Dilution method was used to test the dissolved plant extracts for antimicrobial activity against bacteria: *E.Coli* and *S.aureus*. Plant extract that showed positive results for the streak plate was used. Results were recorded in terms of turbidity. In general, no turbidity indicates inhibition. The use of LB broth as a rich medium to foster or stimulate the growth of the bacteria is noted. Yeast extract and tryptone provide vitamins and amino acids respectively for the bacteria to grow. The result indicates that for *E.Coli* microbe no inhibition (very turbid mixture, T_3) was observed for the hexane and CH_2Cl_2 extract. However, inhibition was observed for the EtOAc extract at a volume of 0.6 ml and for the CH_3CH_2OH extract from volume 0.2 to 0.6 ml. Interestingly, for the dilution method against *S.aureus*, inhibition were seen for CH_2Cl_2, EtOAc and CH_3CH_2OH extract at increasing volume from 0.2 to 0.6 ml. The reference compound ampicillin and the controls showed inhibition and non inhibition respectively as anticipated.

. Fig. 4.0 (a) represent for the Disc diffusion method using the CH_3CH_2OH and EtOAc extract, a bar graph plot of the zone of inhibition vs. type of microorganism, Fig. 4.0 (b) represent for the Well Diffusion method, a bar graph plot of the zone of inhibition vs. Candida albicans. Fig. 5.0 (a) and 5.0 (b) represent plots for the corresponding line graphs. For the disc diffusion technique, a larger zone of inhibition was observed for the CH_3CH_2OH extract as compared with the EtOAc extract. The largest zone of inhibition was observed for *E.Coli* in both cases. For the Well diffusion method, as the volume of plant extract increased in the well so too is the zone of inhibition for the CH_2Cl_2, EtOAc and CH_3CH_2OH extract.

TLC analyses in various solvent system for each solvent type extract revealed the presence of spots that range from two to four, Table 8.0. Each spot is probably due to a pure natural product or phytochemical. Each also has a specific R_f value. The number of spots and R_f value for each spot is recorded in Table 8.0. For example for *Phyllanthus acidus* EtOAc, extract using the solvent system, EtOAc/CH_2Cl_2 (90:10, v/v), three spots at R_f values of 0.048, 0.333 and 0.414 were seen..

Conclusions:

It is clearly seen that *Phyllanthus acidus* has antimicrobial properties. However, antimicrobial activity is selective and solvent dependent with the CH_3CH_2OH extract, the most potent and hexane the least. In general, the order of antimicrobial activity follow the sequence: CH_3CH_2OH extract > EtOAc extract > CH_2Cl_2 extract > hexane extract. Thus, the CH_3CH_2OH and EtOAc extract of *Phyllanthus acidus* can be used as the active constituent of an antimicrobial cream or following clinical trials as herbal medicines. Future work such as isolation and purification of bioactive constituents should target the CH_3CH_2OH and EtOAc extract of *Phyllanthus acidus*.

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Yield And Agronomic Characteristics Of 29 Pigeon Pea Genotype At Otobi In Southern Guinea Savanna Of Nigeria

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ABSTRACT: The yield of pigeon pea (Cajanus cajan (L.) Millsp.) has remained low on the farmers' fields in Southern Guinea Savanna agro-ecological environment of Nigeria, despite its agronomic potentials. In 2002 – 2004, twenty-nine (29) improved pigeon pea genotypes from ICRISAT were evaluated along with a local check for grain yield and other agronomic characteristics with a view to further enhance pigeon pea productivity in the region. The experiment was a randomized complete block design with three replications located at the National Root Crops Research Institute sub-station, Otobi ($07^0 10'$N, $08^0 39'$E, elevation 105.1m), Benue State, Nigeria. All pigeon pea genotypes, including the local check, produced dry grain yield above 1.00 t/ha^{-1} with a mean of 1.37 t/ha except ICEAP 00053. ICP 7343, ICP 7992 (both medium duration), ICP 12746, ICP 7991 and ICP 7186 (long duration) pigeon pea genotypes proved superior to the local check in number of pods produced per plant, dry pod weight and grain yield. However, when farmers' preferences for early-maturing and high-yielding genotypes are considered, ICP 6971 and ICP 88039 may be chosen. [Nature and Science. 2008;6(2):39-50]. ISSN: 1545-0740.

Key Words: Pigeon pea genotypes, yield, Southern Guinea Savanna

INTRODUCTION

Pigeon pea (*Cajanus cajan* (L.) millsp.) is now reported to be grown in 50 countries of Asia, Africa and the Caribbean, where its name "pigeon-pea" is thought to have originated. During 2004, pigeon pea as a field crop was grown on 4.36 ha, with a production of 3.22 million t and an average productivity of 0.74 t/ha^{-1} (FAOSTAT, 2005). The current global estimate of its annual productivity is valued at more than US $1700 million (FAOSTAT, 2005). Pigeon pea is a multipurpose leguminous crop that can provide food, fuel wood and folder for the small-scale farmer in subsistence agriculture and is widely cultivated in Nigeria (Remanandan and Asiegbu, 1993; Egbe and Kalu, 2006). In most locations in the Southern Guinea Savanna ($6^0 20'$ N to $8^0 10'$ W, and $6^0 42'$ E to $10^0 5'$ E) agro-ecological zone of Nigeria, where poverty level is high and income generation status low, resource-poor farmers have through evolutionary history incorporated pigeon pea production into their indigenous cropping systems as field crops, backyard or as field border crops (Egbe and Kalu, 2006). In a survey of pigeon pea farming systems in Southern Guinea Savanna, Egbe and Kalu (2006) found that farmers preferred high-yielding, early-maturing, pest and disease tolerant genotypes to medium or late maturing types. Egbe and Adeyemo (2006) reported that pigeon pea could be intercropped with maize without negative effects on the yield and yield components of maize. Farmers in Southern Guinea Savanna of Nigeria, however, maintain varying degrees of sole and mixed culture with such other crops as sorghum, millet, yam, cassava and sweet potatoes.

Pigeon pea should play an important role in developing new strategic approaches to ensure food security and sustainable increase in agricultural productivity in Southern Guinea Savanna environment of Nigeria. One possible means of doing this is through screening of elite pigeon pea genotypes, which will be discussed in this paper.

MATERIALS AND METHODS

In 2002, 2003 and 2004 planting seasons, field experiments were carried out at the National Root Crops Research Institute Sub-station, Otobi (07^0 10'N, 08^0 39'E, 105.1m elevation) located in Southern Guinea Savanna agro-ecological zone of Nigeria to evaluate 29 new pigeon pea genotypes recently obtained from ICRISAT along with a local check for yield and other agronomic characteristics. Rainfall at the site was 1712.00mm, 1665,60mm and 1675.40mm in 2002, 2003 and 2004, respectively between the months of June and November of each year. The soil at the experimental site was classified as Typic Paleustalf.

The experiment was laid out in randomized complete block design with three replications. The gross plot size was $8m^2$, while the net plot measured $2m^2$, entrimmed. Each gross plot consisted of 4 rows spaced 50cm apart and 4m long. Two seed were planted per hill and spaced 30cm within row. Thinning was done ten days after planting to one plant per hill. All plots received a basal application of 200kg NPK 15:15:15 (30kg N, 12.90kg P and 24.90 kg K) per ha by broadcasting. All plots were hand-weeded twice (at 3 and 7 weeks after planting). The following data were collected: days to 50 flowering, days to maturity, plant height at harvest (m), number of primary branches, number of pods per plant, dry pod weight and dry grain yield (t/ha). All data collected were analyzed using GENSTAT Release 4.23 (Copyright 2003, Lawes Agricultural Trust Rothamsted Experimental Station) following standard analysis of variance procedures (Gomez and Gomez, 1984). Whenever difference between treatment means were significant, means were separated by F-LSD as P = 0.05 (Obi, 1990). It must be noted that data for each trait measured for the 3 years were pooled and analyzed to determine the year effect before being analyzed again for the respective years of 2002, 2003 and 2004.

RESULTS

Year effects were highly significant for all the traits measured. For this reason, pea performance for the 3 years was discussed separately.

During each of the three years of experimentation, the short duration genotypes achieved 50% flowering earlier than the medium, which in turn flowered earlier than the long duration types (Table I). Though classified as late maturity (>180 days), ICP 7193 flowered in 124.00, 121.00 and 119.00 days in 2002, 2003 and 2004 respectively, which were not statistically different from the means for the medium duration (151 – 180 days) genotypes which flowered in 120.71, 123.25 and 124.46 days for the same period. Similarly, the short duration (< 150 days) genotypes reached maturity earlier ($\overline{X}$ = 149.95 days) than medium ($\overline{X}$ = 175.00 days) and the medium matured significantly earlier than the long duration ($\overline{X}$ = 209.08 days)[Table 2]. Although grouped as short duration ICP 6971 matured in 165.00 days (2002), 163.00 days (2003 and 2004), which were not significantly different from those of other genotypes as ICP 7992 and ICP 7338, which have been designated as medium duration. The local check reached maturity in 210.00 days (2002), 199.00 days (2003) and 205.00 days (2004)

Table 3 indicates that, as a group the long duration genotypes were significantly taller (3.58m) than both the medium (3.15m) and the short duration lines (2.98m) in 2002, but not so in 2003 when all groups were equally tall. However, in 2004, both the medium and the long duration genotypes were significantly taller than the short duration types as a group. During the three years of experimentation, all pigeon pea genotypes had mean heights above 3m at harvest, except ICP 88039 (short duration), ICP 6930 (medium) and ICP 6915 (long duration), which

had mean heights of 2.54m, 2.51m and 2.89m, respectively. The local check (farmer's variety) along with ICP 7992 and ICP 7991 had average plant heights above 4m in 2002 and 2004, but no genotype reached the height of 4.0m in 2003 (*Table 3*).

The 3-year average of the number of primary branches per plant of pigeon pea varied between 11.61 (ICP 8997) and 23.06 (ICP 6443) with a mean of 17.77. As a group, the short duration types significantly had higher number of primary branches per plant than the medium during the entire period of the study; and showed a similar superior response over the long duration genotype only in 2003 and 2004 (*Table 4*). As maturity groups, the short and the long duration genotypes had significantly higher number of branches per plant than the local check, but the medium lines were at par with the local check in the number of primary branches produced.

Table 5 shows that the medium and the long duration genotypes consistently had greater number of pods per plant than the short duration types. No significant difference was noticed between the medium and the long duration genotypes in the number of pod yield per plant. On individual genotype basis, ICP 7186 produced the highest number of pods per plant with a 3-year mean of 545.13, while ICEAP 00053 had the lowest with a mean of 58.40. The farmers' variety (local check) consistently produced greater number of pods per plant than the short-, medium- and the long duration genotypes as individual groups in all the years of the study. However, some individual genotypes in the medium and long duration categories produced significantly higher number of pods per plant than the local check, e.g. ICP 7338 (medium), ICP 7343 (medium), and ICP 7186 (long duration).

Mean dry pod weight for the period 2002 – 2005 varied between 2.05 t/ha^{-1} (ICEAP 00053) and 4.5 t/ha^{-1} (ICP 7343)(*Table 6*). On group basis, the dry pod weights of the short-, medium- and long duration pigeon pea were very comparable in each of the years of the trial. However, these improved cultivars in the three groups produced significantly greater quantities of dry pod than the local check only in 2003. On individual genotype basis, ICP 7343 pigeon pea, along with three other genotypes [ICP 12746 (late maturing), ICP 440 (medium) and ICP 6971 (short duration)] gave higher dry pod weights than all other genotypes tested including the local (*Table 6*).

All pigeon pea genotypes, including the local check produced dry grain yield above 1.00 t/ha^{-1}, with a mean of 1.37 t/ha^{-1} in 2002 – 2004, except ICEAP 00053. The dry grain yield of the short-, medium- and long duration pigeon peas were at par during the entire course of the trials. The improved pigeon pea genotypes in the three different maturity groups proved superior to the local check in dry grain production only in 2003. However, ICP 7343 (medium) and ICP 12746 (long duration), consistently out-yielded the local check with mean yields above 2.00 t/ha (*Table 7*). ICP 7992 (medium), ICP 7186 (long) and ICP 7991 (long duration), which had mean yields of 1.93 t/ha^{-1}, 1.82 t/ha^{-1} respectively, also gave significantly higher dry grain yields than the local check in at least two of the three years of the study.

DISCUSSION

Year effects were highly significant for all traits measured probably due to unequal amount of rainfall observed during the growing seasons in the different years. It is known that days to 50% flowering and maturity duration in pigeon pea are very highly and positively correlated. The mean days to physical maturity obtained in this study for the short duration (149.95), medium duration (175.00) and long duration (209.08) compared favourably

with the broad maturity classification of early (<150 days), medium (151 to 180 days) and late (>180 days) adopted by Reddy (1990)., ICP 6971, classified as short duration, may be re-classified as medium since in the Southern Guinea Savanna agro-ecological environment it reached maturity in 163.67 days. Upadhyaya *et al* (2006), however found that days to flower was more reliable in arriving at the maturity duration in pigeon pea, because frequent and heavy pod borer damage triggers fresh flower production in pigeon pea, which delays the days to maturity.

Plant height in pigeon pea is affected by maturity duration, photoperiod, and environment. Pigeon pea genotypes in this work were generally tall, probably due to influence of exposure to long-day conditions of June and July. Reddy (1990) explained that plant height could be substantially increased through prolongation of the vegetative phase by exposure to the long-day situations. The taller plants observed in the long- and medium duration types than the short duration genotypes in this study agreed with the findings of Egbe (2005), which stated that the short duration genotypes of pigeon pea had shorter plants than the medium- and late-maturing genotypes in both sole and intercropping systems. Reddy (1990) observed that late-maturing long-duration varieties are generally tall, because of their prolonged vegetative phase, while the short-duration or early-maturing varieties are comparatively short in stature due to their short vegetative growth phase.

The range of the number of primary branches observed in this study (11.61 – 23.06) with a mean of 17.17 was within the brackets of that recorded by Remanandan *et al* (1988) for over 8,000 world germplasm accessions which had average number of primary branches per plant at harvest to be between 2 – 3 to 66 with a mean of 13.20. The reasons why the short duration genotypes had greater number of primary branches than the medium and the long duration types could not be easily deduced, but this observation seemed to contradict the earlier reports of Baldey (1988) that the semi-spreading types possessed higher branching-habit plasticity than the compact and spreading types.

The results of this work indicate that, although the local check had means of the number of pods per plant, dry pod weight and dry grain yields at par with the means generated by the individual maturity groups of improved pigeon pea genotypes, some individual pigeon pea genotypes proved superior to it. Such genotypes included two medium duration cultivars (ICP 7343 and ICP 7992) and three long duration genotypes, namely: ICP 12746, ICP 7991 and ICP 7186. Since pod number per plant is the component through which variations in seed yield due to growing conditions is predominantly expressed (Lawn and Troedson, 1990), these improved pigeon pea genotypes mainly in the medium and long duration groups proved superior to the others and the local check probably due to greater number of pods produced per plant. The higher dry grain yields recorded by these medium and long duration genotypes over the short duration types agreed with the findings of earlier workers (Sharma *et al*, 1981) in their work on international adaptation of pigeon pea in West Africa. The mean yield of 1.37 t/ha^{-1} reported in this study was higher than the mean of 1.0 t/ha^{-1} obtained by farmers in Nigeria and the world productivity average of 0.74 t/ha^{-1}.

From the foregoing, ICP 7343, ICP 12746, ICP 7991, ICP 7992 and ICP 7186 may be regarded as the best performing genotypes in Southern Guinea Savanna environment of Nigeria. However, when the farmers' preference for early-maturing and high-yielding genotypes are considered, ICP 6971 and ICP 88039 may be chosen, as these varieties gave mean yields of 1.54 t/ha^{-1}, which is even higher than the farmers' average of 1.25 t/ha^{-1}.

