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INSTRUCTIONS FOR AUTHORS

All submitted manuscripts should contain original research not previously published and not under consideration for publication elsewhere. Papers may come from any country but must be written in English or Arabic with two abstracts, one in each language.

Research Paper: We encourage research paper of a length not exceeding 25 double-spaced pages. It should have a set of keywords (up to 6) and an abstract (under 250 words, unreferenced), followed by Introduction, Materials and Methods, Results, Discussion, Acknowledgments, and References.

Short Research Communication: It presents a concise study, or timely and novel research finding that might be less substantial than a research paper. The manuscript length is limited to 10 double-spaced pages (excluding references and abstract). It should have a set of keywords and an abstract (under 200 words, unreferenced), containing background of the work, the results and their implications. Results and Discussion Section should be combined followed by Conclusion. Materials and Methods will remain as a separate section. The number of references is limited to 60 and there should be no more than 4 figures and/or tables combined.

Reviews or mini-reviews should be authoritative and of broad interest. Authors wishing to contribute a manuscript to this category should contact the Editor-in-Chief. Reviews should describe current status of a specific research field with a balanced view. The length of the review manuscript should not exceed 50 double-spaced pages. Mini-reviews should not contain an exhaustive review of an area, but rather a focused, brief treatment of a contemporary development or issue in a single area. The length of the mini-review should not exceed 6 printed pages.

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The accepted papers for publication shall be published according to the final date of acceptance. The editorial board reserves the right to reject a paper for publication without justification.

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Organization of Manuscript:

Manuscripts should be typewritten and double spaced throughout on one side of white typewriting paper with 2.5 cm margins on all sides. Abstract, tables, and figure legends should be on separate sheets. All manuscript sheets must be numbered successively. Authors should submit three copies of the paper and a floppy diskette 3.5 or a CD under (Winword IBM) or by e-mail.

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The title page of manuscript should contain title, author's names of authors and their affiliations, a short title, and the name and address of correspondence author including telephone number, fax number, and e-mail address, if available. Authors with different affiliations should be identified by the use of the same superscript on name and affiliation. In addition, a sub- field of submitted papers may be indicated on the top right corner of the title page.

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The abstract should provide a clear and succinct statement of the findings and thrusts of the manuscript. The abstract should be intelligible in itself, written in complete sentences. Since JJBS is an interdisciplinary journal, it is important that the abstract be written in a manner which will make it intelligible to biologists in all fields. Authors should avoid non-standard abbreviations, unfamiliar terms and symbols. References cannot be cited in the Abstract.

Authors should submit with their paper two abstracts (English and Arabic), one in the language of the paper and it should be typed at the beginning of the paper before the introduction. As for the other abstract, it should be typed at the end of the paper on a separate sheet. Each abstract should not contain more than 250 words. The editorial board will provide a translation of abstract in Arabic language for non-Arabic speaking authors.

Introduction:

This section should describe the objectives of the study and provide sufficient background information to make it clear why the study was undertaken. Lengthy reviews of the past literature are discouraged.

Materials and Methods:

This section should provide the reader with sufficient information that will make it possible to repeat the work. For modification of published methodology, only the modification needs to be described with reference to the source of the method. Information regarding statistical analysis of the data should be included.

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This section should provide hard core data obtained. Same data/information given in a Table must not be repeated in a Figure, or vice versa. It is not acceptable to repeat extensively the numbers from Tables in the text and give long explanations of the Tables and Figures. The results should be presented succinctly and completely.

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Acknowledgements:

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a. The reference is indicated in the text by the name of authors and year of publication between two brackets.
   Example: (Shao and Barker, 2007).

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Embabi NS. 1990. Environmental aspects of distribution of mangrove in the United Arab Emirates. Proceedings of the First ASWAS Conference. University of the United Arab Emirates. Al-Ain, United Arab Emirates.

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Illustrations should be termed "Figures" (not "plates", even if they cover an entire page) and labeled with numbers. All figures should be referred to in the text and numbered consecutively in Arabic numerals (Fig. 1, Fig. 2, etc.). Scales in line drawings must be mounted parallel to either the top or side of the figures. In planning illustrations, authors should keep the size of the printed page in mind, making due allowance for the figure legend. The figures must be identified on the reverse side with the author's name, the figure number, and the orientation of the figure (top and bottom). The preferred location of the figures should be indicated on the margin of the manuscript. Illustrations in color may be published at the author's expense. The legends for several figures may be typed on the same page. Sufficient details should be given in the legend to make it intelligible without reference to the text.

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Jordan Journal of Biological Sciences

EDITORIAL PREFACE

It is my great pleasure to publish the first issue of Volume two of the Jordan Journal of Biological Sciences (JJBS). JJBS is a refereed, peer reviewed quarterly international journal issued by the Jordanian Ministry of Higher Education and Scientific Research in cooperation with the Hashemite University. The journal covers a wide range of research and development concerning biological sciences. Through the publication, we hope to establish and provide an international platform for information exchange in different fields of biological sciences.

Jordan Journal of Biological Sciences aims to provide a highly readable and valuable addition to the literature, which will serve as an indispensable reference tool for years to come. The coverage of the journal includes all new findings in all aspects of biological sciences and or any closely related fields. The journal also encourages the submission of critical review articles covering advances in recent research of such fields as well as technical notes.

The Editorial Board is very committed to build the Journal as one of the leading international journals in biological sciences in the next few years. With the support of the Ministry of Higher Education and Scientific Research and Jordanian Universities, it is expected that a valuable resource to be channeled into the Journal to establish its international reputation.

I have received a good response to this issue of JJBS from biologists in Jordanian universities. I am pleased by this response and proud to report that JJBS is achieving its mission of promoting research and applications in biological sciences. In this issue, there are Seven interesting papers dealing with various aspects of biological sciences.

JJBS will bring you top quality research papers from an international body of contributors and a team of distinguished editors from the world's leading institutions engaged in all aspects of biological sciences. Now, the JJBS invites contributions from the entire international research community. The new journal will continue to deliver up to date research to a wide range of biological sciences professionals. The JJBS will assure that rapid turnaround and publication of manuscripts will occur within three to six months after submission.

I would like to thank all members of the editorial board and the international advisory board members for their continued support to JJBS with their highly valuable advice. Additionally, I would like to thank the manuscript reviewers for providing valuable comments and suggestions to the authors that helped greatly in improving the quality of the papers. My sincere appreciation goes to all authors and readers of JJBS for their excellent support and timely contribution to this journal.

I would be delighted if the JJBS could deliver valuable and interesting information to the worldwide community of biological sciences. Your cooperation and contribution would be highly appreciated. More information about the JJBS guidelines for preparing and submitting papers may be obtained from www. jjbs.hu.edu.jo

Prof. Naim S. Ismail Editor-
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Heavy Metals and Macroinvertebrate Communities in Bottom Sediment of Ekpan Creek, Warri, Nigeria.

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Abstract

The macrobenthic fauna in bottom sediments of Ekpan Creek was studied from January to June 2007. Analyzed heavy metals were Lead, Iron, Zinc, Copper, and Chromium. Variations in chemical parameters showed that station 2 had the highest values recorded in all parameters except for Iron and Zinc, where they were higher at station 1. A total of 1135 individual organisms were recorded. Nineteen (19) macroinvertebrate taxa belonging to four major groups were identified. Mollusca were the most dominant and constituted 92.51% density occurrence, while insecta, crustacean, and polychaeta constituted 1.94, 2.29, and 3.26% respectively. Diversity varied at the study stations, with the highest taxa richness recorded at station 1. Mollusca were positively significantly correlated with lead ($P < 0.05$, $r = 0.836$), and Zinc ($P < 0.05$, $r = 0.96$). Sorenson index indicates similarity in species composition between the stations.

التلخيص

تمت دراسة الحيوانات القاعية الموجودة في خور اكبان في الفترة ما بين كانون ثاني وحزيران 2007. وتم كذلك دراسة تراكيز المعادن الثقيلة وتشمل الرصاص والحديد والزنك والنحاس والكروميوم. وتوضح التحاليل الكيميائية ان المحطة رقم 2 فيها أعلى النسب من المعادن باستثناء الحديد والزنك حيث كان أعلى ما يمكن في المحطة رقم 1. وقد تم جمع 1135 حيوانا في تلك الفترة تنتمي الى 19 نوعا من الحيوانات القاعية في اربع مجموعات وهي الرخويات وتشمل 92.51%. بينما تشمل الحشرات والقشريات وعديدة الاشواك 1.94%و2.29%و 3.26% على الترتيب. اختلف مقياس التنوع بين المحطات وكان اعلاها في المحطة رقم 1. وتبين من مؤشر سورسن ان التشابه في الانواع بين المحطات كبير.

Keywords：Macrobenthic Fauna; Sediment; Heavy Metals; Diversity Creek; Nigeria

1. Introduction

Benthic studies of the brackish aquatic environment in Nigeria have been very scanty. The difficult terrain of the creeks, creeklets, and estuaries has restrained many ecologists from the survey of Nigerian coastal areas. Olomukoro and Egborge(2003) investigated the macrobenthic fauna of Warri River, and reported an array of benthic organisms in their preliminary publication of the fresh / brackish zones of the river catchment area. A total of 138 macroinvertebrate taxa were reported from the River, among the species collected are *Diplogaster* sp*, Naidium bilongata, Nais obstuse, Nais Simplex, Placobdella monifera, Megapus* sp., *Mediopsis* sp., *Baetis bicaudatus,* and *Procladius* sp. The most dominant benthic groups were Decapoda, Ephemeraoptera, Diptera, and Mollusca.

Other notable works of interest were those of Olomukoro and Victor(2001) on a tributary of Ikpoba River, Hart and Chindah (1998), who identified 43 species of benthos from the mangrove forest of the Bonny estuary, Egborge and Okoi (1987) who reported on the biology of a community swamp farm in Odetsekiri, Warri and Victor and Onomivbori (1996) on the effects of urban perturbation on the benthic macroinvetebrates of a southern Nigerian stream.

The structure of benthic communities in running water ecosystem is determined by a dynamic array of abiotic and biotic factors (Kumar, 1995, Austen and Widdicombe 2006). Wharfe (1977) observed that the finer clay/ silk particles had a higher water retaining capacities (42 to 66% water content) compared with more coarse sediments (28 to 49% content). The water retaining capacity can be important for burrowing invertebrates during periods of exposure. Olomukoro and Egborge, 2003 reported that species of polychaeta were restricted to a particular station because their occurrence may be governed by niche preference and feeding habit. Polychaeta are also known to be tolerant to silting and velocity of flow, than most groups of benthic organisms (Bishop, 1973), as they are deposit feeders; and live in the mud.

However, outstanding investigations, which deal with the bottom fauna of some rivers in Southern Nigeria, have also been studied (Victor and Dickson, 1985; Victor and Ogbeibu, 1985; 1991; Olomukoro and Ezemonye, 2000; Olomukoro, Ezemonye and Igbinosun, 2004).

Investigation of macrobenthic fauna of Ekpan-Creek Warri has not been carried out.

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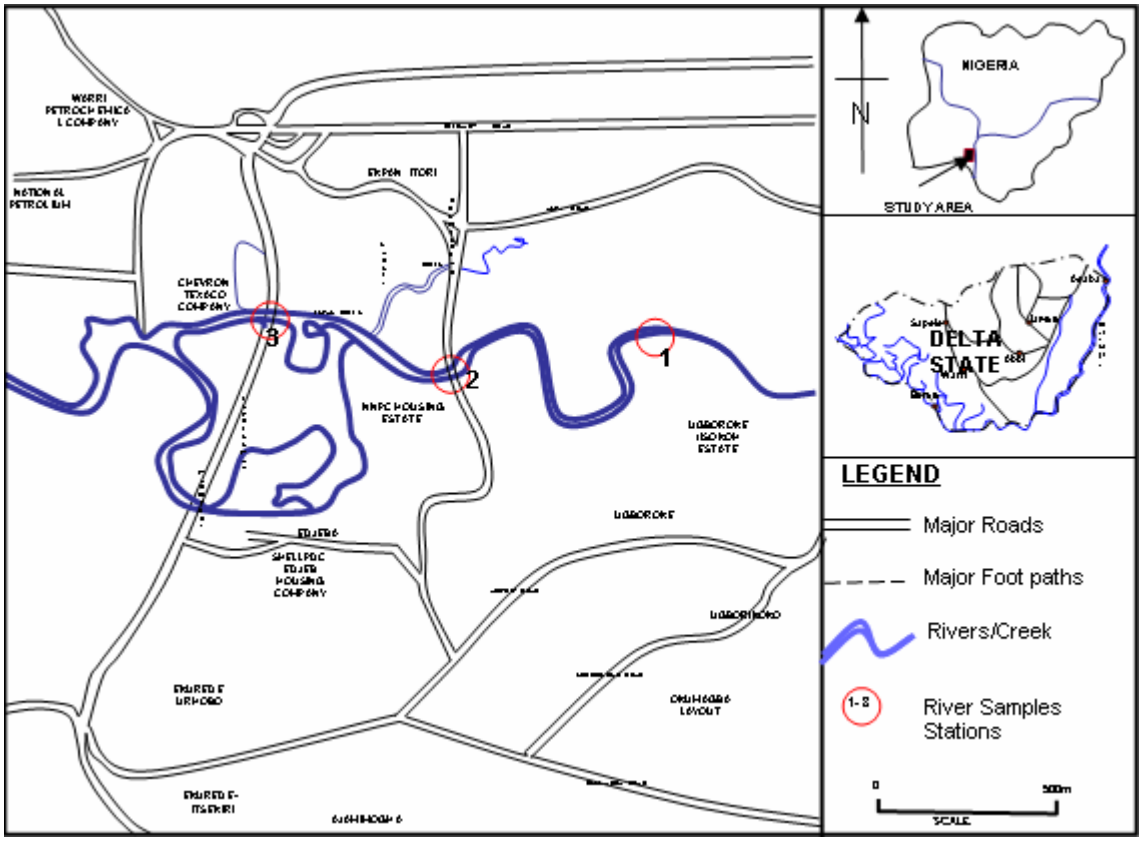

Figure1. Map of Warri Showing the Sampled Stations.

The bottom sediment fauna study is to provide baseline information on which subsequent works would be based. The objectives are to examine the composition, abundance, diversity of benthos, and the heavy metals of the bottom sediment of Ekpan Creek, Warri.