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***TABLE 1*: DAYS TO 50% FLOWERING OF 30 PIGEON PEA GENOTYPES AT OTOBI, SOUTHERN GUINEA SAVANNA OF NIGERIA**

Genotype	Maturity Group	Days to 50% flowering			
		2002	**2003**	**2004**	**Mean**
ICP 6971	S	103.33	116.33	111.67	110.44
ICP 88039	S	70.00	118.33	115.00	101.11
Mean for Short Duration		86.67	117.33	113.34	105.78
ICP 6930	M	126.00	118.67	121.67	122.11

ICP 8997	M	126.00	128.00	132.33	128.78
ICP 7338	M	111.67	121.00	121.33	118.00
ICP 7400	M	111.67	121.00	122.67	118.45
ICP 440	M	108.33	121.00	121.00	116.78
ICP 1	M	128.00	123.33	125.67	125.67
ICP 7992	M	124.00	131.00	130.00	128.33
ICP 7343	M	130.00	122.00	121.00	124.33
Mean for Medium Duration		120.71	123.25	124.46	122.81
ICP 6443	L	177.67	129.33	129.33	145.44
ICP 6912	L	145.33	131.33	134.00	136.89
ICP 00053	L	197.67	131.67	134.33	154.56
ICP 7188	L	158.67	134.00	135.00	142.56
ICP 11916	L	183.00	135.00	135.00	151.00
ICP 6915	L	130.00	141.67	127.67	133.11
ICP 7613	L	199.00	145.00	146.00	163.33
ICP 7193	L	124.00	121.00	119.00	121.33
Farmers' Variety (*Check*)	L	156.00	128.00	140.00	141.33
ICP 7186	L	130.00	122.33	120.33	124.22
ICP 8257	L	170.00	128.67	129.33	142.67
ICP 11953	L	171.33	140.67	145.33	152.44
ICP 11951	L	170.00	128.67	150.00	149.56
ICP 12746	L	156.00	146.67	144.67	149.11
ICP 7991	L	138.00	143.00	138.00	139.67
ICP 6907	L	172.00	128.00	125.67	141.89
ICP 13030	L	170.67	142.67	144.33	152.56
ICP 7315	L	138.00	131.00	131.00	133.33
ICP 8006	L	172.00	143.00	142.00	152.33
ICP 6967	L	172.00	140.00	142.00	151.33
Mean for Long Duration		161.57	134.58	135.65	143.93
Mean		145.80	130.40	131.20	135.80
CV (%)		3.22	3.58	3.09	
FLSD		3.54	3.53	3.29	

S = Short Duration
M = Medium Duration
L = Long Duration

***TABLE 2*: DAYS TO MATURITY OF 30 PIGEON PEA GENOTYPES AT OTOBI, SOUTHERN GUINEA SAVANNA OF NIGERIA**

Genotype	Maturity Group	Days to maturity			
		2002	**2003**	**2004**	**Mean**
ICP 6971	S	165.00	163.00	163.00	163.67
ICP 88039	S	134.00	136.67	138.00	136.22
Mean for Short Duration		149.50	149.84	150.50	149.95
ICP 6930	M	173.00	169.67	171.33	171.33

ICP 8997	M	178.33	174.00	172.67	175.00
ICP 7338	M	165.00	170.67	167.00	167.56
ICP 7400	M	172.33	174.00	170.00	172.11
ICP 440	M	166.67	141.67	173.00	170.45
ICP 1	M	195.00	179.33	190.67	188.33
ICP 7992	M	165.00	168.67	167.00	166.89
ICP 7343	M	193.33	186.67	185.00	188.33
Mean for Medium Duration		176.08	174.34	174.58	175.00
ICP 6443	L	253.33	221.67	223.33	232.78
ICP 6912	L	203.33	197.67	198.67	199.89
ICP 00053	L	255.00	225.00	240.00	240.00
ICP 7188	L	205.00	199.33	201.00	201.78
ICP 11916	L	236.67	225.00	228.33	230.00
ICP 6915	L	186.67	180.67	183.33	183.56
ICP 7613	L	255.00	243.33	248.33	248.89
ICP 7193	L	175.00	170.67	172.67	172.78
Farmers' Variety (*Check*)	L	210.00	199.33	205.00	204.78
ICP 7186	L	190.00	186.00	187.67	187.89
ICP 8257	L	226.67	216.67	215.00	219.45
ICP 11953	L	213.33	211.67	210.00	211.67
ICP 11951	L	230.00	222.67	227.67	226.78
ICP 12746	L	208.33	203.33	200.00	203.89
ICP 7991	L	196.67	190.00	194.00	193.57
ICP 6907	L	205.00	199.33	199.00	201.11
ICP 13030	L	213.33	210.00	211.67	211.67
ICP 7315	L	195.00	188.67	190.00	191.22
ICP 8006	L	213.33	203.00	211.67	209.33
ICP 6967	L	210.00	208.33	213.33	210.55
Mean for Long Duration		214.08	205.12	208.03	209.08
Mean		199.64	193.20	195.28	196.04
CV (%)		3.02	6.40	6.16	
FLSD (0.05)		4.02	5.75	5.67	

S = Short duration
M = Medium duration
L = Long duration

***TABLE 3*: PLANT HEIGHT OF 30 PIGEON PEA GENOTYPES AT OTOBI, SOUTHERN GUINEA SAVANNA OF NIGERIA**

Genotype	Maturity Group	Plant height (m)			
		2002	**2003**	**2004**	**Mean**
ICP 6971	S	4.12	3.40	3.17	3.56
ICP 88039	S	1.83	3.37	2.42	2.54
Mean for Short Duration		2.98	3.39	2.79	3.05
ICP 6930	M	1.43	3.11	2.99	2.51

ICP 8997	M	3.34	3.25	2.77	3.12
ICP 7338	M	3.42	3.23	3.19	3.28
ICP 7400	M	2.67	3.53	3.51	3.24
ICP 440	M	3.06	3.32	3.06	3.15
ICP 1	M	3.53	3.53	3.65	3.57
ICP 7992	M	4.41	3.41	4.23	4.02
ICP 7343	M	3.35	3.37	3.06	3.26
Mean for Medium Duration		3.15	3.34	3.31	3.27
ICP 6443	L	3.99	3.47	2.99	3.48
ICP 6912	L	3.02	3.49	3.73	3.41
ICP 00053	L	3.55	3.18	3.75	3.49
ICP 7188	L	3.99	3.37	3.72	3.69
ICP 11916	L	3.80	3.22	3.34	3.45
ICP 6915	L	2.37	3.23	3.07	2.89
ICP 7613	L	3.23	3.14	3.21	3.19
ICP 7193	L	3.33	3.34	2.86	3.18
Farmers' Variety (*Check*)	L	4.00	3.33	4.09	3.81
ICP 7186	L	3.28	3.40	2.96	3.21
ICP 8257	L	4.26	3.43	3.68	3.79
ICP 11953	L	3.49	3.53	3.44	3.49
ICP 11951	L	3.55	3.50	3.63	3.56
ICP 12746	L	3.42	3.32	3.23	3.32
ICP 7991	L	4.19	3.39	4.00	3.86
ICP 6907	L	3.37	3.57	3.40	3.45
ICP 13030	L	3.63	3.43	3.44	3.50
ICP 7315	L	3.68	3.38	3.12	3.39
ICP 8006	L	3.79	3.34	3.73	3.62
ICP 6967	L	3.59	3.43	3.61	3.54
Mean for Long Duration		3.58	3.37	3.45	3.47
Mean		3.42	3.37	3.37	3.39
CV (%)		7.3	8.0	5.90	
FLSD (0.05)		0.41	0.43	0.32	

S = Short duration
M = Medium duration
L = Long duration

***TABLE 4*: NUMBER OF EFFECTIVE PRIMARY BRANCHES PER PLANT OF 30 PIGEON PEA GENOTYPES AT OTOBI, SOUTHERN GUINEA SAVANNA OF NIGERIA**

Genotype	Maturity Group	Number of effective primary branches per plant			
		2002	**2003**	**2004**	**Mean**
ICP 6971	S	22.55	21.17	22.33	22.02
ICP 88039	S	17.25	16.92	16.33	16.83
Mean for Short Duration		19.90	19.05	19.33	19.43
ICP 6930	M	14.25	14.25	13.58	14.03

ICP 8997	M	17.47	16.25	15.58	16.43
ICP 7338	M	12.92	11.33	10.58	11.61
ICP 7400	M	15.75	15.00	15.33	15.36
ICP 440	M	24.08	22.00	21.67	22.58
ICP 1	M	20.92	19.17	19.25	19.78
ICP 7992	M	15.00	15.33	16.17	15.50
ICP 7343	M	16.42	15.50	15.17	15.69
Mean for Medium Duration		17.10	16.10	15.92	16.37
ICP 6443	L	24.33	23.42	21.42	23.06
ICP 6912	L	15.75	18.17	18.08	17.33
ICP 00053	L	18.23	17.33	16.75	17.44
ICP 7188	L	16.00	14.33	13.50	14.61
ICP 11916	L	21.50	18.50	19.00	19.67
ICP 6915	L	25.17	22.75	21.00	22.97
ICP 7613	L	15.50	15.17	14.75	15.14
ICP 7193	L	20.67	10.33	11.17	14.06
Farmers' Variety (*Check*)	L	15.80	15.33	15.58	15.57
ICP 7186	L	14.83	14.50	14.83	14.72
ICP 8257	L	19.50	20.00	19.63	19.71
ICP 11953	L	18.92	18.33	20.33	19.19
ICP 11951	L	23.17	20.17	22.25	21.86
ICP 12746	L	18.17	17.33	16.50	17.33
ICP 7991	L	15.33	13.75	13.50	14.19
ICP 6907	L	19.83	18.83	19.13	19.26
ICP 13030	L	19.83	20.33	23.33	19.94
ICP 7315	L	20.92	20.17	19.83	2.031
ICP 8006	L	14.42	13.50	13.42	13.78
ICP 6967	L	21.08	22.33	22.58	21.99
Mean for Long Duration		18.95	17.73	17.83	18.17
Mean		18.52	17.38	17.42	17.77
CV (%)		19.60	6.60	6.90	
FLSD (0.05)		1.92	1.88	1.97	

S = Short duration
M = Medium duration
L = Long duration

***TABLE 5*: NUMBER OF PODS PER PLANT OF 30 PIGEON PEA GENOTYPES AT OTOBI, SOUTHERN GUINEA SAVANNA OF NIGERIA**

Genotype	Maturity Group	Number of pods per plant			
		2002	**2003**	**2004**	**Mean**
ICP 6971	S	174.00	154.00	170.00	166.00
ICP 88039	S	120.70	101.70	112.70	111.70
Mean for Short Duration		147.35	127.85	141.35	138.85
ICP 6930	M	161.50	164.70	165.00	163.73

ICP 8997	M	125.70	133.50	123.70	127.63
ICP 7338	M	363.70	306.20	303.30	324.40
ICP 7400	M	158.00	161.00	162.70	160.57
ICP 440	M	191.70	194.70	201.20	195.87
ICP 1	M	153.30	149.00	161.00	154.43
ICP 7992	M	186.00	172.70	181.70	180.13
ICP 7343	M	295.70	280.20	292.00	289.3
Mean for Medium Duration		204.45	195.25	198.83	199.51
ICP 6443	L	168.80	165.70	162.70	165.73
ICP 6912	L	87.80	82.00	91.00	86.93
ICP 00053	L	74.70	47.00	53.50	58.40
ICP 7188	L	180.00	170.80	173.80	174.87
ICP 11916	L	264.00	249.70	226.30	167.33
ICP 6915	L	203.30	189.80	185.20	192.77
ICP 7613	L	148.00	138.50	166.00	150.83
ICP 7193	L	199.50	186.50	175.80	187.27
Farmers' Variety (*Check*)	L	293.30	242.30	233.30	256.30
ICP 7186	L	622.80	508.30	504.30	545.13
ICP 8257	L	243.30	239.20	236.80	239.77
ICP 11953	L	156.00	212.50	133.80	137.10
ICP 11951	L	273.00	253.80	262.30	263.03
ICP 12746	L	247.20	234.20	229.50	236.97
ICP 7991	L	184.80	155.80	152.50	164.37
ICP 6907	L	106.50	100.50	123.20	110.07
ICP 13030	L	217.00	217.70	223.20	219.30
ICP 7315	L	243.80	214.30	268.80	242.30
ICP 8006	L	151.30	128.50	152.50	144.10
ICP 6967	L	170.20	168.20	170.30	169.57
Mean for Long Duration		211.77	190.72	196.24	119.58
Mean		205.50	187.70	193.30	195.50
CV (%)		9.60	6.80	5.40	
FLSD (0.05)		32.30	20.95	17.01	

S = Short duration
M = Medium duration
L = Long duration

***TABLE 6*: DRY POD WEIGHT OF 30 PIGEON PEA GENOTYPES AT OTOBI, SOUTHERN GUINEA SAVANNA OF NIGERIA**

Genotype	Maturity Group	Dry pod weight (t/ha)			
		2002	**2003**	**2004**	**Mean**
ICP 6971	S	4.16	2.56	2.28	3.00
ICP 88039	S	3.36	2.56	2.60	2.84
Mean for Short Duration		3.76	2.56	2.44	2.92

ICP 6930	M	3.49	1.69	1.89	2.36
ICP 8997	M	3.58	2.94	2.72	3.08
ICP 7338	M	2.71	2.44	2.24	2.46
ICP 7400	M	2.00	2.91	2.92	2.61
ICP 440	M	5.12	2.79	3.16	3.69
ICP 1	M	2.23	2.87	1.94	2.35
ICP 7992	M	4.53	2.58	4.26	3.79
ICP 7343	M	4.69	4.05	4.77	4.50
Mean for Medium Duration		3.54	2.78	2.99	3.10
ICP 6443	L	2.47	2.73	2.45	2.55
ICP 6912	L	2.71	3.59	2.53	2.94
ICP 00053	L	1.66	3.03	1.47	2.05
ICP 7188	L	2.68	2.43	2.59	2.57
ICP 11916	L	2.31	2.26	2.19	2.25
ICP 6915	L	3.03	2.63	2.55	2.74
ICP 7613	L	1.78	3.12	1.77	2.22
ICP 7193	L	2.46	1.99	2.22	2.22
Farmers' Variety (*Check*)	L	3.74	1.63	2.70	2.69
ICP 7186	L	3.90	3.26	3.52	3.56
ICP 8257	L	2.92	2.53	3.01	2.82
ICP 11953	L	4.15	0.93	4.03	3.04
ICP 11951	L	2.95	2.90	2.90	2.92
ICP 12746	L	5.27	2.48	4.77	4.17
ICP 7991	L	4.09	2.12	3.77	3.33
ICP 6907	L	2.33	2.49	2.92	2.58
ICP 13030	L	3.23	2.22	3.18	2.88
ICP 7315	L	3.08	2.53	2.92	2.84
ICP 8006	L	3.39	3.34	3.58	3.44
ICP 6967	L	2.45	1.34	2.56	2.12
Mean for Long Duration		3.03	2.48	2.88	2.79
Mean		3.22	2.56	2.88	2.89
CV (%)		10.30	17.30	10.10	
FLSD (0.05)		0.54	0.73	0.48	

S = Short duration
M = Medium duration
L = Long duration

***TABLE 7*: DRY GRAIN YIELD OF 30 PIGEON PEA GENOTYPES AT OTOBI, SOUTHERN GUINEA SAVANNA OF NIGERIA**

Genotype	Maturity Group	Dry grain yield (t/ha)			
		2002	**2003**	**2004**	**Mean**
ICP 6971	S	2.11	1.32	1.13	1.52
ICP 88039	S	1.82	1.40	1.42	1.55
Mean for Short Duration		1.97	1.36	1.28	1.54
ICP 6930	M	1.64	0.59	0.82	1.02

ICP 8997	M	1.88	1.13	1.26	1.42
ICP 7338	M	1.05	1.26	1.05	1.12
ICP 7400	M	1.11	1.93	1.73	1.59
ICP 440	M	1.47	1.34	1.64	1.48
ICP 1	M	1.21	1.63	0.77	1.20
ICP 7992	M	2.08	1.62	2.08	1.93
ICP 7343	M	2.71	1.92	2.19	2.27
Mean for Medium Duration		1.64	1.43	1.44	1.50
ICP 6443	L	0.96	1.16	1.11	1.08
ICP 6912	L	1.17	1.70	1.16	1.34
ICP 00053	L	0.84	0.84	0.66	0.78
ICP 7188	L	1.22	0.82	1.18	1.07
ICP 11916	L	1.25	1.08	0.91	1.08
ICP 6915	L	1.63	1.25	1.30	1.39
ICP 7613	L	0.85	1.60	0.98	1.14
ICP 7193	L	1.18	1.02	1.02	1.07
Farmers' Variety (*Check*)	L	1.67	0.69	1.38	1.25
ICP 7186	L	1.88	1.75	1.82	1.82
ICP 8257	L	0.99	1.05	1.31	1.12
ICP 11953	L	1.94	0.28	1.59	1.27
ICP 11951	L	1.11	1.42	1.36	1.29
ICP 12746	L	3.06	1.23	2.13	2.14
ICP 7991	L	2.24	1.23	1.82	1.76
ICP 6907	L	1.03	1.05	1.38	1.15
ICP 13030	L	1.16	0.98	1.58	1.24
ICP 7315	L	1.46	1.21	1.41	1.36
ICP 8006	L	1.61	1.53	1.83	1.66
ICP 6967	L	1.09	0.69	1.23	1.00
Mean for Long Duration		1.42	1.13	1.36	1.30
Mean		1.51	1.22	1.37	1.37
CV (%)		4.60	18.10	12.90	
FLSD (0.05)		0.41	0.36	0.29	

S = Short duration
M = Medium duration
L = Long duration

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Ultrastructural study of some helminth parasites infecting the Goatfish, *Mullus surmuletus* (Osteichthyes: Mulldae) from Syrt coast, Libya

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ABSTRACT: In this study a total of 68 live fishes caught from Syrt Coast, Libya dissected and examined for helminth parasites, out of them 46(67.6%) showed the presence of parasites. Four species of helminth parasites have been collected and identified during the present investigation. First species of them belongs to Digenea (*Opecoelides furcatus* Odhner, 1928), one Cestode in larval stage (*Nybelinia* sp.), one species of Acanthocephala (*Echinorhynchus gadi* Zoega in Muller, 1776) and one to Nematoda (*Cucullanus longicollis* Stossich,1899). Light and SEM observations revealed some differences between the present species and other related helminthes detected before. [Nature and Science. 2008;6(2):51-63]. ISSN: 1545-0740.

Key words: Opecoelidae- *Nybelinia* sp - *Mullus surmuletus*- Echinorhynchidae - *Cucullanus* - Syrt Coast, Libya- Marin fish

INTRODUCTION

Although, the striped red mullet *Mullus surmuletus* Linnaeus, 1758 (Order: Perciformes; Family: Mullidae) is very common in Syrt Gulf and is a commercially important species its parasites are scarcely known. In this regard, Jousson & Bartoli (2000) recorded decapod crustaceans as second intermediate host of two species of Opecoeloides parasitic on goatfishes from the Mediterranean Sea. While, the high diversity of digenean species in goatfishes was documented by Le pommelet et al. (1998), who listed 18 digenean species parasitic in goatfishes, however, many of these species have a restricted distribution to the western Mediterranean and the Adriatic Seas.

According to Williams and Bunkley (1996), adult forms of tapeworms are not very common in bony fishes, but larval forms of cestodes use bony fishes as intermediate host. Many species of larval tapeworms are found in the intestinal tract, often in large numbers, and few are encapsulated in tissues of marine bony fishes including big game fishes (Schmidt, 1986 and Williams and Bunkley, 1996). Palm & Walter (1999) and Palm (1999) described or re-described 17 species from genus *Nybelinia* and erected two new genera, *Heteronybelinia* and *Mixonybelinia*. Forty-eight species of *Nybelinia*, *Heteronybelinia* and *Mixonybelinia* are considered valid (Palm, 1999).

Acanthocephalans have few stable characters that are useful for family-level taxonomy. Recently, the specificity of the intermediate hosts was shown to be useful in taxonomy but, at genus-level (Nickol et al. 1999). Generally, no single character state serves to define echinorhynchidan families alone.

Cucullanidae Cobbold, 1864 includes intestinal nematodes characterized by a highly developed buccal cavity formed by the oesophagus (oesophastome) (Berland 1970), and males with or without a precloacal sucker and harboring 10–15 pairs of caudal papillae (Maggenti 1971). Most of about 100 species of *Cucullanus* have been described from fishes of different orders of which at least 70 were collected from marine or brackish-water fishes (González-Solís et al. 2007). Williams and Bunkley (1996) reported *Cucullanus carangis* from the intestine of a Crevalle jack at the New York Aquarium that was described as *Dacnitis cangris*, and *Cucullanus pulcherrimus* Barreto from a Black Jack in Brazil. In addition, In Egypt, the same genus was recorded in fresh water fishes like *Barbus bynni* and *Mormyrus kannume* (El- Naffar, 1970; 1983; Fahmy et al. 1976 and Soliman, 2000).

Parasites of the commercial inshore fishes of the Syrt Coast were rarely studied; hence, the present investigation aims to give some information on some helminthes infecting the goatfish *Mullus surmuletus* by light and SEM microscopy.

MATERIALS AND METHODES

Sixty-eight fish were collected in Syrt Coast, from May to October 2006. Fish were taken to the laboratory alive where they were serially pithed and autopsied. All fish were examined for helminth infection within 24 hrs of capture. The isolated helminthes were washed in saline solution (0.9%) and fixed in 4% buffered formalin. Except nematodes, specimens were stained in Acetic-Carmine, dehydrated and mounted in Canada balsam. The collected nematodes were cleared in glycerin for examination. Drawings were made with the aid of a Camera Lucida, connected to a Wild bright field microscope. All measurements are given in millimeters except otherwise mentioned.

For SEM studies, recovered species were washed in phosphate buffered saline and fixed overnight in 2.5% gluteraldehyde (pH 7.4) at 4 ºC. Specimens were washed three times in phosphate buffer and post fixed in 1% Osmium tetraoxide in 0.1% M Phosphate buffer and dehydrated through a graded series of ethanol. Complete dehydration was performed in two changes of absolute ethyl alcohol. Specimens were then mounted on stubs with double adhesive tape, coated with gold. Coated samples were examined with a high-resolution scanning electron microscope (JEOL SEM T330) operating at 20 Kev.

RESULTES

Out of 68 fishes examined, 46 showed the presence of parasitic helminth (67.6%). The distribution of such parasitic was as follows: *Opecoeloides furcatus* in 9 specimens (2.9%), *Nybelini*a sp. in 10 specimens (5.9%), *Echinorhynchus gadi* in 15 specimens (16.2%) and *Cucullanus longicollis* in 12 specimens (8.8%); The collected parasites were described and classified up to the level of species as follows:

I- *Opecoeloides furcatus* Odhner, 1928 (Fig.1A-B and 2)

Family: Opecoelidae Ozaki, 1925

Site of infection: intestine

The body elongate, slender, unspined and measures 6.3 –7.4. It has a ventral sucker (V.S.) with four papillae, separated from the body on peduncle, which is close to the anterior end of the body. It appeared as a projection arising from the body, measuring 4.5 –6.2 X 1.7 –2.2 length by width. The oral sucker (O.S.) and pharynx (Ph.) are similar in size and the ventral sucker slightly larger, 0.53-0.61X0.41-0.49, 0.31-0.41X0.21-0.24 length by width, respectively. The prepharynx is short, but the oesophagus long. The caeca extend to near the posterior end of the worm; the vitellaria fill the posterior 3/4 of the body. The testes tandem in the posterior body in-between the cecae, the anterior testis (A.T.) tetra-lobed, inter-caecal and well separated and measures 0.47-0.45X0.23-0.25 length by width. While the posterior testes (P.T.) penta –lobed, larger and measure 0.57-0.61X0.30-0.33 length by width. The ovary (O.) is of median position, situated at a considerable distance in front of the anterior testis, and measures 0.21- 0.25X0.19-0.23 length by width. The uterus is relatively long, intercaecal, winding between the ovary and acetabulum. The eggs (E.) are few in number, oval, moderately large, and measure 130-160 X50-80 µm length by width.

II- *Nybelinia* sp. (Figs. 1 C-D and 3).

Order: Trypanorhyncha Dollfus, 1942

Family: Tentaculariidae Poche, 1926

Genus: *Nybelinia* Poche, 1926

Site: gills, oesophagus, stomach wall.

The post larvae were found free in the gills or encapsulated in the oesophagus and the stomach wall. The present post larvae possess long and slender bothridia with free lateral margins and characteristic marginal, hook-like microtriches in a V –shaped pattern the presence of sensory fossettes on the bothridia. A basal tentacular swelling is absent. The tentacle sheaths straight, the armature homeoacanthous, homeomorphous and a characteristic basal armature present, consisting of about 10 rows. The tentacular hook form changes towards the metabasal part of the tentacle from compact to rounded rose-thorn shaped hooks (Plate 1d), with a slight anterior extension of the basal plate (uncinate). Bothridia 4, widely spaced, lateral margins free, longer than half scolex length. Measurements of the postlarva as follows, Scolex length 0.75-0.88., width 0.35-0.47; proboscis 0.826-0.938 (0.770-1.005); basal 0.052-0.055, 0.038-0.041. Tentacles (T) emerging pairwise, 0.29 long, slender, diminishing in size toward tip; TW 25 (basal) and 17 (apical); Tentacle sheaths straight; Prebulbular organs lacking, muscular rings around basal part of tentacle sheath not visible. Retractor muscles were originating in basal part of bulbs.

III – *Echinorhynchus gadi* Zoega in Muller 1776. (Figs.1 F-H and 4).

Class: Palaeacanthocephala Meyer, 1931

Order: Echinorhynchidea South well and Macfie, 1925

Family: Echinorhynchidae South well and Macfie, 1925

All worms had a milk-white color, the sex of adult *E. gadi* clearly distinguishable. The trunk elongate, sub-cylindrical, somewhat swollen anteriorly, aspinose.

Male: 7- 9 mm length by 0.3-0.5 mm width, Proboscis cylindrical, stout, claviform, densely armed with numerous rows of strongly recurved hooks, which vary in size according to their position, they are bigger on the top of the proboscis but become progressively smaller as we go backwards. Worms's posses 18-19 longitudinal rows, with 4 hooks per row, In the middle of the trunk, two elliptical testes arranged linearly. Posterior to the testes, 6 cement glands, psyriform in shape arranged linearly, with ducts leading into the sperm duct. Proboscis measures 0.8X0.2, proboscis sheath 0.9-1.1X0.2-0.3 length by width, lemnisci length 1.1- 1.5, testis 0.63 X0.16 length by width.

Female: usually larger than the males, possess 14- 18 longitudinal rows, with 6-10 hooks per row. Each worm contained a uterine bell. Body 8- 11 by 0.4-0.6, proboscis 1.1 by 0.4, proboscis sheath 1.3-1.5 by 0.4-0.5, lemnisci length 1.6 –1.8, eggs were 0.27 by 0.06. Generally length of apical blade 70- 80 µm, prebasal 38-40µm and the basal 45-50µm. Proboscis followed by a short neck measuring 0.08-0.10µm in length. Proboscis receptacle double walled, about twice as long as proboscis. The hooks are terminals, very sharp and their roots simple and round. In each worms, two lateral claviform protrusions, lemnisci, from the body wall at the base of neck. The shapes of the proboscis hooks are similar to those of the males. The body cavity of the female worms filled with fusiform shape eggs, where each showing a polar prolongation of the middle shells. The uterine bell and the uterus present in the posterior portion of body.

IV- *Cucullanus longicollis* Stossich, 1899 (Figs.5 - 7).

Order: Ascaridida
Superfamily: Seuratoida
Family: Cucullanidae Cobbold, 1864
Genus: *Cucullanus* Muller, 1777

They are medium sized nematodes, with very thick cuticle and longitudinal lateral alae absent. Oral opening dorsoventrally elongated, surrounded by narrow membranous ala (collarette) supported by raw of numerous minute teeth. Four submedian cephalic papillae and pair of small lateral amphids present. Muscular oesophagus consisting of two distinct portions: anterior with sclerotized lining expanded anteriorly to form a pseudobuccal capsule, narrowing immediately below nerve ring; and posterior with a claviform aspect and strong muscular structure, opening into intestine through valves. Deirids and excretory pore situated at level of posterior half of oesophagus. Cuticular transverse striae are occurring along the entire body seen posterior to the excretory pore (Plate, 7A &B). The width of these striae was 20 µm. They appear highly corrugated towards the posterior end giving the body a general rough appearance.

Male (Figs. 5 A-C and 6): length of body 12.3, width 0.17; entire oesophagus 0.93; oesophastome 0.08 X0.16 length by width. Nerve ring (N.R.) 0.36, excretory pore (Ep) and deirids (D) 0.91, 0.89, respectively; from anterior extremity, equal alate spicules observed, length of spicules 0.64. Gubernaculum (G) is well developed and sclerotised (S), 0.06 is long. Preanal sucker (P.S) well developed and without a chitinous rim measured 0.17 long and 0.57 away from cloaca. Caudal alae was absent. Caudal papillae (P) were distributed as follows: 5 pairs preanal, 1 pair adanal and 5 pair postanal (3 subventral and 2 lateral). Tail is smooth and pointed. Cloaca was 0.31 from posterior extremity. Males with a spirally coiled posterior portion, demonstrated on the ventral surface of the coils, a rugose area, cloacal aperture and caudal papillae (Plate5 B). The rugose area is a modification of the cuticular striation pattern, composed of long stripes of cuticular ridges obliquely placed in a longitudinal band at the ventral posterior coiled region.

Female (Figs. 5 D-F and 7): Body long and slender 16.76 long and 0.21wide. Dorsoventral elongate stoma; pseudobuccal capsule (oesophastome) 0.087 X 0.176 length by width. Total oesophagus 1.4 distances of nerve ring, excretory pore and deirids from anterior extremity 0.44, 0.96 and 1.1 respectively. Vulva (V.) near the middle of the body, vulvular lips prominent. Vagina directed anteriorly; uteri opposed, containing immature eggs. Anterior ovary almost extends beyond junction of oesophagus and intestine (I), posterior ovary almost reaches anus. Tail conical in shape, Apair of phasmids (Ph.) situated midway between the anus and posterior end. Eggs 114 X68 µm, thin- shelled and with small polar protruberance.anus 0.51 from posterior extremity. Phasmids measured 0.12 from tip of tail.

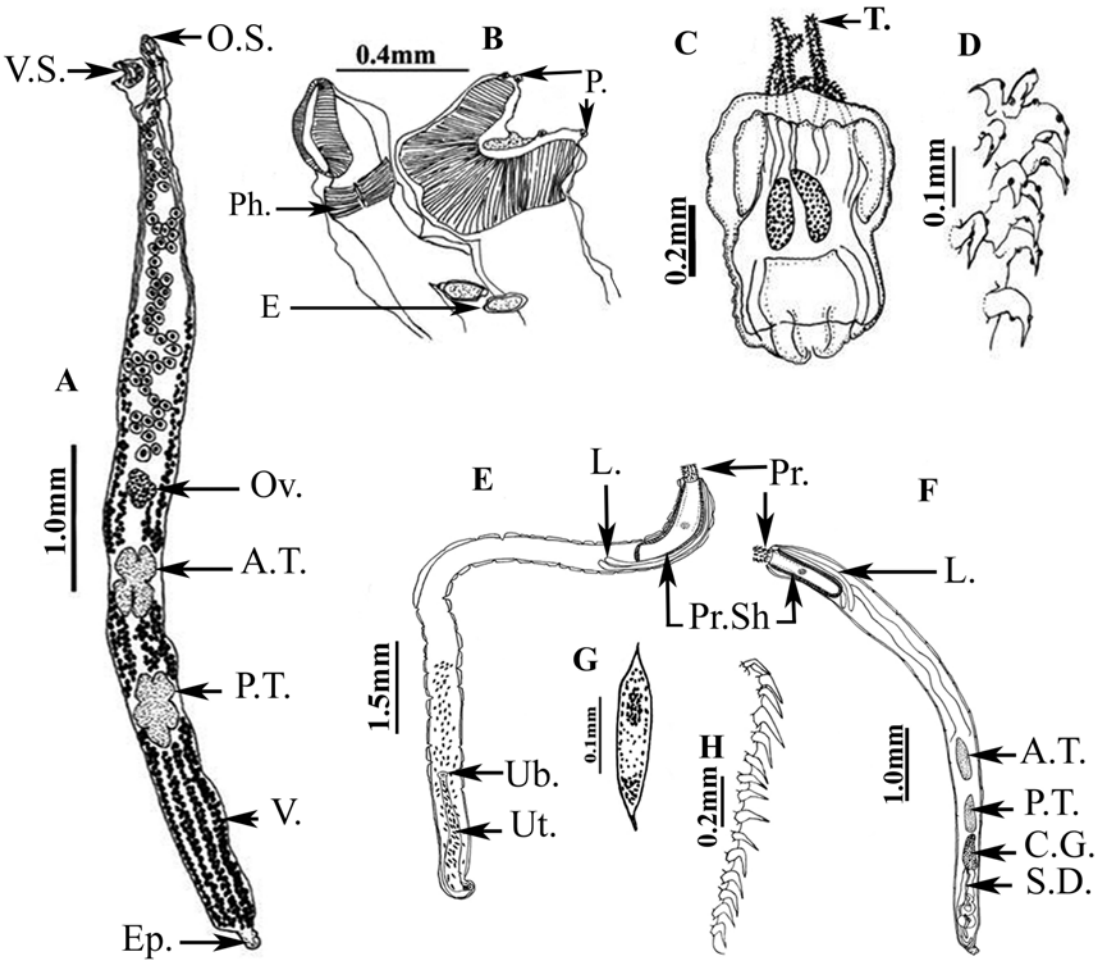

Fig. 1: Camera lucida drawing of **A**: *Opecoelides furcatus*. **B**: ventral and oral suckers of *O. furcatus*. **C**: Larval stage of *Nybelinia* sp. **D**: hooks of tentacles of *Nybelinia* sp. **E**: female *Echinorhynchus gadi*, **F**: male *E. gadi*, **G**: egg **H**: hooks

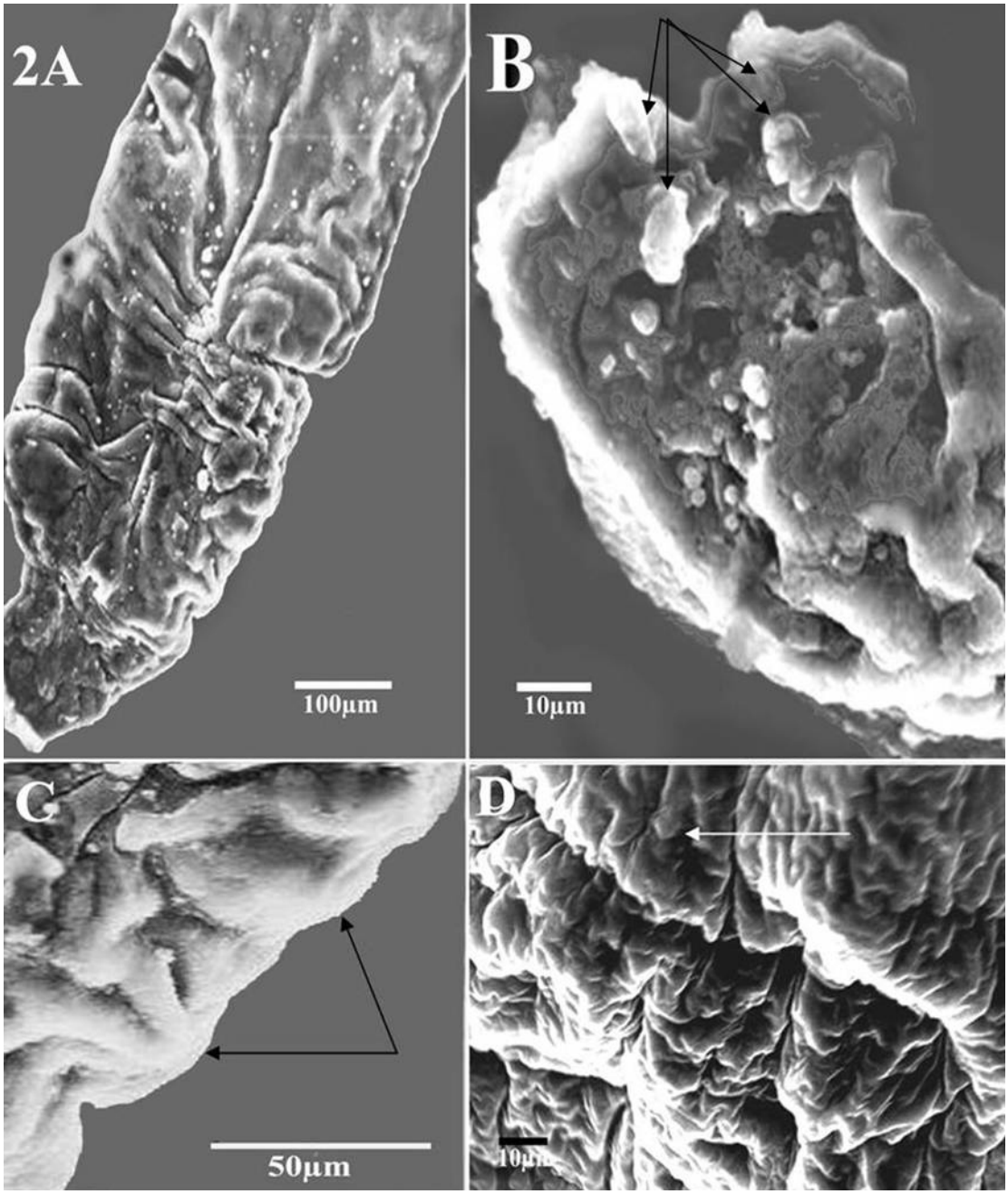

Fig. 2: SEM. Micrographs of *Opecoelides furcatus* **A**- posterior end **B**- ventral sucker with 4 papillae (arrows); separated from the body on peduncle; **C**- cuticular projections in the form of needle-like spines (arrows). **D**- Tegument of the body with longitudinal ridges;