2. Study Area

Ekpan Creek is located within Effurun-Warri of Delta State in Southern Nigeria (Latitude $5^{0}3^{011}$-$5^{0}3^{011}$ and longitude $5^{0}40^{11}$- $5^{0}44^{11}$E). The Creek, which is about 12km long, takes its source from Effurun. It flows through the city (westernly) into Tori Creek at NNPC Jetty, and empties into Warri River at Bennet Island.

a. Sampling Location

Three sampling stations were chosen (Figure1) for their proximity to facilities, structures or human activities that could potentially affect water quality and biodiversity. Station I is located close to the creek source. Water depth is 2.20 ± 3.74m, and the velocity of flow (1.07 ± 0.46m/S) is minimal and the bank is flanked with red mangrove, *(Rhizophora racemosa*), plantain tree (*Musa* sp.) and some shrubs. This water is murky and turbid, and the substratum is made of clay and mud. Human activities include fishing, bathing, and laundry.

Station II is about 4km from station one. It is located at the bridge, close to NNPC housing complex. Water depth is 4.10 ± 7.72, and the flow rate (1.49 ± 0.11m/S) is faster than station I. The substratum is a combination of sand, silt, and clay. Marginal vegetation consists of *Rhizophora recemosa* (red mangrove), few grasses. Human activities include fishing, and the use of the water for construction.

Station III is located at the Chevron-Texaco company bridge site, 5km from station II. The substratum is a mixture of sand and silt. Water depth is 6.50 ± 8.46m/S, and the velocity of flow is very fast, about 1.48 ± 0.14m/S. Oil film dots the water surface.

3. Material and Methods

a. Sampling Techniques

Benthic samples were collected fortnightly from the study stations from January to June 2007, using an Ekpan grab operated by hand in shallow water. It is recommended for sand and silt (Hynes, 1971). A 6-inch Ekman grab was forced into the substratum to depth of 15-20cm. Contents trapped by the grab were processed using the technique, earlier described by Hynes 1971. Sieved and sorted organisms were preserved in 4% formalin. Bottom sediment samples were collected with grab at monthly intervals in polythene bags for heavy metals analysis.

b. Digestion and Sediment Analysis

Sediment samples were digested, using the nitric acid, perchloric acid method (APHA 1997). An atomic absorption spectrophotometer (model pycunicam sp. 2900) was also used for the analysis of lead, Iron, Zinc, copper and Chromium. Identification of organisms was possible by using appropriate keys and works of Mellanby, 1963; Needham & Needham, 1962; Pennak 1978, and Olomukoro, 1996.

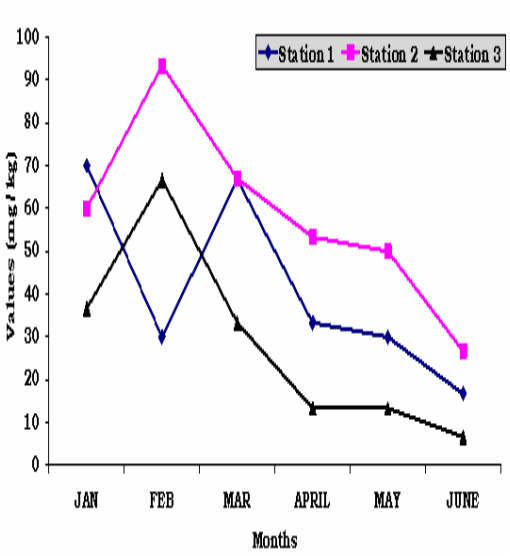

Figure2. Monthly variation in iron concentrations in Ekpan Creek.

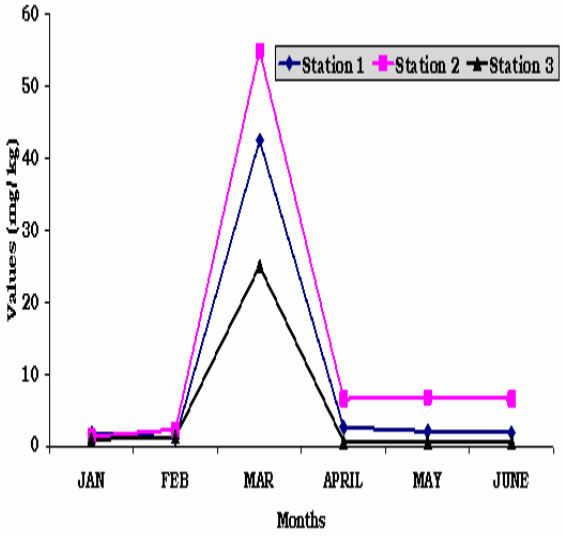

Figure 3. Monthly variation in lead concentrations in Ekpan Creek.

c. Statistical Analysis

Biological indices, Margalef's index (d); Shannon–Weiner index (H), and Evenness E were used in the calculation of taxa richness, general diversity. and evenness (Green, 1971 and Robinson and Robinson, 1971). The faunal of the stations were compared. using Sorenson's quotient (Sorenson 1948) of similarities. Both, the correlation coefficient of chemical variables and benthic organisms were computed. ANOVA DUNCA combined test was used in calculating the mean value of the heavy metals of the stations. One way Analysis of variance and Pearson's correlation coefficient were used in the statistical analysis of chemical variables at 5% level of significance.

4. Results

a. SEDIMENT

The summary of the heavy metals concentration values of the sediment of Ekpan creek study stations is presented in Table 1.

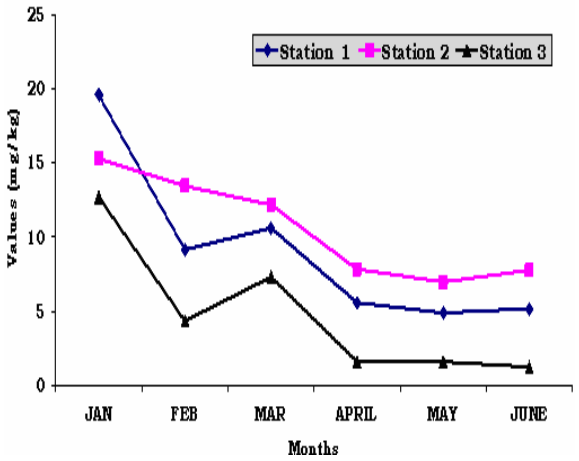

Figure 4.Monthly variation in zinc concentrations in Ekpan Creek.

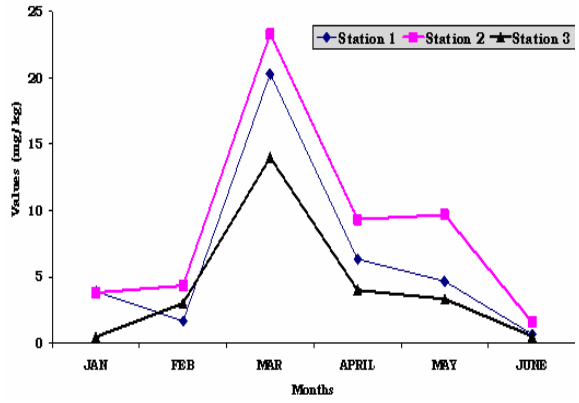

Figure 5.Monthly variation in copper concentrations in Ekpan Creek.

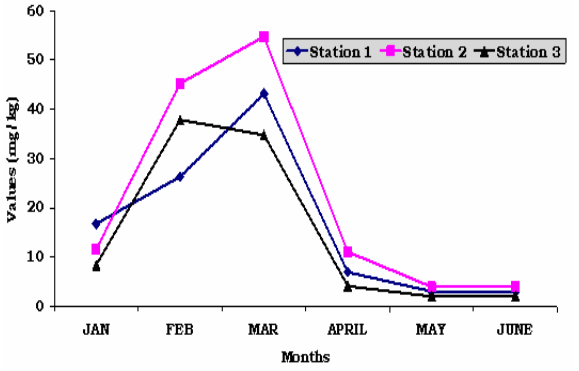

Figure 6. Monthly variation in chromium concentrations in Ekpan Creek.

Iron and Zinc concentration values were higher in January at stations II and I than in station III (Figs 2 and 3). The values of these metals ranged from 6.67 to 93.33 mg/kg and 1.21 to 19.60mg/kg in the stations.

However, the increases in Lead (ranged from 0.59 to 55.00mg/kg), Copper (0.47 to 23.30mg/kg) and Chromium (02.02 to 54.74mg/kg) concentration values were high in the month of March in all the stations, but very low in May, particularly at station III (Figs. 4, 5 and 6)

b. Macrobenthic Invertebrate Fauna

A total of 19 macro invertebrate taxa comprising 1,135 individuals include 3 species of polychaeta, 1 species of Decapoda, 3 species of Diptera, 1 species of Lepidoptera and 11 species of Mollusca.

Table 1. The summary of chemical parameters in the three study stations of Ekpan Creek.

			STATION 1		STATION 2		STATION 3	
Parameter	**Units**	N	Mean ± S. E	Min - Max	Mean ± S. E	Min – Max	Mean ± S. E	Min – Max
Lead	mg/kg	6	8.52 ± 6.79	– 42.50	13.09 ± 8.44	1.30 – 55.00	4.85 ± 4.03	0.59 – 25.00
Iron	mg/kg	6	41.11 ± 8.93	16.67 – 70.00	58.39 ± 8.94	26.67 – 93.33	28.33 ± 9.10	6.67 – 66.67
Zinc	mg/kg	6	9.16 ± 2.29	4.87 – 19.60	10.58 ± 1.43	6.96 – 15.30	4.78 ± 1.85	1.21 – 12.70
Copper	mg/kg	6	6.26 ± 2.93	0.69 – 20.30	8.67 ± 3..21	1.59 – 23.30	4.22 ± 2.05	0.47 – 14.00
Chromium	mg/kg	6	16.51 ± 6.49	2.98 – 43.16	21.79 ± 9.10	4.00 – 54.74	14.89 ± 6.86	2.02 – 37.89

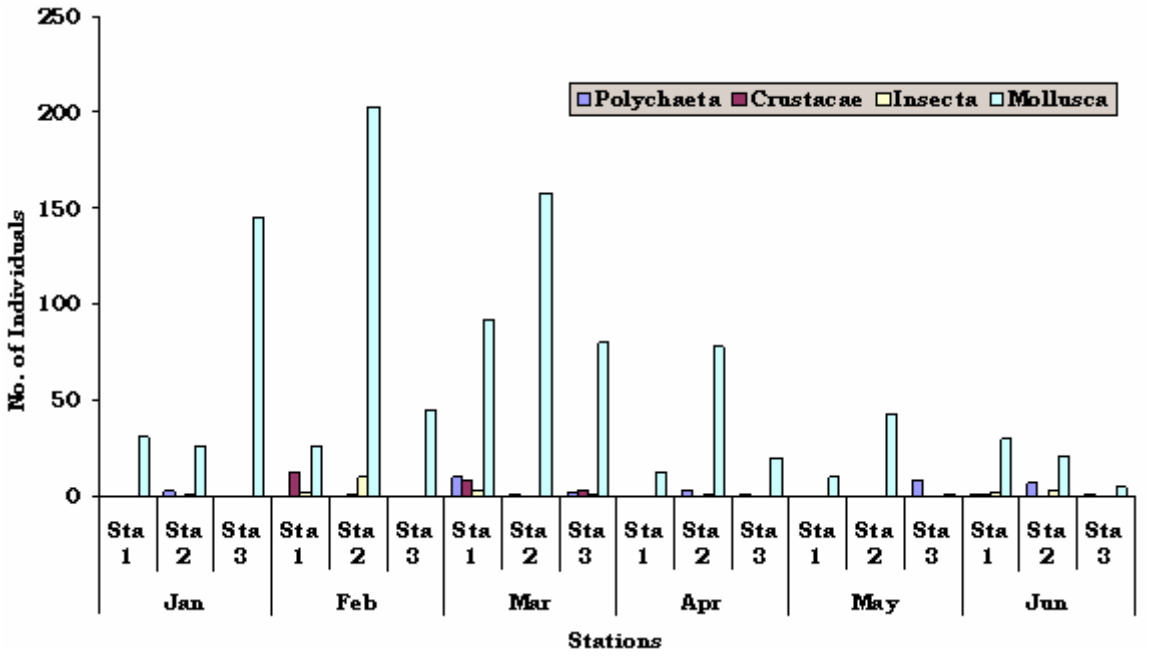

Figure 7. Monthly variation in the distribution of macrobenthic invertebrate groups.

The overall taxa composition, distribution, and abundance of macrobenthic invertebrates collected during the study period are presented in Table 2. Individuals organisms were dominated by Mollusca, which constituted 92.51% density occurrence, while polychaeta, crustacea and insecta made up of 3.26, 2.29 and 1.94% respectively Table 3. as also found in station 2.

Ampullariidae (Pilidae) had Pila ovata at station 3 in the month of February as a single record.

Assimineidae was represented by the *Assiminea hessei*

Polychaeta was represented by *Namanereis hawaiiensis, Lycastopsi* sp and *Nereis* sp. This was represented in the months of March, June, January, April, and May in all the stations (Figure 7)

Potamalpheops monodi (Apheidae, Crustacea) occurred in all stations with highest occurrence in station I, and it was recorded in February, March, and June (Figure 7).

Chironomidae (Diptera) was represented by *Chironomus* sp. *Tanytarsus* sp. and *Tanypus* sp., and were recorded in all stations in February, March, April, and June.

A single Lepidopteran larva was found in the month of February in station 2.

Gastropoda recorded in the Creek consists of five families viz Neritidae, Hydrobiidae., Potamididae, Pilidae, and Assimineidae. Neritidae was represented by *Neritina glabrata* and *Nerita senegalensis*, and was recorded in all stations throughout the sampling period (Figure 7).

Hydrobiidae has its highest occurrence in station 2, although it was recorded in all the sampling stations in February and March. Representatives of this group are *Hydrobia* sp., *Potamopyrgus* sp. and *Argyronecta aquatica* (Figure7). Potamididae (*Tympanotonus* sp) w in station 3 in the month of March.

Lamelibranchia (Ancylidae) was represented by *Macoma cumana*. It occurred in all stations in the months of January, February, and May (Figure 7).

i. Diversity

Figure 8 shows taxa richness (D), Shanon – Weinner's index (H), and Evenness (E) estimated for the study stations. The highest taxa richness was recorded in station 1, while the least was recorded in station 3.

Figure 9 shows spatial and temporal variation in species richness of benthic macroinvertebrates in the study area. The highest general diversity was recorded in station 3 while the least in Station 2. Also the highest evenness was recorded in station 3, and the least was in station 2.

The faunal similarity showed that all the three stations were similar in species composition, but the highest similarity was between stations II and I, and the lowest was between III and II (Table 4).

ii. Correlation Coefficient Analyses

The correlation coefficient analyses of Mollusca variables and chemical parameters of station 1 were computed. Mollusca were inversely insignificantly correlated with chromium, but positively insignificantly correlated with copper. However, they were positively significantly correlated with lead ($P < 0.05$, $r = 0.836$), and Zinc ($P < 0.05$, $r = 0.96$) but inversely significantly correlated with iron ($P < 0.05$, $r = -0.58$).