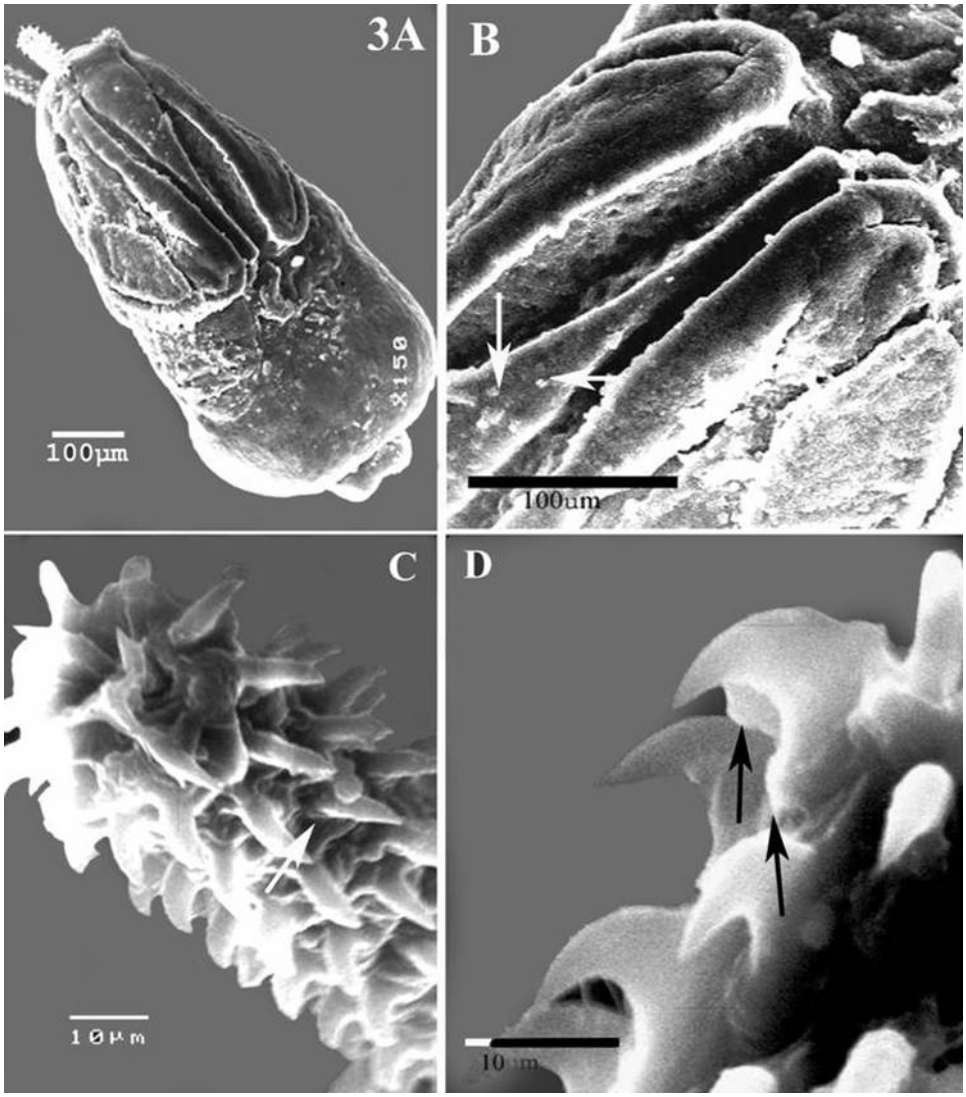

Fig. 3: SEM .Micrographs of larval stage of *Nybelinia* sp. **A-** larva with 4 bothridia;**B**- hooks supported by small nodules or buds ;**C**- sensory fossettes; **D**- slender rose-thorn-shaped metabasal hooks.

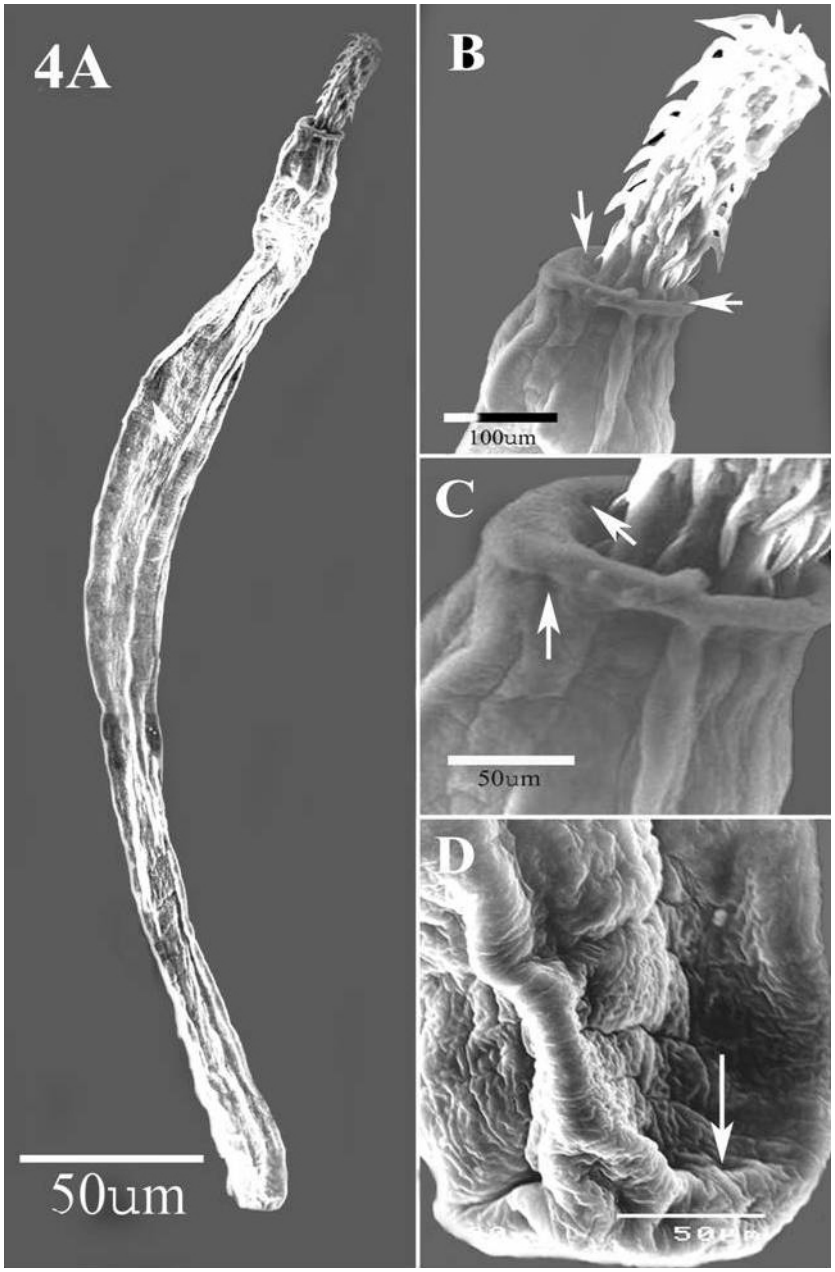

Fig. 4: *Echinorhynchus gadi*. SEM micrographs **A**-entire body, **B**- proboscis with numerous rows of hooks, **C**-neck showed 2 small pits, **D**- tail tip, e-excretory pore.

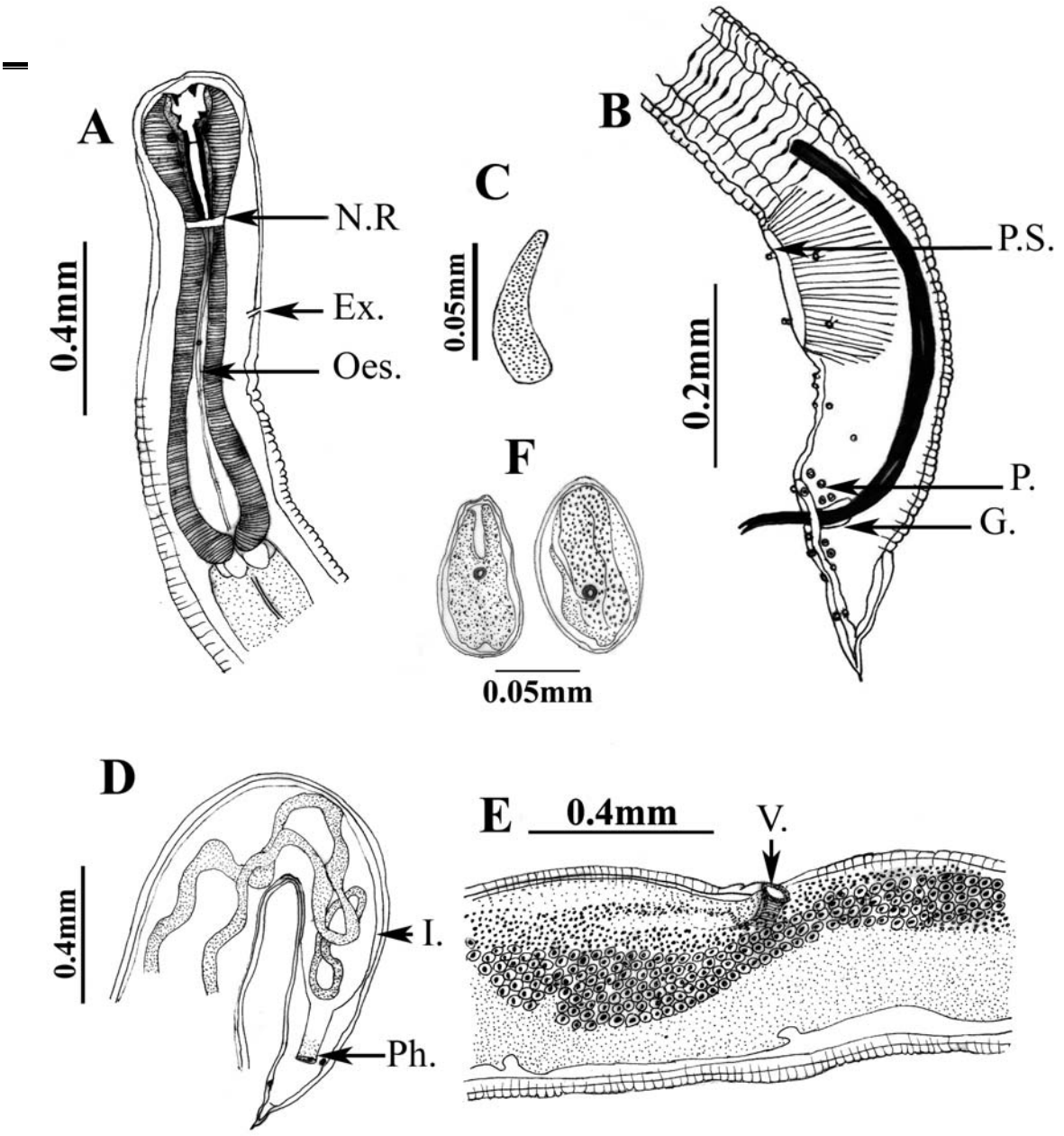

Fig. 5: Camera lucida drawing of *Cucullanus longicullus* **A & B**: anterior and posterior ends of male. **C**: gubernaculums. **D**: posterior end of female. **E**: vulvular region. **F**: egg.

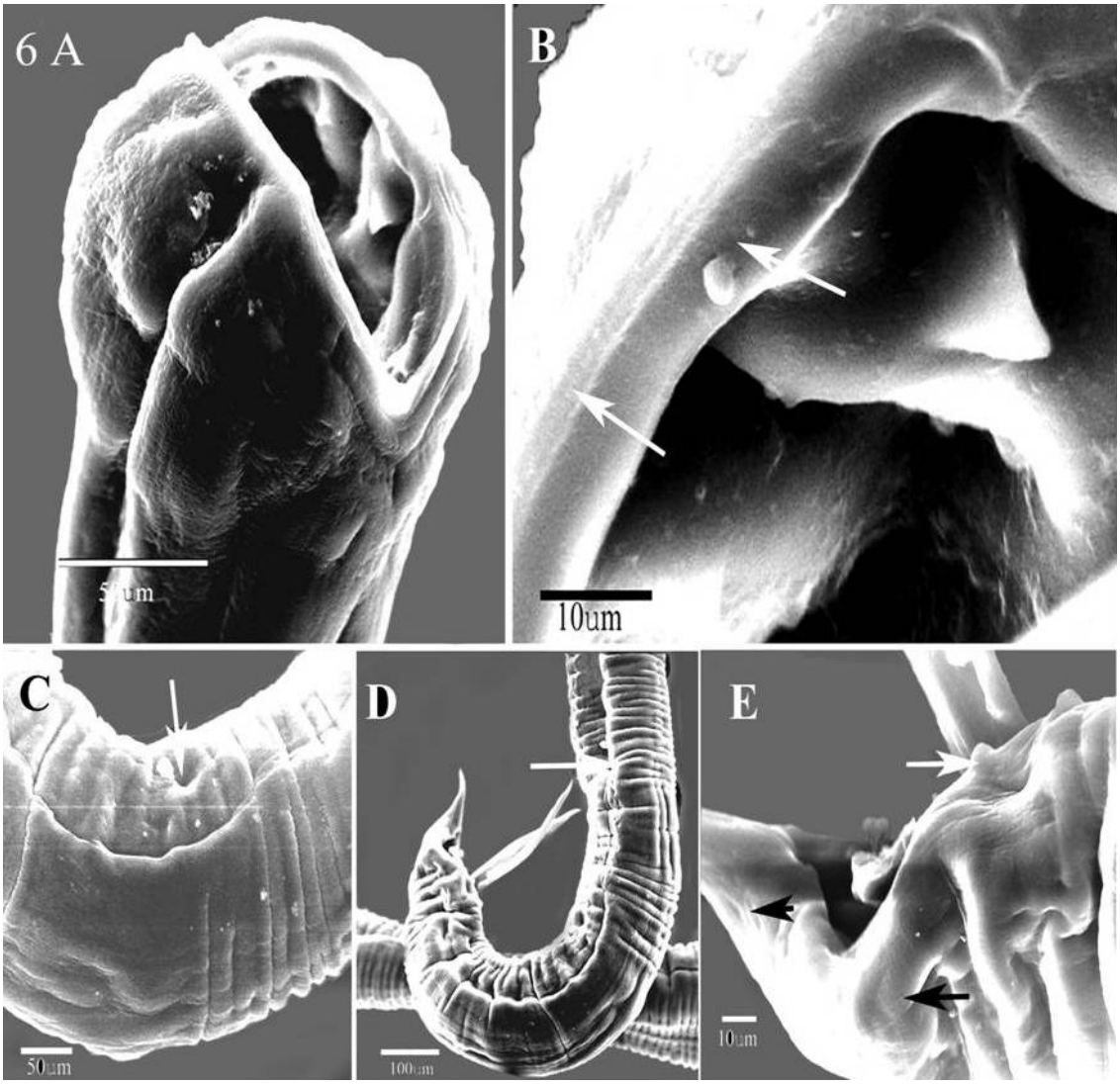

Fig. 6: SEM micrographs of male *C. longicollis*. **A** – cephalic end showing triangular teeth and cephalic papillae; **B**- detail of cephalic teeth, papillae; **C**- preanal sucker; **D**- posterior end, lateral view; **D**- detail of caudal papillae.