Table 2. Composition, Distribution and Abundance of Macrobenthic Invertebrates in Ekpan Creek, Warri January – June 2007.

	STATION 1	STATION 2	STATION 3
POLYCHAETA			
Namanereis hawaiiensis		1	
Lycastopsi sp	1	2	
Nereis sp	10	11	
DECAPODA			
Potamalpheops monody	22	1	3
DIPTERA			
Chironomus sp	3	2	
Tanytarsus sp	2		
Tanypus sp	2	10	1
LEPIDOPTERA			
Lepidopteran larvae		2	
ARCHAEOGASTROPODA			
Neritina glabrata	14	69	72
Nerita senegalensis	5	20	18
MESOGASTROPODA			
Hydrobia sp	4		
Potamopyrgus sp		1	
Potamopyrgus ciliatus	87	103	22
Argyronecta aquatica			1
Tympanotonus furscatus radula	138	271	85
Tympanotonus furscatus furscatus	5	64	36
Pila ovata			1
Assiminea hessei			2
Macoma cumana	9	2	21
Total	**302**	**559**	**274**

5. Discussion

The high heavy metals concentration in the bottom sediment of Ekpan creek confirms the previous reports on some aquatic studies (Gibbs, 1977; Ezemonye 1992). Metal enrichment of sediment is reflected by the sedimentation of metals ions when they compete with H^+ ions sorption sites in the aquatic environment (Oguzie, 2002). The physical process in the area could help the release of solutions rich in heavy metals into the bottom sediment of the creek, similar to what was reported for Canadian waters by Sly (1977). These metals, according to Edginton and Callender (1970) and Choa (1977), have high content of detrital mineral bonds and forms complexes that's precipitate at river bottom.

The high concentration of iron in the sediment might suggest the influx of industrial effluents. The observed higher concentration of heavy metals such as iron, zinc, and chromium in the sediment at the stations during the dry season than the rainy season shows the possible dilution effects by run-off. Thus there was a clear pattern of seasonal variation. However, lead and copper did not reflect the two seasons; they fluctuated throughout the duration of the study.

Table 3. Percentage abundance of species and individuals of the major groups.

Groups	Taxa	% Taxa	Individual	% Individual
Polychaeta	3	15.79	37	3.26
Crustacean	1	5.26	26	2.29
Insecta	4	21.05	22	1.94
Mollusca	11	57.89	1050	92.51

Macrobenthic fauna of Ekpan creek appear to be unique in its community structure. A total number of 1, 135 individuals, belonging to four major groups of organisms, included polychaeta (3 taxa), crustaceans (1 taxon), insecta (4 taxa) and Mollusca (11 taxa). The low number of taxa recorded is not surprising. According to Victor and Victor (1992), the number of taxa in brackish waters has been known to be fewer than that of freshwater and marine habitat. The configuration of immediate substrate of occupation, both as a refuge and more critically, as a source of food, is often the paramount factor governing distribution of macroinvertebrate fauna, and the bottom sediment of aquatic ecosystems are known to serve as shelter for macrobenthic invertebrates and direct or indirect food source for detritus and grazers (Bishop, 1973).

Table 4. Faunal similarities in the study stations of Ekpan Creek, Warri.

	STATION I	STATION II	STATION III
STATION I	*	81.48	72.00
STATION II	81.48	*	69.23
STATION III	72.00	69.23	*

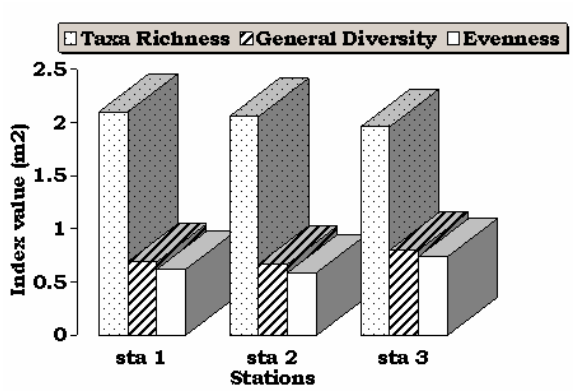

Figure 8. *Diversity of macrobenthic invertebrates in the study stations.*

Recorded Polychaetes, except for Nereis sp, were restricted to station 1 and 11, which have muddy bottom compared to the more sandy station III. The restriction of these species is known to be associated with muddy substratum rich in organic matter (Carter, 1981). They are also deposit feeders and are known to be tolerant to silting and velocity of flow than most groups of benthic organisms (Bishop, 1973).

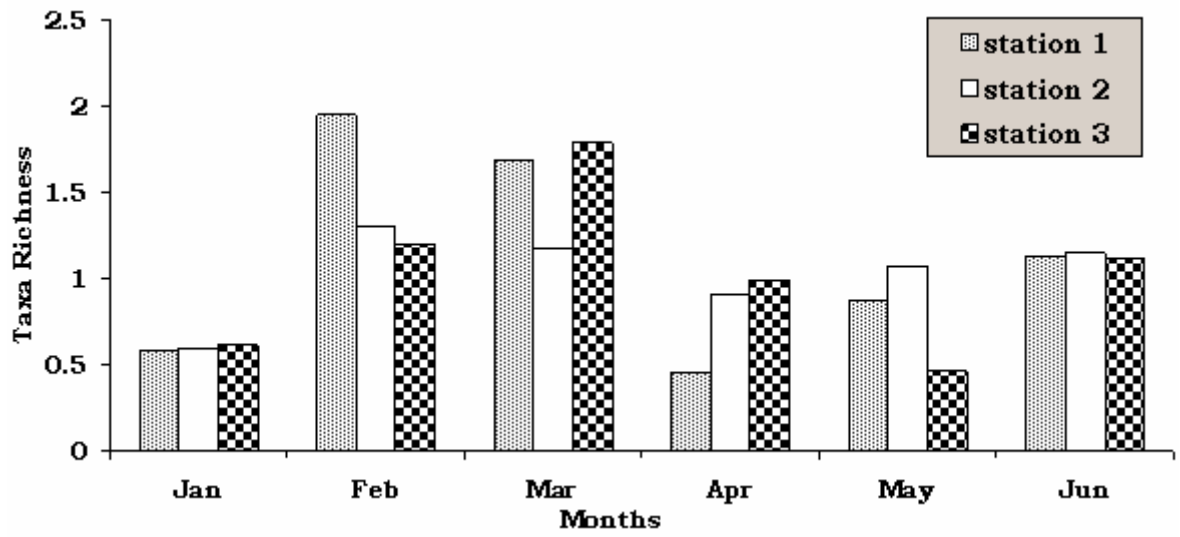

Figure 9. Spatial and temporal variation in species richness of benthic macroinvertebrate in the study area.

High abundance and distribution of mollusc in Ekpan creek could be attributed to the level of pH in the creek. Slight decrease in acidity and the corresponding slight increase in alkalinity may account for the abundance of mollusc. Beadle (1994), has reported that acidity is one of the major factors limiting the distribution of the mollusc in water bodies and in support of this, Nwadiaro (1984), reported that the distribution of mollusc in the lower Niger Delta was limited to the neutral to slightly alkaline brackish water zone. Of all the molluscan recorded in this study, six species; *Neritina glabrata, Nerita senegalensis, Potamopyrgus ciliatus, Tympanotonus furscatus radula, Tympanotonus furscatus furscatus* and *Macoma cumana* were present in all the stations. Mollusca were positively significantly correlated with lead, and zinc.

The distribution pattern of insecta shows that they were restricted to stations 1 and 2. Awachie (1981) observed that the Insecta usually does not show habitat restrictions, but the dominance of chironomids at station 2 compared to other stations may indicate pollution stress in the station.

The crustaceans were represented by single specie, *Potamalpheops monodi*. This was mostly found at station 1 and its abundance in that station may be due to factors other than food and shelters.

The seasonal variation of the macroinvertebrates reveals that, mollusca occurred in all the months. They were more abundant in the months of January to March (dry season) compared to April to June (rainy season). Other species occurred randomly throughout the duration of the study.

6. Conclusion

The effects of human activities on heavy metals concentration values were predominant during the dry season, and much of water dilution in the rainy season lowered the concentrations of metals. On the abundance and diversity of invertebrates, only few representative taxa of Polychaeta, Lepidoptera, Diptera, and Mollusca were recorded in the bottom sediment of the creek.

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Datura Aqueous Leaf Extract Enhances Cytotoxicity via Metabolic Oxidative Stress on Different Human Cancer Cells

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Abstract

This study was designed to evaluate the cytotoxic effect of aqueous *Datura stramonium* leaf extract on different human cancer cell lines *in vitro*. Breast (MDA-MB231), head, neck (FaDu), and lung (A549) cancer cell lines were treated with 1 mg/ mL of *Datura* aqueous extract for 24 and 48 hours. Exposure of MDA-MB231 and FaDu cells to the extract for 24 hours resulted in a significant decrease in cell survival. Same effect was seen with all cell lines exposed to the *Datura* aqueous extract for 48 hours. Treatment with *Datura* aqueous extract also caused perturbations in parameters indicative of oxidative stress, including increased glutathione disulfide (GSSG) in FaDu cells treated for 48 hours. Additionally, an increase on the redox sensitive enzymes was seen in MnSOD and HO-1 on A549 cells, treated with *Datura* aqueous extract for 24 and 48 hours. The results may suggest therapeutic potential of *Datura* aqueous leaf extract for the treatment of different types of cancer. Further investigations are needed to verify whether this cytotoxic effect occurs *in vivo*.

الملخص

صممت هذه الدراسة لتقيم مدى سمية المستخلص المائي لأوراق نبتة الداتورة استرامونيوم (*Datura stramonium*) على العديد من سلالات خلايا الإنسان السرطانية , لتحقيق هذا الهدف قمنا بتعريض خلايا سرطان الثدي (MDA-MB231), الرأس والرقبة (FaDu) وسرطان الرئة (A549) في المختبر لتركيز (1 مغم\مل) من مستخلص هذه النبتة لمدة 24 و48 ساعة.

اتضح من النتائج انه بعد تعرض خلايا MDA-MB231 وFaDu للمدة 24 ساعة انخفاض ملحوظ في عدد الخلايا الحية , وقد تم ملاحظة نفس التأثير على جميع أنواع الخلايا بعد تعرضها للمستخلص لمدة 48 ساعة.

وأسفر معالجة الخلايا السرطانية بهذا المستخلص باضطرابات في معايير جهد الأكسدة بما في ذلك زيادة نسبة الجلوتاثيون المؤكسد (glutathione disulfide (GSSG)) , بالإضافة إلى زيادة إنزيمات جهد الأكسدة مثل MnSOD, HO-1 .

تشير هذه النتائج إلى أنه يمكن إستخدام المستخلص المائي لأوراق نبتة الداتورة استرامونيوم (*Datura stramonium*) لعلاج أنواع مختلفة من السرطان في الإنسان بعد إجراء المزيد من التجارب لمعرفة حيثية عمل هذا المركب في الإنسان .

Keywords: *Datura Stramonium*; Glutathione; Mnsod; HO-1.

1. Introduction

1.1. Plant

Datura stramonium, more commonly known as jimson weed or thorn apple, is a wild-growing flowering plant belonging to the family Solanaceae and is a medicinal plant with antinociceptive (Abdollahi et al., 2003) antioxidant (Couladis et al., 2003), hypolipidemic (Rasekh et al., 2001), anti-inflammatory, anti-rheumatoid (Tariq et al., 1989), and hypoglycemic (Gharaibeh et al., 1988) properties. Therefore, this study was carried out to evaluate the therapeutic potential of the aqueous *Datura stramonium* leaf extract in the treatment of different types of cancer.

1.2. Oxidative Stress

Mammalian cells continuously produce reactive oxygen species (ROS) through various metabolic pathways. Reactive oxygen species are molecules that contain oxygen and have higher reactivity than ground-state molecular oxygen. These species include not only the oxygen radicals (like $O_2^{\bullet-}$, $^{\bullet}OH$, and peroxyl radicals), but also non-radical molecules such as H_2O_2 and $^1O_2$. Superoxide is formed during the reduction of O_2 by the mitochondrial electron transport system (Boveris and Cadenas, 1982). Eukaryotic cells are equipped with an antioxidant system capable of converting ROS to H_2O *via* different cytosolic enzymes. Oxidative stress results when the balance between the production of ROS exceeds the antioxidant capability of the target cell. It is generally thought that low levels of ROS are not harmful to cells, and indeed even perform useful signaling functions, whereas high levels of ROS are detrimental through covalent reactions with cellular proteins, lipids, and DNA that results in altered target molecule function. The accumulation of oxidative damage has been implicated in

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both acute and chronic cell injury, including possible participation in the formation of cancer. Acute oxidative injury may produce selective cell death or sublethal injury, such as mutations, chromosomal aberrations or carcinogenesis (McCord *et al*., 1971; Klaunig *et al*., 1998). In contrast, chronic oxidative injury may lead to a non-lethal modification of normal cellular growth control mechanisms. Cellular oxidative stress may modify intracellular communication, protein kinase activity, membrane structure and function, and gene expression, and it may result in modulation of cell growth (Klaunig *et al*., 1998).

Cells are protected against oxidative stress by different intracellular antioxidant compounds, mainly Glutathione (GSH) and thioredoxin, and by other antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and heme oxygenase-1 (HO-1) (Tsan, 1989; Guo *et al*., 2001).These antioxidant enzymes were shown to be up-regulated by various physical, chemical, and biological agents and oxidative stress (Tsan, 1989, Wong *et al*., 1989; Bianchi *et al*., 2002).

Little information is available on the antioxidant or pro-oxidant properties of the herbal preparations of *Datura stramonium.* The purpose of this study was to evaluate the therapeutic potential of aqueous leaf extract of this plant in the treatment of cancer *in vitro.*

2. Material and Methods

2.1. Cell Culture

Breast (MDA-MB231) cells were routinely kept in RPMI 1640 medium supplemented with 10% fetal bovine serum, and head, neck (FaDu), and lung (A549) cancer cell lines were routinely kept in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. All cells were obtained from the European Collection of Cell Cultures (ECACC) and were kept at 37 °C in a humidified 5% CO_2 incubator.

2.2. Preparations of Extract

Datura stramonium, a wild-growing flowering plant belongs to the family Solanaceae, was collected during the flowering period in August 2005 in Jordan. The leaves were separated and dried in the shade in green house for several days; and was deposited in the Herbarium of the Department of Biology at the Hashemite University. The procedure was as follows**:** Leaf part of *Datura stramonium* (150g) of dried plant was ground and the obtained powder was mixed with 1 L of boiling distilled water for 1 hour. The obtained mixture was filtered twice through a funnel by using suction pump. Water was concentrated under vacuum by using a rotary evaporator at a temperature of 50°C. The extract was evaporated under a reduced pressure till it dried by using a lyophilizer (or by using fume hood). The extract was stored in glass flasks to protect them from humidity and light. 1 mg/ mL of the extract was prepared by dilution of the stock with sterile phosphate-buffered saline (PBS) solution.