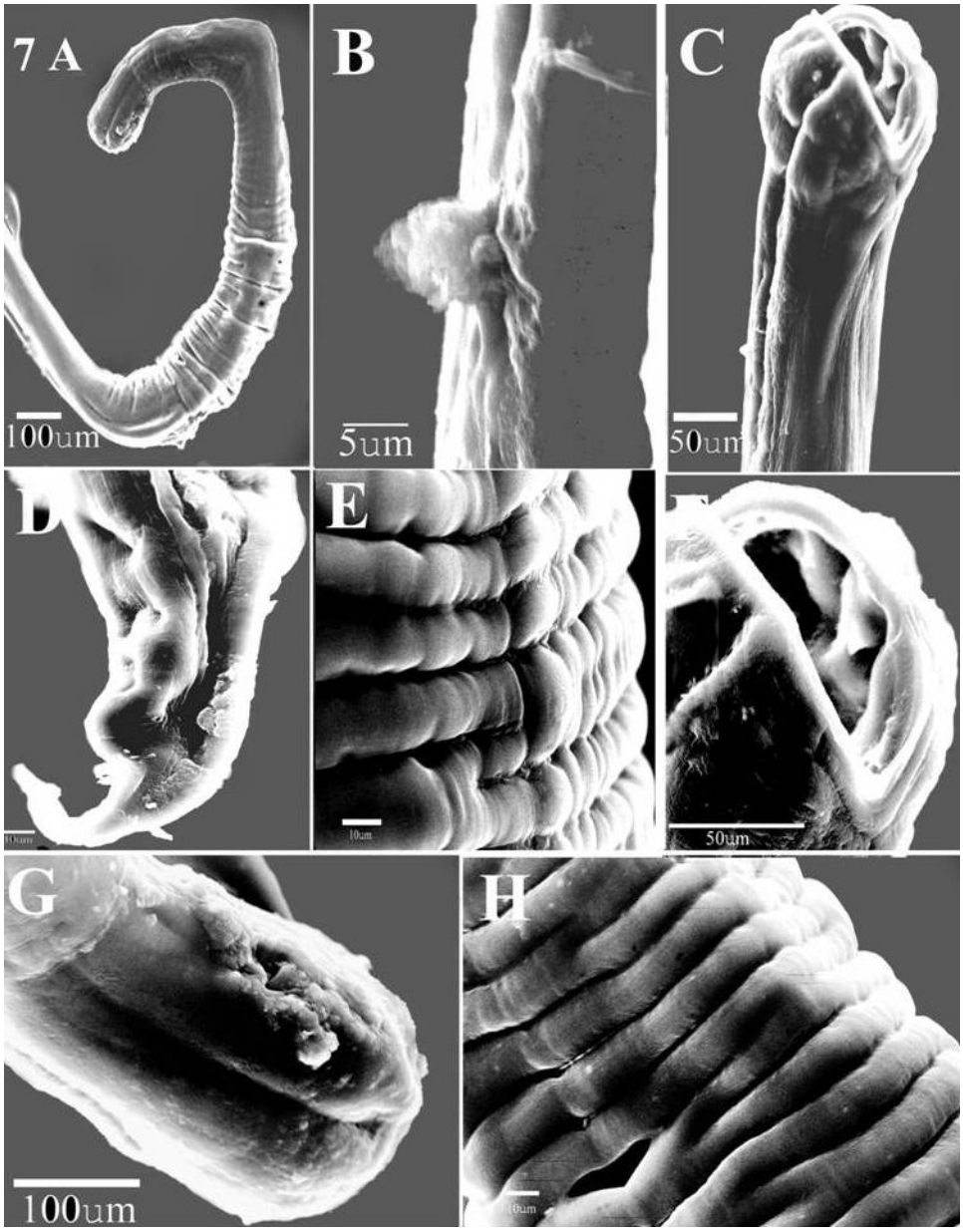

Fig. 7: SEM micrographs of female *C. longicollis*. **A-** anterior portion; **B-** deirids; **C-** anterior extremity, ventral view; **D-** caudal extremity,phasmide; **E-** sensitive papillae along the body; **F-** pseudobuccal capsule with collarette, apical view; **G-** anterior end with amphids; **H-** valvular region, branched and interrupted cuticle.

DICUSSION

The Opecoelidae Ozaki, 1925 constitute a large and cosmopolitan family of digeneans, which includes about 51 genera and 465 species (Yamaguti, 1971). SEM showed that tegument is provided with longitudinal ridges, sharp cuticular projections in the form of needle-like spines and corrugations, which spread on the outer surface, these modifications might explain the tight attachment and penetration of the flukes to the intestinal mucosa of infested fishes.

Dollfus (1960) described a specimen of *Nybelinia riseri* with incompletely evaginated tentacles. The present specimens had completely evaginated tentacles revealed a characteristic armature consisting of compact, rounded rose-thornshaped basal hooks, lacking an anterior extension of the basal plate, and more slender rose-thorn-shaped metabasal hooks semelar observations were reported on *Nybelinia riseri* (Dollfus, 1960**).** However, in *N. lingualis*, the apical hook form remains similar to that seen on the metabasal part of the tentacle, and the hooks increase slightly in size. SEM observations revealed the presence of a sensory receptor"sensory

fossettes" or "ciliated pits," within tufts of microtriches on the bothridial surface. Also, hooks are supported by small nodules or buds. These structures possibly enabling the worm to attachement and orientate within the host may explain the abundance of the ciliated receptors within the trypanorhynch bothridial tegument. These results are in agreement with Palm et al. **(1998**). Palm and Overstreet (2000) renamed these structures bothridial pits, as they could not detect any cilia but documented microtriches within the pits of *O. cysticum.* In addition, *Heteronybelinia estigmena* and *H. microstoma* burrowed their scoleces deeply in the mucosa of the host and attached via hooked tentacles and unciniform microtriches of the scolex (Bounska and Caira, 2006).

However, the functional specialization of the cestode tegument for sensory reception is not well understood and the sensory nature of these receptors has not been demonstrated in experimental and behavioral studies (Palm et al. 1998). Nevertheless, several different functions have been attributed to ciliated receptors, such as chemoreception (Allison, 1980), osmoregulation, (Hess and Guggenheim, 1977), and mechanoreceptor, (Webb & Davey, 1974 and Andersen, 1975). Furthermore, Lumsden and Murphy (1980) proposed that the tapeworm tegument might be an example of an "epidermal" tissue exerting a modulating effect on muscle tissue. On the other hand, retractile sensory receptors on the bothridial surface of the trypanorhynch *Bombycirhynchus sphyraenaicum* had a mechano-receptory function (Palm et al. 1998).

Echinorhynchus gadi Zoega in Muller, 1776 is the most common acanthocephalan infecting marine fish and is found in more than 60 species (Arai, 1989 and Omar, 1987). In the present work, the morphological characteristic features of each organ of the worms corresponded to those listed before. The size, arrangement, number and morphological characters of the body, eggs, and hooks were also correspond to those listed ones (Yamaguti, 1963; and David, 1986 and Arai, 1989). In the present species, the neck showed 2 small pits which could be openings of solitary gland cell ducts and 2 sensory papillae, helping the proboscis in participating in the attachment and nourishment as well as a defensive role.

The nematode family Cucullanidae Cobbold, 1864, comprises several genera, including many species parasitizing various fresh, brackish and marine fishes worldwide. Most cucullanids are representatives of the genera *Cucullanus* Miiller, 1777 and *Dichelyne* Jiigerskiold, 1902. The genus *Cucullanellu*s Tornquist, 1931 is characterized mainly by a ventral intestinal caecum and the ventral sucker lacks the prominent papilliform projection with description given by Yamaguti (**1961**).

An Ultrastrucure study revealed that pseudobuccal capsule (oesophastome) as wide as posterior part of oesophagus and contains 2 pairs of triangular large teeth. The lips of the vulva are Y shaped and considerably elevated. The surface of parasites seems to be important in the intricate relationship between these organisms and their hosts. The cuticle has rugae or folds that described as transverse ridges and external raised incomplete annulations; branched and interrupted on the cuticle surface. The female tail has a pair of sensory papillae situated in a ventro lateral position, which represent the phasmids and they are considered to be comparable to the amphids seen on the head and may have both a glandular and sensory function (**Melarn, 1976**). The ventral sucker helps in attachment during copulation.

Johnston & Mawson (1945) described *Cucullanellus cnidoglanis* from catfish *Cnidoglanis mega-stomus*. Brunsdon (1956) recorded this parasite from *Pagrus auratus* in New Zealand. According to Petter (1974), the genus *Dichelyne* includes three subgenera: *Dichelyne* Jiigerskiold, 1902 (precloacal sucker absent; 11 pairs of caudal papillae), Cucullanellus Tornquist, 1931 (precloacal sucker present; 11 pairs of caudal papillae), and *Neocucullanellus* Yamaguti, 1914 (more than 11 pairs of caudal papillae).

According to Moravec et al. (1993), the genus *Cucullanus* Müller, 1777 includes several species that bear many similarities. The original descriptions of these are often poor, making comparisons between them difficult.

The main distinguishing characters of *C. heliomartinsi* are markedly short spicules that correspond to 2.5% of the total body length; deirids and excretory pore situated posterior to the oesophago-intestinal junction; marked sclerotizations in the oesophago-intestinal junction; marked sclerotizations in the oesophegastome, and oesophagus divided in two distinct portions(Moreira et al. 2000). Although possessing a similar ratio of spicule/body length to the present species, *C. brevispiculus* (Moravec et al. 1993) it differs mainly from *C. heliomartinsi* in the position of deirids and the excretory pore. Both are located in the posterior half of the oesophagus.

The present *Cucullanus longicollis* species has markedly longer alate spicules; 2 pairs of triangular teeth and row of sensitive papillae along the body, which not observed in the parasites recorded from freshwater fishes (González-Solís et al. 2007).

The chitinous triangular teeth are probably used during penetration into and migration through the intestinal wall of the fish host; while the raw of sensitive papillae served for orientation during sexual intercourse.

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Cellulase Production by some Fungi Cultured on Pineapple Waste

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Abstract: Cellulase production from cellulosic pineapple waste using *Trichoderma longibrachiatum, Aspergillus niger* and *Saccharomyces cerevisiae* was assessed. The wastes were dried, pre-treated with alkali and steam, re-dried and then blended. The powdered wastes were then used as substrates in separate shake-flasks which contained mineral salts medium (MSM) and inoculi of *Trichoderma longibrachiatum, Aspergillus niger* and *Saccharomyces cerevisiae.* Fermentations were carried out in flasks containing the MSM, the waste substrate and the inoculum at pH 5.0, 1% substrate concentration, 10% inoculum size and cultured on a rotary shaker at 29±1ºC initially for 5 days to verify cellulase production by the organisms from the waste substrates, then for 7 days or 9 days while varying different fermentation parameters. Cellulase activity and amount of glucose produced by the three test organisms from the waste substrates were determined and compared. The amount of glucose produced was optimized by varying the fermentation parameters: Time, pH, Substrate concentration, Inoculum size and Temperature. The results obtained from the fermentations showed that *Trichoderma longibrachiatum* produced the highest amount of glucose among the cultures tested (0.92mg/0.5ml). This was produced from pineapple pulp at pH 4.5 and temperature of 45ºC on Day7 of fermentation. The highest amount of glucose produced by *Aspergillus niger* was also from pineapple pulp (0.63mg/0.5ml) at pH 3.5 and temperature of 40ºC on Day5 of fermentation. The highest amount of glucose produced by *Saccharomyces cerevisiae* was from pineapple pulp (0.54mg/0.5ml) at pH 4.5 and temperature of 45ºC on Day5 of fermentation. [Nature and Science. 2008;6(2):64-79]. ISSN: 1545-0740.

Keywords: Waste, Pineapple, Cellulase,

INTRODUCTION

Cellulose is the principal constituent of the cell wall of most terrestrial plants. The source of cellulose is in plants and it is found as micro-fibrils (2-20nm in diameter and 100 – 40,000nm long). These form the structurally strong frame work in the cell walls.

Despite a worldwide and enormous utilization of natural cellulosic sources, there are still abundant quantities of cellulose-containing raw materials and waste products that are not exploited or which could be used more efficiently. The problem in this respect is however to develop processes that are economically profitable. Cellulose-containing wastes may be agricultural, urban, or industrial in origin, sewage sludge might also be considered a source of cellulose since its cellulosic content provides the carbon needed for methane production in the anaerobic digestion of sludge. Agricultural wastes include crop residue, animal excreta and crop processing wastes slashing generated in logging, saw dust formed in timber production and wood products in forestry originated activities.

The previous negative attitude in which wastes were viewed self consciously as valueless and even offensive and for disposal only has been replaced in large part by a positive view in which wastes are recognized as raw materials of potential value (Pranner, 1979).

Currently, there are two major ways of converting cellulose to glucose: chemical versus enzymatic. Enzymatic hydrolysis of cellulose is an important reaction in nature for it marks the first step in the decay of cellulose, the most abundantly occurring organic material. In the early 1970s, the oil crisis generated interest in using cellulose as a chemical and energy resource. One promising approach was to hydrolyze the cellulose to glucose with fungal enzymes and then to ferment the glucose to ethanol which could be used as a liquid fuel (Mandels *et al.,* 1974).

The research on both methods has for decades occupied the attention of many investigators world wide because each cellulose molecule is an unbranched polymer of 1000 to 1 million D- glucose units, linked together with β-1, 4 glucosidic bonds. Cellulose from various sources is all the same at the molecular level. However, they differ in the crystalline structures and bindings by other biochemicals (Nam, 1979).

Pineapple fruits are compound, oval fruits, six to eight inches long with spiky robust leaves at the top of the fruit. The tough waxy rind is green, brown and yellowish in colour with a scale-like appearance. The flesh of the pineapple is juicy and yellow to white in colour.

Pineapple is a tropical plant and fruit. Its scientific name is *Ananas comosus* and it belongs to the class: Liliopsida and family Bromeliaceae (Morton, 1987). Pineapple is the source of the proteolytic

enzyme bromelain which is used in commercial meat tenderizers and which constitutes practice as a soft tissue anti-inflammatory and for topical debridement.

In addition to its nourishing uses, the pineapple has long served medical purposes in folk medicine. It is used to arouse appetite, effective as diuretic and contraceptive and in the expulsion of internal worms. It has been used to prevent ulcers, enhance fat excretion, among many other uses.

MATERIALS AND METHODS

Microorganisms and Waste substrates

The organisms used for this study were isolated from three sources: *Aspergillus niger* was isolated from rotten wood (RW) picked up on the premises of University of Ilorin campus, *Saccharomyces cerevisiae* was isolated from palm wine (PW) bought from a palm wine tapper at Offa garage area in Ilorin metropolis and *Trichoderma longibrachiatum* was collected from the Faculty of Agriculture, University of Ilorin. The microorganisms were identified in the Microbiology laboratory, University of Ilorin, Nigeria.

The waste substrates used were in this study were pineapple peel (**ppe**) and pineapple pulp (**ppu**). Pineapple fruit was washed and peeled. The pineapple peel was drained to remove excess water. The pineapple pulp was prepared from the after extraction of the juice from the pineapple fruit. The juice was removed with the aid of a juicer in order to separate the juice from the pulp. The pulp was then washed in water to remove excess juice. Both the peel and pulp were dried in the oven at 70ºC for 3 days. After drying, the pineapple wastes were pounded into small pieces using a mortar and pestle. A modified method of Ali *et al*., (1991) which involved alkali and steam treatments was used in the pretreatment of the waste substrates.

The pounded pieces of the samples were autoclaved for one hour at 121ºC with 5% (w/v) NaOH (20ml per gram of substrate) in separate conical flasks for delignification. The autoclaved ppe and ppu waste substrates were filtered through muslin cloth. They were then washed thoroughly with water and neutralized with 1M HCl. The wastes were finally washed with distilled water and dried at 70ºC. After drying, the treated wastes were ground in a blender for 10 minutes each.

Enzyme assays

The three selected test fungi were cultivated using the submerged culture technique and later maintained on PDA slants. A 10ml three to four day old spore suspension was made from PDA slants of

each culture using sterile distilled water. The fermentation media used was Mary Mandels' mineral salts solution and it was used along with different carbon and nitrogen sources. The medium (**M1**) contained the following (per L) Cellulose,10g ; Peptone,1g ; $(NH_4)_2SO_4$, 1.4g ; KH_2PO_4, 2g ; $CaCl_2$, 0.3g ; $MgSO_4.7H_20$, 0.3g ; Urea, 0.3g ; *trace metal solution (2.5g $FeSO_4$; 0.98g $MnSO_4.H_2O$; 1.76g $ZnSO_4.H_2O$; 1.83g $CoCl_2.6H_2O$ dissolved in 495ml of distilled water and 5ml of conc. HCl), 1ml ; pH 4.8 (Jeffries, 1996).

*Trace metal stock solution (1ml is used per L).

Mineral salts glucose medium was prepared and approximately 2.8×10^6 spores/cells of each culture were inoculated into 500 ml flasks containing 100 ml of medium each. The spores/ cells were counted using a Neubauer counting chamber. The flasks were incubated for 24 hours at 29±1ºC on a Gallenkamp (England) rotary shaker at 250rpm to develop the inoculum.

The waste substrates provided the carbon sources in the fermentation media. These were combined with M1 to give Mineral salts glucose medium (MSGM), Mineral salts pineapple peel medium (MSppeM) and Mineral salts pineapple pulp medium (MSppuM).

All the media mentioned above were prepared separately and dispensed in conical flasks. They were sterilized in the autoclave at 121ºC for 15 minutes.

The pH of the fermenting media containing the waste substrates at a level of 10g/L was adjusted to 5.0. The suspension of germinated spores was inoculated at a level of 10% (v/v) into the production medium contained in flasks. These were incubated at 29±1ºC on a shaker at 100rpm. Glucose production in the medium was measured on Day 5 of fermentation (Srivastava *et al*. 1987; Jeffries, 1996; Bukoye, 2001).

Cellulase activity was determined colorimetrically by measuring the increase in reducing groups by the hydrolysis of a carboxymethylcellulose (CMC) substrate (Jeffries, 1996). The procedure followed the 0.5ml assay described by Jeffries (1996).

Samples were withdrawn from the culture at 2-day intervals over a period of 7-9 days and the supernatant that resulted following centrifugation at 3000 rpm for 15 minutes to remove solids, were assayed for total reducing sugars using DNSA method of Miller (1959). Enzyme solutions were diluted in 0.05M citrate buffer, pH 4.8. The enzyme diluted in buffer and one percent CMC (0.5ml each) was mixed well and incubated for 30 minutes at 50ºC. Three milliliters of the DNSA was added and the tubes were placed in boiling water bath for five minutes. The tubes were cooled and the reducing sugar, glucose was determined (Jeffries, 1996). The sample, enzyme blank, glucose standard and control were boiled together and absorbance was read at 540nm using a spectrophotometer. A control (substrate and buffer) was used to set the spectrophotometer at zero absorbance. During the course of the experiments, the absorbance of the

sample tube, corrected by subtraction of the enzyme blank was translated into glucose during the reaction using a glucose standard. The linear glucose standard was used to translate the absorbance values of the sample tubes into glucose i.e. mg glucose produced during the reaction. For a 30-minute assay, 1mg of glucose equals 0.185unit $\left(\frac{1}{30 \times 0.18}\right)$.

Determination of Optimal Conditions for Enzyme Production

Effect of varying time: Cellulase activity was measured at regular intervals while fermentation was observed at 29±1ºC for a period of 9 days and the period of maximum enzyme production was determined.

Effect of varying pH: The pH of the fermentation media were adjusted to various values ranging from 2.0-6.0 with 0.1N NaOH or 0.1N HCl. The pH was determined using the pH meter

Effect of varying substrate concentration: Different concentration of the waste substrates ranging from 1.0% to 5.0% were used in the fermentation media.

Effect of varying Temperature: The fermentation was carried out at different temperatures ranging from 30ºC to 45ºC.

Effect of varying inoculum size: Each cellulosic waste was fed with varying sizes of inoculum of the organisms. The inoculum size was varied from 2% to 10%.

Optimization experiments were carried out and each of the organisms were grown on each of the substrates and hydrolyzed using parameters that produced maximal activity of the enzyme from all the earlier experiments. In accord with the International Union of Biochemistry, one enzyme unit equals 1micromole (μ) of substrate hydrolyzed per minute (Ghose, 1987).

RESULTS AND DISCUSSION

Table 1. Fermentation of Pineapple wastes substrates by Test Fungi

WASTE SUBSTRATE	GLUCOSE PRODUCED (mg/0.5ml)		
	T. longibrachiatum	*A. niger*	*S. cerevisiae*
Pineapple peel	0.52	0.41	0.18
Pineapple pulp	0.43	0.49	0.43

Fermentation conditions: Substrate concentration 1%, pH: 5.0, inoculum size: 10%, temp: 29±1ºC, time: 5 days

Table 2: Effect of Substrate Concentration on the Fermentation of Pineapple waste by test fungi

Fermentation conditions: pH: 5.0, inoculum size: 10%, temp: 30ºC, time: 5 days.

Substrate concentration	Glucose produced (mg/0.5ml)					
	T. longibrachiatum		*A. niger*		*S. cerevisiae*	
	ppl	ppu	ppl	Ppu	ppl	ppu
1%	0.28	0.30	0.21	0.25	0.19	0.21
2%	0.29	0.31	0.29	0.27	0.26	0.268
3%	0.34	0.36	0.25	0.28	0.31	0.294
4%	0.31	0.36	0.26	0.26	0.29	0.326
5%	5.87	0.33	0.27	0.27	0.31	0.296

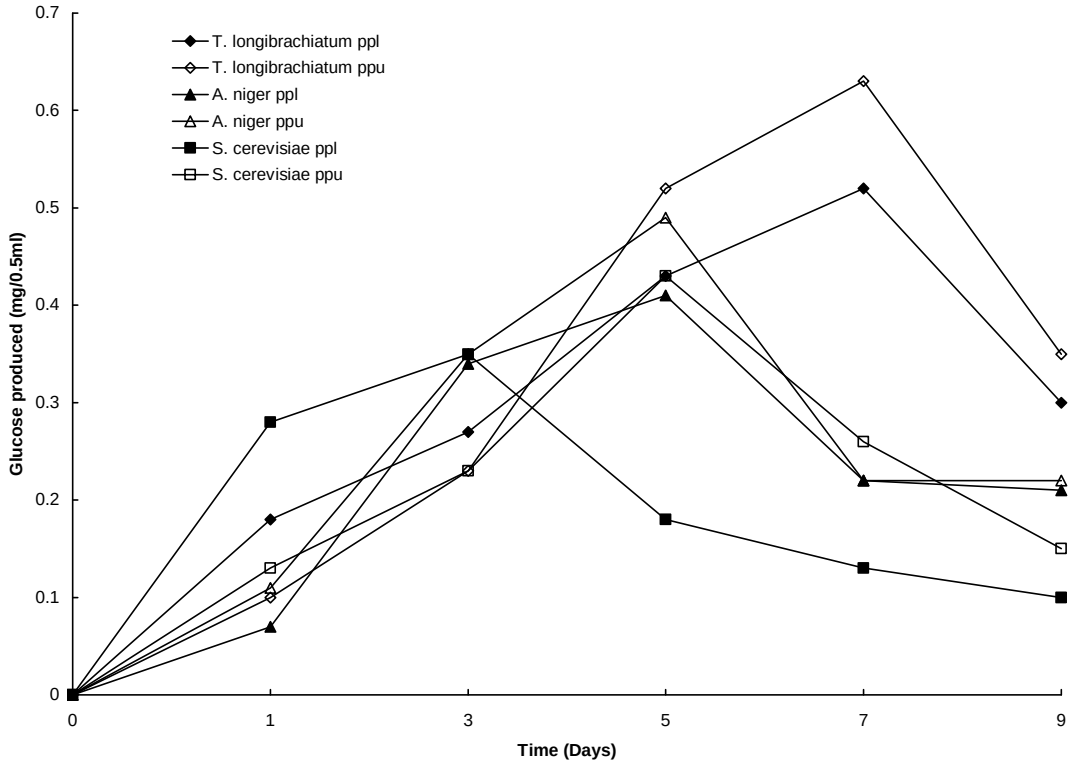

Figure 1. Effect of varying time on the fermentation of Pineapple waste substrates using test fungi.

Table 3. Effect of pH on the Fermentation of Pineapple waste by test fungi

WASTE SUBSTRATE	pH2.0	pH2.5	pH3.0	pH3.5	pH4.0	pH4.5	pH5.0	pH5.5	pH6.0
T. longibrachiatum									
Pineapple peel	0.200_e	0.178_e	0.208_d	0.200_e	0.242_c	0.312_f	0.296_f	0.210_e	0.212_e
Pineapple pulp	0.152_b	0.192_c	0.262_c	0.250_c	0.338_f	0.366_f	0.280_c	0.292_f	0.234_c
Aspergillus niger									
Pineapple peel	0.162_b	0.164_b	0.218_b	0.290	0.240	0.232_b	0.208_b	0.252	0.236_c
Pineapple pulp	0.184_b	0.170b	0.240	0.316	0.292	0.300	0.246	0.294	0.264
Saccharomyces cerevisiae									
Pineapple peel	0.146	0.146	0.150	0.198	0.158	0.198	0.188	0.142a	0.154
Pineapple pulp	0.146	0.152	0.168	0.216	0.188	0.248_b	0.210	0.214	0.234_b

(Fermentation conditions: Substrate concentration: 1 %, Temp: 29±1ºC, inoculum size: 10%)

Values are presented as mean.

All groups are compared to each other at $p<\alpha=0.05$.

Values with different subscripts are statistically different.

TABLE 4. Effect of Substrate Concentration on the Fermentation of Pineapple waste by test fungi

n	1%	2%	3%	4%	5%
SUBSTRATE					
Pineapple peel	0.280	0.288	0.338	0.314	5.874a
Pineapple pulp	0.296	0.314	0.360	0.360	0.334
Aspergillus niger					
Pineapple peel	0.208	0.288	0.250	0.256	0.268
Pineapple pulp	0.246	0.266	0.278	0.264	0.274
Saccharomyces cerevisiae					
Pineapple peel	0.188	0.262	0.308	0.286	0.314
Pineapple pulp	0.210	0.268	0.294	0.326	0.296

(Fermentation conditions: pH :5.0, Temperature : 29±1ºC

Values are presented as mean.

All groups are compared to each other at $p<\alpha=0.05$.

Values with different subscripts are statistically different.

TABLE 5. Effect of Inoculum size on the Fermentation of Pineapple waste by test fungi

WASTE SUBSTRATE	2%	4%	6%	8%	10%
Trichoderma longibrachiatum					
Pineapple peel	0.144	9.200_{e}	0.134	0.198	0.280
Pineapple pulp	0.136	0.140	0.192	0.166	0.296_{a}
Aspergillus niger					
Pineapple peel	0.124	0.216	0.302_{a}	0.288_{a}	0.208
Pineapple pulp	0.122	0.230	0.286_{a}	0.280	0.234
Saccharomyces cerevisiae					
Pineapple peel	0.232	0.228	0.284_{a}	0.264	0.188
Pineapple pulp	0.264	0.268	0.292_{a}	0.272	0.210

(Fermentation conditions: Temperature: 29±1ºC, pH: 5.0, Substrate concentration: 1%)

Values are presented as mean.

All groups are compared to each other at $p<\alpha=0.05$.

Values with different subscripts are statistically different.

TABLE 6**.** Effect of Temperature on the Fermentation of Pineapple waste by test fungi

WASTE SUBSTRATE	30ºC	35ºC	40ºC	45ºC
Trichoderma longibrachiatum				
Pineapple peel	0.280^{b}	0.242^{b}	0.300^{b}	0.368^{c}
Pineapple pulp	0.296^{b}	0.252^{b}	0.302^{b}	0.360^{c}
Aspergillus niger				
Pineapple peel	0.208^{b}	0.200^{a}	0.282^{b}	0.304^{c}
Pineapple pulp	0.246^{b}	0.206^{a}	0.328^{c}	0.324^{c}
Saccharomyces cerevisiae				
Pineapple peel	0.188	0.192	0.258^{a}	0.276^{a}
Pineapple pulp	0.210	0.214	0.270^{a}	0.296^{c}

Values are presented as mean.

All groups are compared to each other at $p<\alpha=0.05$.

Values with different subscripts are statistically different.

Table 7. Fermentation of Pineapple wastes using optimized fermentation parameters using the test fungi

WASTE SUBSTRATE	*T.longibrachiatum*	*A. niger*	*S. cerevisiae*
Pineapple peel	0.453_c	0.375_b	0.297_a
Pineapple pulp	0.565_d	0.375_b	0.323_a

Values are presented as mean.

All groups are compared to each other at $p<\alpha=0.05$.

Values with different subscripts are statistically different.

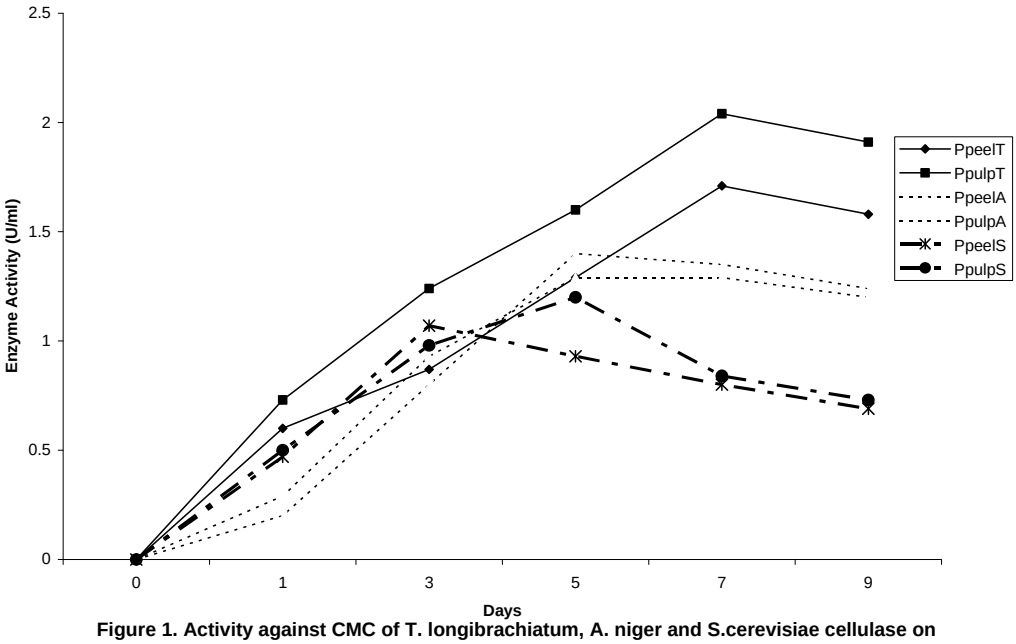

Figure 1. Activity against CMC of T. longibrachiatum, A. niger and S.cerevisiae cellulase on Pineapple Wastes

Figure 2.

The three test organisms are capable of producing cellulase as evidenced by the production of glucose (Table 1). Optimal glucose production from *T. longibrachiatum* was observed on Day 7 of fermentation, Day 5 for *A. niger* and Day 3 for pineapple peel and Day 5 for pineapple pulp for *S. cerevisiae*. Pineapple pulp released higher amount of glucose from the hydrolysis than pineapple peel (Table 2). Hydrolysis rates decline with time due to depletion of the more amorphous substrates, product inhibition and enzyme inactivation (Ghose, 1987). Caritas and Humphrey (2006) and Narasimha *et al*. (2006) also gave similar time course reports of maximum glucose yield on 5th day of fermentation using *A. niger*. Effect of pH on glucose production from the two waste substrates by the three microorganisms was shown on Table 3. This supports the findings of Lee *et al*. (2002) who reported that CMCase, Avicelase and FPase activities exhibit a pH optimum of approximately 4, while the pH optimum of β- glucosidase was between pH 5 and 6.

The effect of substrate concentration was shown on Table 4. Further increase in cellulose concentration beyond the level that gave the optimum glucose did not result in proportionate increase in glucose yield. Haapela *et al*., (1995) and Jeffries, (1996) reported that maximum endoglucanase activity was recovered on the medium with cellulose at 10g/l. Mandels and Reese (1959) also reported that maximal yields of cellulase were obtained on one percent substrate (cellulose, lactose, cellobiose and glucose) using *T. viride* and *Myrothecium verrucia.* These reports support the findings of this study as substrate concentration of 10g/L gave the highest amount of glucose from *T. longibrachiatum* on pineapple pulp.

Since the substrates contain different minerals apart from carbon which may serve as nutrient supplements, increase in substrate concentration leads to increase in these nutrients which may adversely affect the cell concentration. The increase in glucose production until the optimum that was obtained was due to the availability of cellulose in the medium; while a decrease in production beyond optimum concentration is explained to be as a result of an inhibitory effect of accumulated cellobiose and cellodextrins of low degree of polymerization to the growth medium. It might also be due to the specific binding of the enzymes with the substrates (Gilkes, *et al*., 1984). Low glucose production after optimum very probably highlights sugar depletion from the substrates into the medium (Brien and Craig, 1996).

Effect of inoculum size was shown on Table 5. Decrease in amounts of glucose production resulted at inoculum sizes above 6% and 8% for fermentations using *A. niger*. This decrease in glucose production with further increase in inoculum might be due to clumping of cells which could have reduced sugar and oxygen uptake rate and also, enzyme release (Srivastava, *et al*., 1987). Effect of Temperature was shown on Table 6. The optimum temperature for the synthesis of enzymes for saccharification of agrowaste in all cases to enzymatic hydrolysis can be attributed to lignin content of the material. Pretreatment of lignocellulosic material enhances enzyme activity and maximum saccharification was achieved within the range 30-45^{o}C coinciding with the characteristics of mesophiles (Baig *et al*., 2004).

Optimum glucose from the waste substrates using *T. longibrachiatum,*was produced at 10% inoculum size at 45ºC on Day7 but at pH 5.0 and 3% substrate concentration for pineapple peel and pH 4.5 and 1% substrate concentration for pineapple pulp.

Optimum glucose from the waste substrates using *A. niger* was produced at pH 3.5, 2% substrate concentration, 6% inoculum size on Day5 but at 45ºC for pineapple peel and 40ºC for pineapple pulp.

Optimum glucose from the waste substrates using *S. cerevisiae* was produced at pH 3.5, 3% substrate concentration, 6% inoculum size, 45ºC on Day3 for pineapple peel and pH 4.5, 4% substrate concentration, 2% inoculum size and 45ºC on Day5 for pineapple pulp.

These optimal conditions were combined in single fermentations for each organism and cellulase activity was measured (figure 1). Cellulase activity from pineapple peel was 1.71U/ml when hydrolyzed by *T. longibrachiatum*, 1.29 U/ml when hydrolyzed by *A. niger* and 1.07U/ml when hydrolyzed by *S. cerevisiae.* Cellulase activity from pineapple pulp was 2.04U/ml when hydrolyzed by *T. longibrachiatum*, 1.40U/ml when hydrolyzed by *A. niger* and 1.20U/ml when hydrolyzed by *S. cerevisiae*. Cellulase activity of *S. cerevisiae* was lowest. The exo-β-1, 3-glucanases produced by *S. cerevisiae* yield glucose as the end product, whereas endo-β-1, 3-glucanase releases a mixture of oligosaccharides with glucose as the minor product. Because β-1, 3-glucan is the main structural polysaccharide responsible for the strength and rigidity of the yeast cell wall, β-1, 3-glucanases have been suggested to play a role in important morphogenetic processes involving the controlled autolysis of β-1, 3 glucan. During vegetative growth, several endo- and exo-1, 3- β- glucanases are synthesized, some of which are secreted only to remain entrapped in the cell wall whereas others are released to the surrounding medium (Lee *et al*., 2002).

In conclusion, this study revealed that pineapple peel and pulp, which are examples of domestic and industrial agro-wastes, produce large amounts of cellulase enzymes when hydrolyzed by cellulolytic microorganisms and instead of being left behind for natural degradation can be utilized effectively under these conditions, to produce cellulase.