2.3. Cell Survival Experiments

Cells were plated in 60 mm tissue culture dishes at low density (300 per dish) and grown for 3 days in the presence of antibiotics (Gentamycin). At the beginning of each experiment, the cells were placed in DMEM or RPMI-1640 supplemented with 10% fetal bovine serum. Control cultures were treated identically. Cells were then treated with *Datura* aqueous extract (1 mg/mL). Cultures were then placed in an incubator. At each time point (24 and 48 hours), cells were trypsinized, counted, diluted, and plated at low density (300-1000 per plate) for clonogenic cell survival assay as previously described (Spitz *et al*., 1990). Surviving colonies were fixed and stained with Coomassie Blue stain after 14 days of incubation; and were counted under a dissecting microscope. Colonies containing 50 cells or more were scored.

2.4. Measurement of Glutathione Levels

The intracellular levels of reduced glutathione (GSH) and GSSG in cancer cells were measured. Total glutathione content was determined according to (Anderson, 1985). The total intracellular GSH was determined by the colorimetric reaction of DTNB (5, 5-dithio-*bis*- (2-nitrobenzoic acid)) with GSH to form TNB (5-thio-2-nitrobenzoic acid). The rate of formation of TNB, which is proportional to the total GSH concentration (GSH + GSSG), was measured spectrophotometrically at 412 nm. Cellular GSSG is reduced to GSH by glutathione reductase (GR), using NADPH as a cofactor. Briefly, cell pellet was lysed in 5 % 5-sulfosalicylic acid (SSA); the total GSH was measured by mixing 50 µL sample with 100 µL water, 700 µL working buffer [0.298 mM NADPH in stock solution (0.143 M sodium phosphate, 6.3 mM EDTA)], and 100 µL DTNB (6 mM DTNB in stock solution). The assay was initiated by the addition of 50 µL GR (266 U/mL), and the rate of TNB formation was followed spectrophotometrically at 412 nm, every 15 seconds for 2.5 min. The total GSH of a sample was extrapolated from a standard curve of glutathione concentration as a function of the change in absorbance over time. The cellular GSSG level was determined using the same DTNB assay when the reduced GSH is masked by 2-vinylpyridine (2-VP) (Griffith, 1980). 2-VP (2 µL of a 50% solution in EtOH per 50 µL aliquot of media) was added to 30 µL of sample for 1.5 h to block all reduced GSH, and then 30 µL SSA were added, and this was subjected to DTNB assay as described for total GSH. GSH was determined by subtracting the GSSG content from the total GSH content. All biochemical determinations were normalized to the protein content using the Bradford method (Bradford, 1976).

2.5. Western Blotting Analysis

Cell lines were grown to near confluence, washed with ice-cold PBS, and then collected by scraping and centrifugation. Cells were lysed by sonication in 10 mM phenylmethanesulphonylfluoride or phenylmethylsulphonyl fluoride (PMSF). The cell lysates were then mixed with one volume of 2X sample buffer containing 6% SDS and 10% mercaptoethanol, denatured by heating to 95ºC for 5 min, and separated on 3% acrylamide stacking, and 12% Laemmli running gels for SDS polyacrylamide gel electrophoresis. After separation, the proteins were electrophoretically transferred to nitrocellulose membranes (Bio Rad, Hercules, CA). The membrane was blocked with 5% skim milk in TBST (Tris

buffered saline with 0.1% Tween) for 1 hr, and then incubated with the primary antibody for 1–2 hr. The blot was washed with TBST and incubated with secondary antibody (horseradish peroxidase-conjugated anti-IgG (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). The immuno-reactive protein was detected using an enhanced chemiluminescence (ELC) detection kit (Amersham Pharmacia Biotech, USA). Primary antibodies were anti-Heme Oxygenase-1 (Stressgen Biotech Serologies, USA), and Rabbit anti-MnSOD (kind gifts from Dr. Larry Oberley, University of Iowa, USA).

2.6. Statistical Analysis

All results are expressed as mean ± 1 standard deviation (S.D). Student's t test was employed ($p<0.05$) for two groups analysis.

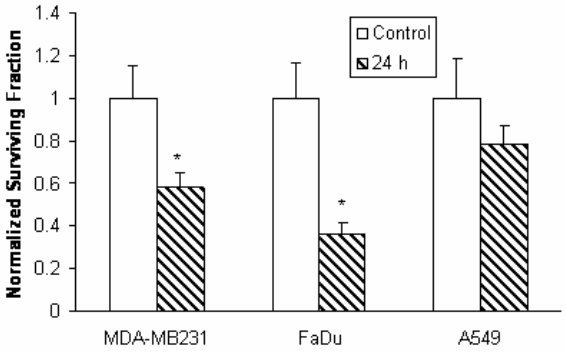


Figure 1. Clonogenic cell survival showed increased susceptibility of human cancer cells (MDA-MB231 and FaDu) to *Datura* aqueous leaf extract -induced cytotoxicity.
Cells were grown for 24 hours in RPMI (MDA-MB231) and DMEM (FaDu and A549) medium in the presence of 1mg/mL *Datura* aqueous leaf extract. Error bars represent ±1 SD of the mean of N=3 experiments performed with at least three cloning dishes taken from one treatment dish. Asterisks indicate significant differences between treated group and their prospective control ($p<0.05$, t-test, n = 3). Data were normalized to sham-treated cultures from each cell line.

3. Results

3.1. Datura Aqueous Leaf Extract -Induced Cytotoxicity and Oxidative Stress in Human Cancer Cell Lines

A variety of human cancer cells derived from breast (MDA-MB231), head and neck (FaDu), and lung (A549) human cancer cell lines were exposed for 24 and 48 hours to *Datura* aqueous leaf extract and clonogenic cell survival as well as parameters indicative of oxidative stress were assayed. MDA-MB231 and FaDu cells significantly enhanced ($p<0.05$) clonogenic cell killing following 24 hours exposure to the extract, relative to each respective control (Figure 1). However, A549 cells were found to be resistant to cell killing induced by exposure to the extract for 24 hours. Some variability in responses between the cell lines was also noted with MDA-MB231 showing 40% killing, and FaDu showing 65% cell killing during 24 hours of *Datura* aqueous leaf extract exposure (Figure 1). Exposure of these cells to the extract for 48 hours showed that all cancer cell lines were sensitive to cell killing induced by *Datura* aqueous leaf extract exposure with some variability ($p<0.05$) (Figure 2). MDA-MB231 showing 61% killing, FaDu showing 63% cell killing, and A549 showing 22% cell killing during 48 hours of *Datura* aqueous leaf extract exposure. Figure 3 shows the results of the glutathione analysis done on co-cultures obtained from the same experiments, shown in Figure 1 and 2. Glutathione is a major intracellular redox buffer such that the ratio of GSH to GSSG can be used as a reflection of intracellular redox status (Schafer *et al*., 2001). Exposure of FaDu cells for 24 and 48 hours to *Datura* aqueous leaf extract caused a ~2-fold increase in total GSH and GSSG as well, whereas a minimal change was seen in MDA-MB231 exposed to *Datura* aqueous leaf extract for 24 hours. However, A549 cells exposure to *Datura* aqueous extract for 24 or 48 hours did not seem to significantly alter GSSG level (Figure 3 A, B).