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Synthesis, Characterisation and Preliminary Anion complexation studies of a nuetral novel 2,7-diacetamido fluorene molecular tweezer receptor

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Abstract: *A novel 2,7-diacetamido fluorene receptor has been synthesized and characterized via ^{1}H NMR, ^{13}C NMR, DEPT 135, ^{1}H-^{1}H COSY, HMQC, HMBC experiments. Preliminary anion binding studies via ^{1}H NMR indicate recognition and complexation of bromide anion. There are only a few neutral amide receptors reported to date*. [Nature and Science. 2008;6(2):80-89]. ISSN: 1545-0740.

Research in Anions coordination Chemistry is increasing, considering the versatile role anions play in nature, medicine, laboratory and industrial processes etc. Several concepts of anion binding motifs have been explored over the years. These include the synthesis of acyclic and macrocyclic ligands incorporating a positively charged centre or a Lewis acidic centre in close proximity and in tandem with amide hydrogen bonds[1-4]. For example, porphyrin (1) was shown spectrally and electrochemically to sense, Cl^-, Br^-, NO_3^-, HSO_4^- and $H_2PO_4^-$ anions with selectivity trend: $H_2PO_4^- > HSO_4^- > Cl^- > Br^- > NO_3^-$. A similar selectivity trend was noted for compound (2) [4]. Other receptors have use neutral urea motifs to complex anions with large binding constants and excellent selectivity[4-8]. For example, a series of highly potent and remarkably highly selective free base "Picket fence" porphyrin urea receptors (3)-(6) and their Zn(II) complexes (7)-(9) were synthesized, characterized[5-6] and anion binding studies investigated. The *Cis*-5,10,15,20-*tetrakis*(2-(arylurea)phenyl)porphyrins bind strongly (K (M^{-1}) > 10^3-10^5) to chloride anion in DMSO-d_6 and also in the more competitive solvent system DMSO-d_6/D_2O (88:12, v/v) as revealed by ^{1}H NMR titration studies. To the very best of knowledge, it is the largest stability constant reported to date for any anion receptors complexing chloride in a highly competitive DMSO-d_6 solvent and also the best chloride selective receptor reported. The selectivity trend $Cl^- > Br^- > H_2PO_4^- > HSO_4^- > NO_3^-$ is also novel for any neutral urea-anion binding system. Of great significance, X-ray crystallography revealed *Cis*-5,10,15,20-*tetrakis*(2-(4-chlorophenylurea)phenyl)porphyrin to be the first coordination complex of an anion (chloride and bromide) bound by a neutral free-base porphyrin. Still other anion binding motifs include the use of Lewis acid centres such as boron and mercury to complex anion such as fluoride[8]. The design and synthesis of neutral receptors is receiving increasing attention, considering the prevalence of amide hydrogen bond in nature[7-8]. There are only a few neutral amide receptors reported to date.

Anion binding and transport has a unique role in nature: In membrane transport for example, the selective flow of ions into and out of cells occurs via anion transport mechanisms. Such processes have been regulated by ion binding proteins whose main mechanism of transport rely on extensive hydrogen bonding in the binding sites complementary to the anion being transported. Phosphate chelation is said to involve the formation of twelve complementary hydrogen bonds with the protein, five from the main chain and seven from side chain residues. Amide hydrogen bonding are also involved in sulphate binding[9,10] with selectivity ratios of phosphate over sulphate or sulphate over phosphate greater than 10^5. Also, in nature, the selective binding for anion is achieved via the positional alignment of amide hydrogen bonds[4]. In Biochemistry, 70% of naturally occurring enzymes require an anion either as a substrate or as a cofactor[12]. For example, the enzyme carbonic anhydrase has as its cofactor Zn^{2+}, a Lewis acid which coordinates OH^-

and thus allowing it to perform its catalytic function. The genetic encoded material DNA and RNA, essential in cell replication and organism growth and ATP which provides the energy required for growth and metabolism are polyanions. Their negative charges are conferred by phosphate ester groups[13]. In the laboratory, anions act as nucleophiles (CN^-), redox active agents ($S_2O_8^{2-}$) in titrations, bases (^-OR) and as phase transfer catalysts. In pursuit of neutral receptors, exhibiting strong and selective complexation for anions in highly competitive solvents, receptor (11), 2,7-diacetamido fluorene was synthesized and characterised. Such a receptor present convergent amide binding sites to host anions, Fig. 2.0 i.e –CO-NH---- anion hydrogen bonds. In such a neutral system, it might also be possible that the positive induced carbonyl carbon may assist in some form of induced dipole---anion interaction, Fig.3.0. Compound (11) was synthesized via the addition of the requisite acid chloride, acetyl chloride to the amine: 2,7- diamino fluorene, dissolved in CH_2Cl_2 in the presence of ET_3N and stirred under nitrogen, Scheme 1.0. The crude sample after work up was purified via flash column silica gel chromatography to gave compound (11) as a white solid in 65 % yield . Compound (11) was characterized via ^{1}HNMR, ^{13}CNMR, , Dept 135, ^{1}H-^{1}H COSY, HMBC, HMQC, FAB MS spectra and IR spectroscopy and these are presented in the experimental sections.

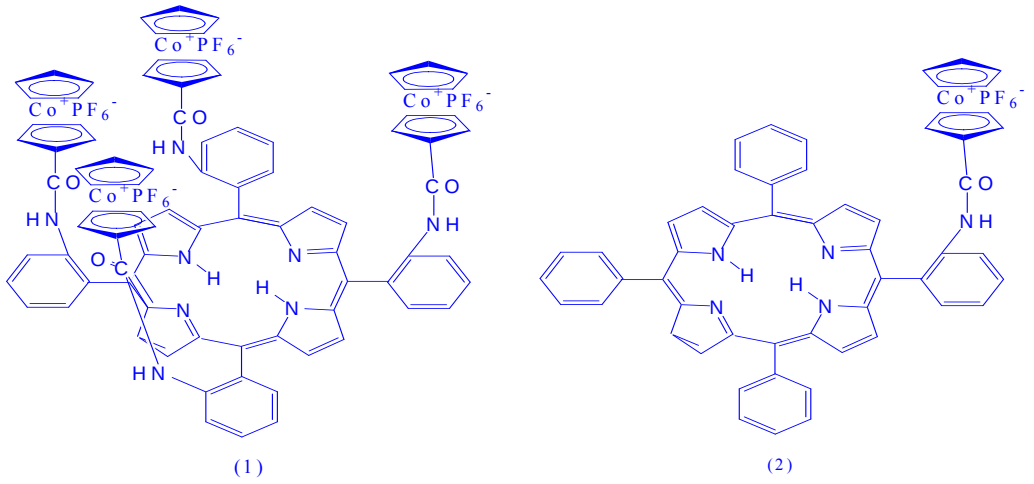
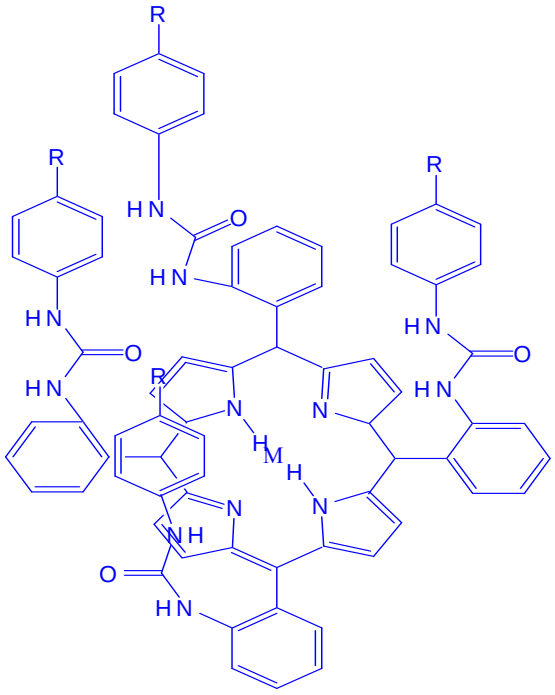


Fig. 1.0. Positively charged cobalticinium amido phenyl functionalised porphyrins: *Cis*-5,10,15,20-*meso tetrakis* (*ortho* cobaltocenium) amido phenyl porphyrin(1) and 5-(*ortho*-(cobaltocenium amido phenyl)-10,15,20-triphenyl porphyrin(2)



R = H, (3)

R = Cl, (4)

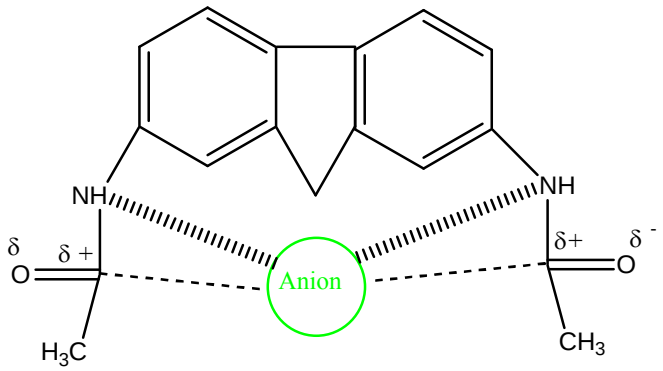
R = F, (5)

R = NO_2 (6)

M = Zn

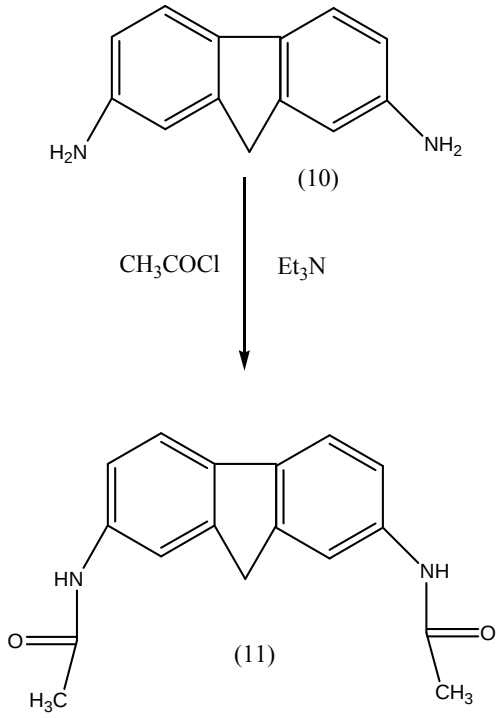
R =H (7), R=Cl (8), R = F (9)

Fig. 2.0. Neutral "Picket fence" Porphyrin urea anion receptors: (α,α,α,α)-5,10,15,20-*meso tetrakis*(2-(arylurea)phenyl) porphyrins.



(11)

Fig. 3.0. Proposed mode of anion binding by the molecular Tweezer (11)



Scheme 1.0. Synthesis of compound (11)

The ^{1}H NMR spectrum, Fig. 4.0, recorded in DMSO-d_6 indicates that the amide protons H-14 and H-17 resonate as a broad singlet downfield at 9.98 ppm. Aromatic protons resonate as a singlet, doublet and double of doublets in the region 7.47 to 7.86 ppm. The singlet at 7.86 ppm is due to H-1=H-8 protons. H-4 = H-5 protons couple with H-3 =H-6 protons. As such they resonate as a doublet at 7.69 ppm. However, H-3=H-6 protons are split into a doublet by H-4 protons which is further split by H-1(J=1.6Hz) resulting in a doublet of doublets. The CH_2 protons that bridge the two benzene rings resonate as a singlet at 3.85 ppm. The CH_3 protons of the acetyl group is seen as a conspicuous sharp singlet at 2.05 ppm, Fig. 4.0. The ^{13}C NMR spectrum indicates the presence of nine different signals due to nine different carbons of the structure. DEPT-135 experiments were used to differentiate the CH_3, CH and CH_2 protons. Accordingly, the only CH_3 proton resonate at 24.42 ppm, whereas the bridged methylene CH_2 protons resonate at 36.9 ppm. The CH aromatic carbons resonate at 119.76, 118.06 and 116.11 ppm. Quaternary carbons were seen at 136.46, 138.13, 143.79 ppm whereas the carbonyl carbons resonate at 168.48 ppm. The complete spectral assignment was furnished via ^{1}H NMR, ^{13}CNMR, ^{13}CNMR-DEPT -135, HMQC, HMBC and ^{1}H-^{1}H COSY experiments and are summarized in Table 1.0.

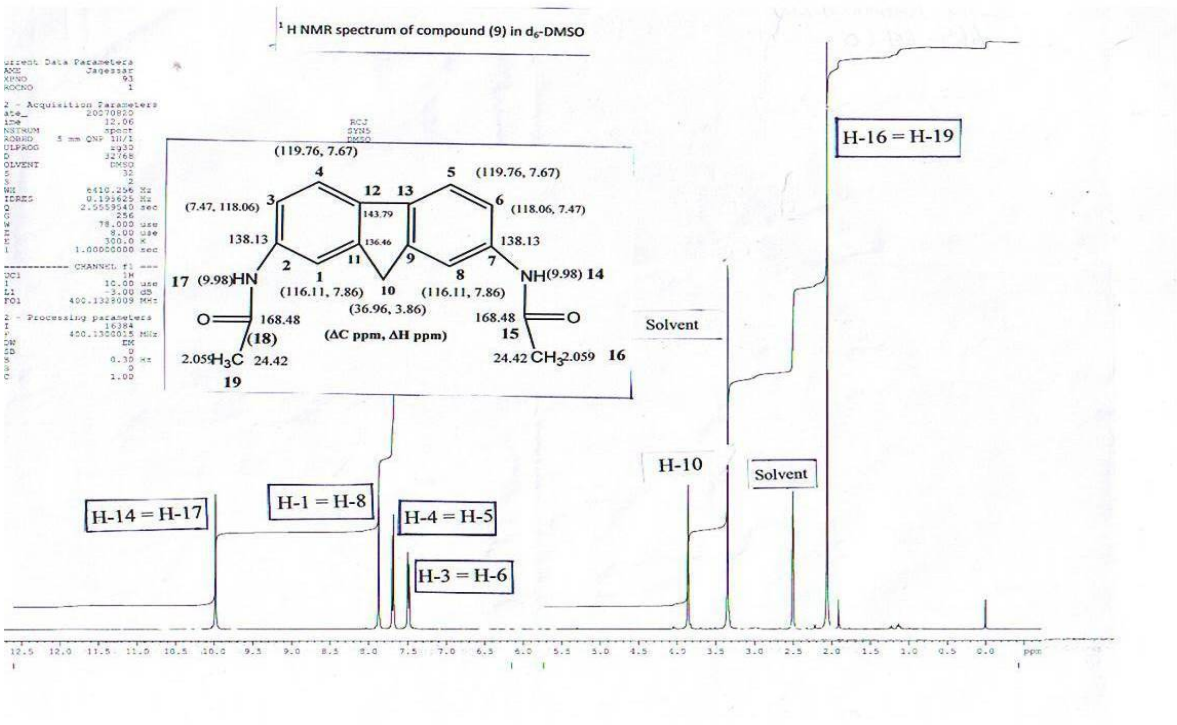


Fig. 4.0 [1] H NMR spectrum of compound (11) in DMSO-d_6

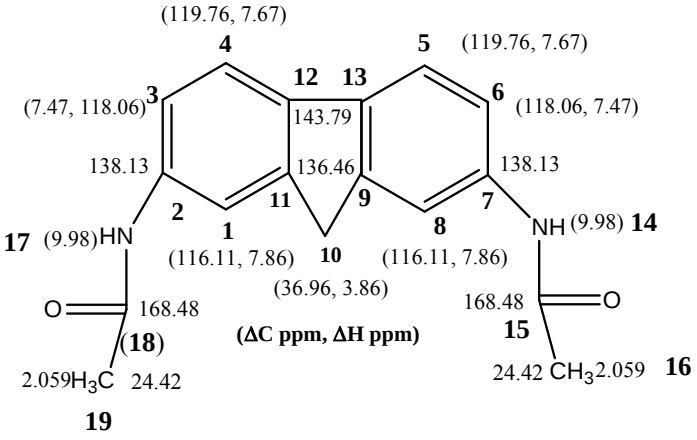


Fig. 5.0 ([13] CNMR and [1] H NMR chemical shifts for respective carbons and protons of compound (11)

Table 1. NMR DATA for compound 11 ($CDCl_3$) [a]

Position	δC	δH (jHH, HZ)	^{1}H-^{1}H COSY	HMBC
1 = 8	116.11	7.86 (s)	H-3, H-4, H-10	118.06(C-3), 136.46(C-11)
2 = 7	138.13	QC	QC	QC
3 = 6	118.06	7.47 (dd, J =1.6Hz)	H-1, H-4	116.11(C-1), 118.06(C-3), 119.76(C-5), 136.46(C-11)
4 = 5	119.76	7.67 (d, J =8.4 Hz)	H-3, H-1, H-10	118.06(C-3), 136.46(C-11), 143.79(C-12)
5 =4	119.76	7.67 (d, J =8.4 Hz)	H-1, H-3, H-10	118.06(C-3), 136.46(C-11), 143.79(C-12)
6 = 3	118.06	7.47 (dd, J=1.6Hz),	H-1, H-4	116.11(C-1), 118.06(C-3), 119.76(C-5), 136.46(C-11)
7 = 2	138.13	QC	QC	QC
8 = 1	116.11	7.86	H-3, H-4, H-10	118.06(C-3), 136.46(C-11)
9 = 11	136.46	----------	QC	
10	36.96	3.86	H-1, H-3, H-4	136.46(C-11), 116.11(C-1), 143.79(C-12)
11= 9	136.46	QC	QC	QC
12=13	143.79	QC	QC	QC
13=12	143.79	QC	QC	QC
14=17	---------	9.98 (s)	---------	116.11(C-1), 118.06(C-3), 168.48(C-15)
15=18	168.48	QC	QC	QC
16 = 19	24.42	2.059	---------	--------

17 =18	NH	9.98 (s)	---------	--------
18 =15	168.48	---------	---------	--------
19 =16	24.42	2.059 (s)	---------	168.48(C-15)

[a] 400 MHz for ^{1}H NMR and 100 MHz for ^{13} C NMR. All Chemical shifts (relative to TMS) are given in δ(ppm) and coupling constants expressed in Hertz.

QC: Quaternary carbon

Preliminary Anion complexation studies:

Preliminary anion binding studies for (11) were investigated via the stepwise addition of tetrabutylammonium halide to solution of compound (11) in deuterated DMSO- d_6 at room temperature. Before, any addition took place, the ^{1} H NMR spectrum of the free ligand was recorded . Addition of stepwise equivalents of anion resulted in significant shifts in the host protons. The ^{1} H NMR spectrum was recorded after each addition. For example, after the addition of 1 equivalent of bromide, the amide protons, -NH-CO- at 9.994 ppm exhibited downfield shift of δS = 0.13 ppm suggesting -CO-NH---Anion complexation. Aromatic protons have also responded to complexation. A new peak at 8.3 ppm is evident. The singlet resonance due to aromatic proton, H-1 shifted downfield from 7.86 to 7.98 ppm (δS = 0.12 ppm) after the addition of two equivalents. Also, the aromatic doublet at 7.64, H-5 exhibited an upfield shift of 0.07 ppm and remained broad. The other doublet at 7.496 exhibited a downfield shift of 0.06 ppm. However, the fluorene methylene protons, H-10 exhibited an insignificant downfield shift of 0.007 ppm.. These shifts are significant, considering that complexation was done in the polar DMSO-d_6 solvent. These results are summarized in the table below:

Table 2.0 ^{1} HNMR chemical shifts of ligand (10) protons after the addition of two equivalents

Protons	Chemical Shifts Free Ligand	Chemical Shifts After the addition of two equivalents of Br-	δS
Methyl protons, H-16 = H-19	2.061	2.088	0.027 ppm
Fluorene methylene protons, H-10	3.858	3.851	0.007 ppm
Aromatic doublet, H-6	7.496	7.56	0.06 ppm
Aromatic doublet, H-5	7.639	7.71	0.07 ppm
Aromatic singlet, H-1	7.862	7.98	0.12 ppm
Amide NH proton, H-14= H-17	9.994	10.13	0.13 ppm

Conclusions: A novel 2,7-diacetoamido fluorene receptor has been synthesized and characterized via ^{1}H NMR, ^{13} C NMR, DEPT 135, ^{1}H-^{1}H COSY, HMQC, HMBC experiments. Preliminary anion binding studies via ^{1} H NMR indicate recognition and complexation of bromide anion. There are only a few neutral amide receptors reported to date. Future work will investigate coordination with other anions.

Acknowledgement: This research was funded through a grant to purchase chemicals to Dr. R.C. Jagessar from the University of Guyana Publication Agency. NMR spectroscopic characterization using a 400MHZ NMR spectrometer was done overseas. The syntheses, characterization and preliminary anion complexation studies were all done by Dr.R.C.Jagessar.

General: 2,7-diaminofluorene and acetyl chloride were purchased from Aldrich in the USA. Melting points were measured on a Geahaka model PF 1500 version 1.0 apparatus and are uncorrected. ^{1}H and ^{13}C NMR COSY, HMQC and HMBC spectra were recorded on a Bruker DRX-500 spectrophotometer, using $CDCl_3$ as the solvent. Chemical shifts are quoted in δ ppm and coupling constants expressed in Hertz (Hz). Silica gel 60A (70-230 mesh) was used for flash column chromatography. TLC analyses were done on precoated Kieselgel 60 F_{254} plates.

Experimental: 2,7-diamido fluorene receptors: The amine(0.22 g, 1.1 x 10^{-3} mol) was dissolved in dried CH_2Cl_2 and stirred for 15 minutes under nitrogen. This was followed with the addition of Et_3N, leaving a brown solution . To that solution was added acetyl chloride, CH_3COCl (0.19g, 2.4 x 10^{-3} mol)stepwise. The reaction mixture was left stirring for 24 hours under nitrogen. Solvents were removed in *vacuo*, yielding a brown crude product. After workup, a pale brown solid was obtained. The latter was purified via flash column chromatography on silica gel to yield compound (2) as the major product in yield of 65% (0.2g).

Compound (11) $C_{17}H_{16}O_2N_2$:M.P: 43.4-43.6˚C ^{1}H NMR ($CDCl_3$) δ: 9.978 (s, br; 2H; NH), 7.864 (s, 2H; ArH), 7.68 (d, 2H; J = 8.4Hz; ArH), 7.49 (dd, 1H; J =1.6Hz, ArH), 7.47(dd, J = 1.6Hz, 1H; ArH) 3.85 (s, 2H; CH_2), 2.06(s, 6H; CH_3); ^{13} CNMR ($CDCl_3$, 400MHz) □: 168.47, 143.79, 138.14, 136.48, 119.76, 118.05, 116.11, 38.95, 24.42. DEPT 135: C=O (168.48) QC: 143.75, 138.13, 136.46, ArH CH: (119.76, 118.05, 116.11), 36.958 (CH_2), 24.42(CH_3); COSY:H-1/H-3,H-4,H-10; H-3/H-1,H-4;H-4/H-3,H-1,H-10; H-5/H-1,H-3,H-10;H-6/H-1,H-4;H-8/H-3,H-4,H-10;H-10/H-1,H-3/H-4; HMBC correlations: H-1/C-3, C-11;H-3/C-1,C-3,C-5,C-11;H-4/C-3,C-11,C-12,H-5/C-3,C-11,C-12,H-6/C-1,C-3,C-5,C-11,H-8/C-3,C-11,H-10/C-11,C-1,C-12,H-14/C-1,C-3,C-15;H-19/168.48.

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Thrombin

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Abstract: Thrombin is most widely recognized for its role in blood coagulation but the diversity of this protein's functions in the body are far more widespread. There are thousands of articles related to thrombin published each year. This review article describes the major functions of thrombin in the body, considering the different processes separately. [Nature and Science. 2008;6(2):90-93]. ISSN: 1545-0740.

Keywords: coagulation; fibrinogen; thrombin

1. Introduction

Thrombin is most widely recognized for its role in blood coagulation but the diversity of this protein's functions in the body are far more widespread. Indeed, it has been noted by one group of workers that almost every cell type tested (except erythrocytes) was responsive to thrombin (Fenton, et al, 1998). This review article describes the major functions of thrombin in the body, considering the different processes separately.

2. Thrombin in Coagulation

The blood coagulation system comprises a cascade of proteases that cleave precursor enzymes to form active enzymes, forming an extremely effective amplification system that culminates in the formation of thrombin. Thrombin, once formed, then catalyses the conversion of fibrinogen to fibrin to form a clot and, importantly, regulates the system by providing stimulatory and inhibitory feedback. The centrality of thrombin to this system makes it an extremely powerful enzyme in coagulation and makes it the most suitable enzyme to be a target for anticoagulant drug therapy (Fenton, 1998), as to control the action of thrombin would be to control the entire coagulation system.

3. Fibrin formation

The main role of thrombin in coagulation is the cleavage of soluble fibrinogen to form insoluble fibrin, the basis of the haemostatic clot. Fibrinogen cleavage is an orderly process comprising of two distinct steps, both catalysed by thrombin (Mosesson, 1998). The fibrinogen molecule is made up of a central "E" domain, attached to two "D" domains, one on either side. In the first step of fibrinogen cleavage, thrombin cleaves fibrinogen at the "E" domain, releasing fibrinopeptide A (FPA) and exposing a polymerisation site, so-called EA. The exposure of this site allows relatively weak association between this site and a D domain of another fibrin molecule; the molecules are then arranged in an end-to-middle, cross-linking fashion and fibrin polymerisation thus begins to occur. In the second step, thrombin cleaves the E domain again, releasing fibrinopeptide B (FPB) and exposing another fibrin polymerisation site, EB. EB associates with another site on D domains of other molecules, and the cross-linking is strengthened (Mosesson, 1998).

Thrombin's role in fibrin formation does not quite end there, however. The fibrin matrix is strengthened by more cross-linking by factor XIII, a transglutaminase generated by thrombin formed after the formation of the fibrin clot, which cross-links the fibrils, thus stabilizing the clot (Dahlbäck, 2000).

4. Positive feedback

Once the coagulation cascade is initiated and thrombin is generated, the initial stimulus is often "turned off", and the cascade is maintained by the feedback effects of thrombin. This stimulatory feedback comes in the form of activation of factors V, VIII and XI (Narayanan, 1999). Factor V, when activated, associates with activated factor X and cleaves prothrombin to thrombin. Factor VIII, when activated, associates with activated factor IX and activates factor X. Factor XI, when activated, activates factor IX. In this way, the cascade is up-regulated, and a very large amount of product (i.e. fibrin) can be formed from a relatively

small initial stimulus. This role of thrombin makes it an extremely powerful procoagulant and causes it to be the target of many anticoagulant drug therapies.

To complement its role in fibrin clot formation, thrombin also plays a part in inhibition of lysis of that clot (Broze, 1996). This action is carried out by a plasma carboxypeptidase enzyme, which circulates in the blood as an inactive proenzyme and is activated by thrombin. This enzyme, termed "thrombin activatable fibrinolysis inhibitor" (TFPI) inhibits fibrinolysis by cleavage of carboxy-terminal lysine residues on the fibrin polymers (Tilburg, Rosendaal & Bertina, 2000). These residues are important in assembling components of the fibrinolytic system, so their removal inhibits fibrinolysis.

5. Inhibitory feedback

Thrombin regulates its own production by being part of an inhibitory system. This is achieved via binding to a vascular endothelial cell protein called thrombomodulin. This leads to activation of protein C (causing inactivation of coagulation factors V and VIII and thus down-regulation of thrombin generation), and inhibition of thrombin's ability to form fibrin and activate factor XIII, platelets and coagulation feedback stimulatory proteins (Esmon, 2000).

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The protein C system of thrombin inhibition is extremely powerful under normal circumstances, and so must be confined to the site of injury. This confinement is achieved by the necessity of binding to thrombomodulin, which is expressed on damaged endothelial cell walls.

As well as inhibiting fibrinolysis, as seen earlier, thrombin also has indirect effects that are stimulatory for this phenomenon. Thrombin is a chemoattractant for neutrophils, which play a role in degradation of the fibrin clot (Sonne, 1988) and thrombin releases plasminogen activators from endothelial cells which generate plasmin. Plasmin then initiates fibrinolysis and inactivates prothrombin (Fenton, et al, 1998). This apparent anomaly, that thrombin plays a role in both enhancement and inhibition of fibrinolysis, may seem confusing but is an example of the high degree of regulation which is integral to maintaining the fine balance of haemostasis. This regulation must be extremely rigorous in order to prevent the catastrophic consequences of both excessive haemorrhage and excessive coagulation. Thrombin is central to this regulation.

6. Thrombin and Platelets

Supplementary to its role in coagulation, thrombin also plays a vital role in primary haemostasis, as an extremely potent activator of platelets. This thrombin-induced platelet activation is critical for adequate haemostasis (Hung, et al, 1992). Platelet activation by thrombin, as by any other platelet agonist, results in intra-platelet events such as activation of phospholipase C, inhibition of adenylate cyclase, and mobilisation of calcium, culminating in platelet aggregation (Hayes, et al, 1994). This activation is initiated by interaction of the thrombin molecule with a receptor on the surface of the platelet, with subsequent activation of various secondary messenger systems, of which the inositol triphosphate/calcium system is probably the most important (Harrison, 2000). The interaction of thrombin with the platelet receptor has been extensively studied. The receptor, a member of the seven-transmembrane domain family, has been cloned (Hung, et al, 1992). The interaction of these two molecules is interesting because the kinetics of the reaction suggest that what actually occurs is not simple ligand-receptor interaction, but something more akin to an enzyme-substrate interaction where thrombin enzymatically cleaves the receptor/substrate (Hayes, et al, 1994). The amino terminal extracellular domain of the receptor contains a cleavage site for thrombin, which is structurally similar to the anticoagulant hirudin and is therefore able to bind thrombin (Liu, et al, 1994). Thrombin cleaves this site between Arg 41 and Ser 42 exposing a new amino terminal domain, which acts as a ligand for the receptor itself - termed a "tethered ligand" (Liu, et al, 1994).

The role of thrombin in platelet activation is not an isolated one, but is closely associated with thrombin's other roles in coagulation, particularly fibrin formation. As platelet aggregation and coagulation go hand-in-hand during bleeding, it is fitting that the molecule that is central to and regulates one system also regulates the other. Whenever fibrin formation is required to achieve haemostasis, platelet aggregation will also be required, and vice versa.

7. Thrombin and Inflammation

Thrombin has various actions in inflammation, a few of which will be discussed. Firstly, thrombin is a chemoattractant for neutrophils (Esmon, 2000) and monocytes (Becker, et al, 1998), that is it induces the cells to move down a chemical gradient to where the thrombin is most concentrated, i.e. the site of injury. This allows the neutrophils and monocytes to carry out their phagocytic role if there is invading bacteria present.

Thrombin stimulates the production from Weibel-Paladi bodies in endothelial cells, of the cell-anchoring protein P-selectin, which is then expressed on their membrane (Esmon, 2000). This molecule is important in the process of leucocyte "rolling", in which leucocytes are loosely bound to the vessel wall and therefore begin to slow down their flow rate, and roll along the endothelium, eventually stopping where they are required. Thrombin also stimulates endothelial cells to produce platelet activating factor (PAF). Although not implied in its name, PAF is a potent activator of neutrophils especially those bound to P-selectin.

In addition to P-selectin, thrombin induces the production of other pro-inflammatory and pro-coagulant substances from endothelial cells including von Willebrand factor, growth factors and cytokines, and induces changes in the endothelial cell itself including shape change and increased permeability (Coughlin 1999).

Thrombin also acts on monocytes to induce production of proinflammatory cytokines such as interleukin 6 (IL6) and IL8, and on endothelial cells to produce other inflammatory cytokines (Cate, 2000). These cytokines, as well as initiating an inflammation response, have been shown to induce thrombin production by mononuclear cells, and thus induce a coagulation response also (Cate, 2000).

In this way, the processes of coagulation and inflammation are linked, with thrombin at the centre of the interaction between the two.

8. Other Roles of Thrombin

As mentioned previously, it has been suggested that thrombin affects almost all cell types in some way, so it is not difficult to imagine that there are more roles of thrombin in the body than we are currently aware of. It has been suggested recently that thrombin plays a part in nervous development, and as such in the pathophysiology of Alzheimer's disease (Turgeon & Houenou, 1997). Alzheimer's disease pathology centres around vascular and cerebral plaques which are composed largely of a protein called amyloid beta protein. Thrombin has been shown to cause the secretion from endothelial cells of the precursor to this protein - amyloid precursor protein (APP) (Ciallella, et al, 1999) and so has been implicated in Alzheimer's disease pathology.

Thrombin causes cell proliferation in a number of cell types including smooth muscle cells and macrophages. This function of thrombin causes it to be implicated in the disease process of atherosclerosis and the build-up of the atherosclerotic plaque which contains many macrophages and smooth muscle cells (Becker, et al, 1998).

9. Conclusion

The biological roles of thrombin are diverse. It is evident that thrombin is a molecule with an extremely wide range of biological roles, and an extremely high amount of regulation associated with it due to its potency as a pro-coagulant and pro-inflammatory mediator. This essay described the main roles of thrombin, and in particular those which interest the haematologist. New functions of thrombin are being discovered at a rapid rate and research is continuing into how we can use and control those functions to our advantage, for example with the development of thrombin-specific anticoagulants such as hirudin.

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MAASTRO, Maastricht Radiation Oncology, is a co-operation between MAASTRO clinic, the University of Maastricht (UM) and the University Hospital Maastricht (azM) (see www.maastro.nl). MAASTRO consists of several division, including Maastro Clinic, which offers state-of-the-art radiotherapy to more than 3500 cancer patients each year from the Mid and South Limburg area in the Netherlands. MAASTRO clinic is also world-wide reference centre for Siemens Medical. In addition, research and training at Maastro is carried out in Maastro Physics, Maastro Trials, Maastro School, and Maastro Lab.

MAASTRO Lab is a basic and translational research laboratory embedded within the GROW research institute of the Faculty of Health, Medicine and Life Sciences at Maastricht University. Research carried out in the past has been focused on the tumour microenvironment and EGFR signalling pathways, both of relevance to radiation oncology. MAASTRO Lab has made several important discoveries in these fields, including demonstration that EGFR is up regulated by radiation and that hypoxia inhibits the initiation step of mRNA translation. In addition, we have initiated translational and clinical studies based on these results including both phase I novel treatment and molecular imaging trials as well as a Biobank project with more then 1500 patients included.

The lab has 4 permanent scientists, 5 technicians, more then 5 PhD students and is fully equipped for cell culture, molecular biology, flow cytometry, hypoxia, gene expression, proteomics and microscopy. Maastro lab has set up the necessary infrastructure for controlled exposures to hypoxia and hypoxia/reoxygenation, including development of novel equipment that allows rapid and precise changes in oxygenation. Access to expertise, equipment and resources within the much larger GROW research institute and other facilities in the University are also readily available, including the genome centre, advanced microscopy, and the animal facility with its imaging facility (Optical imager, MRI 7Tesla and micro CTPET to come). MAASTRO has a structural collaboration with the VU in Amsterdam on molecular PET biomarkers, with the TU/Eindhoven on Systems Biology and is initiating a new collaboration with the University of Toronto on research related to the Unfolded Protein Response and tumour hypoxia.

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