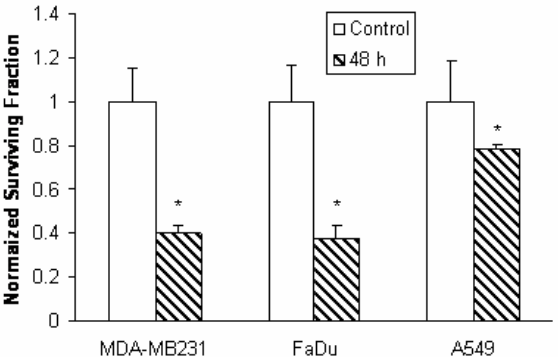


Figure 2. Clonogenic cell survival showed increased susceptibility of human cancer cells (MDA-MB231, FaDu and A549) to *Datura* aqueous leaf extract -induced cytotoxicity.
Cells were grown for 48 hours in medium in the presence of 1mg/mL *Datura* aqueous leaf extract. For more details, see legend of Figure 1.

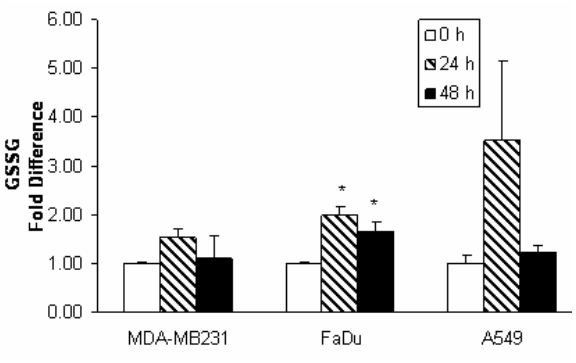
3.2. Datura Aqueous Leaf Extracts Activity and Antioxidants Levels

The protein level of antioxidant enzymes MnSOD and HO-1 was determined by immunoblotting. As shown in Figure 4, there was a significant ($P<0.05$) increase in the protein expression of MnSOD and HO-1 in A549 cells exposed to 1mg/mL of *Datura* aqueous leaf extract for 24 & 48 hours, while no changes were seen in MDA-MB231 and FaDu cells. These results explain the resistance of A549 cells to cell killing–induced by the *Datura* aqueous leaf extract for 24 hours and the minimal toxicity seen for 48 hours.

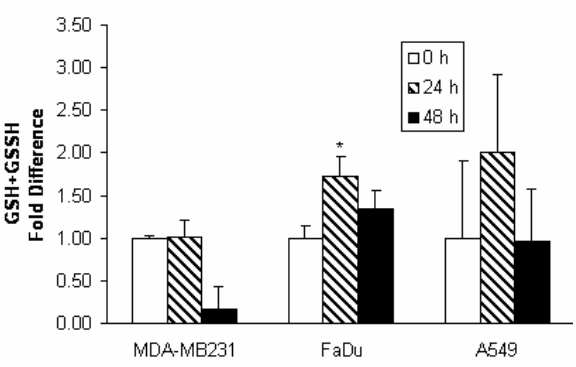
4. Discussion

It has been reported that all parts of the plant *Datura stramonium* are poisonous if ingested by humans or livestock (Radford et al., 1964). However, it could be used for medicinal purposes (King 1984, Mann 1992). The effect of *Datura* extracts on the oxidative stress has not been studied well. In this study, we investigated the toxic effect of *Datura stramonium* aqueous leaf extract on different cancer cells and how this exposure might affect the oxidant/antioxidant status of the cells.

Using clonogenic assay, and after incubation for 24 hours, both MDA-MB231 and FaDu cells showed significantly reduced growth ($P<0.05$) when compared to



(a)



(b)

Figure 3. Effect of *Datura* aqueous leaf extract on total GSH (a) and GSSG levels (b) in human cancer cells (MDA-MB231, FaDu, and A549). Cells were treated with 1 mg/mL *Datura* aqueous leaf extract for 24 and 48 hours. Cells were then harvested for glutathione analysis, using the spectrophotometric recycling assay. For more details, see legend of Figure 1.

control. The situation was different with A549 cells (Figure 1). The killing ability of the *Datura* aqueous leaf extract could be attributed to the imbalance of the internal oxidant/antioxidant capability of the cells caused by this exposure. This can be seen clearly by the level of GSSG changes in FaDu cells. Taken together, the data in Figure 1 and 2 suggest that the cytotoxic effect of the extract in FaDu cells was mediated by disruptions in thiol metabolism consistent with oxidative stress. Although, the extract exposure in MDA-MB231 induced significant cytotoxicity, the level of GSSG was not highly up-regulated. This indicates that the extract might have induced different mechanism of cytotoxicity (pro-apoptotic characteristics). Further studies are still to be done. The higher levels of GSH in A549 cells could be protecting cells from oxidative stress induced by *Datura* aqueous leaf extract exposure for 24 hours (Figure 3).

Upon 48 hours exposure, the picture was different. The level of oxidative stress induced by exposure conditions was causing more killing in the three cell lines. Again looking at the GSH levels, it is clear that exposure to the extract induced GSH response to lesser extent this time. The inability of cells to induce more GSH production could be due to the toxic effect of the *Datura* aqueous leaf extract.

GSH and GSSG are the major redox pair involved in cellular redox homeostasis. A change in the cellular GSH or GSSG is regarded as a representative marker for oxidative stress; and is directly responsible for the

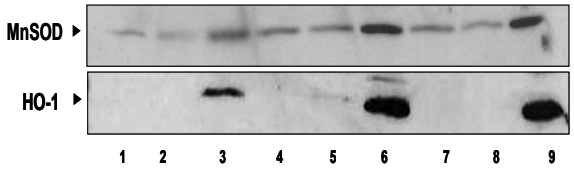


Figure 4. *Datura* aqueous extract produced an increase in MnSOD and HO-1 protein in A549 cells. The effect of *Datura* aqueous leaf extract on the redox sensitive enzymes MnSOD and HO-1 for 24 h and 48 hours, using immunoblot determination technique in MDA-MB231, FaDu and A549 cells. Lane 1, MDA-MB231 control; Lane 2, FaDu cells control; Lane 3, A549 cells control; Lane 4, 5, and 6, MDA-MB231, FaDu and A549 cells treated for 24 hours with the extract respectively; Lane 7, 8, and 9, MDA-MB231, FaDu and A549 cells treated for 48 hours with the extract, respectively.

perturbation of cellular function (Schafer *et al*., 2001). This includes activation of antioxidant defense pathways, as well as induction of cytotoxic responses.

Our results above motivsted us to study the expression of certain antioxidant enzymes such as: MnSOD and HO-1. As we can see the levels of MnSOD or HO-1 were not changed upon exposure to the *Datura* aqueous leaf extract in both MDA-MB231 and FaDu cells, whereas A549 cells showed clear up-regulation on both 24 and 48 hours exposure (Figure 4). This interesting result shows that different cancer cells have different inherent response to oxidative stress. This response will affect the ability of different cancer cells to respond to compounds and chemicals that can induce oxidative stress. Studying signal pathways, involved in different activation processes, could evolve and explain the different responses seen.

In this study, we have demonstrated that *Datura stramonium* aqueous leaf extract induced oxidative stress in different human cancer cell lines. In response, these cells exhibit up-regulating the expression of certain antioxidant compounds and enzymes such as: GSH, HO-1 and SOD. Further studies are still needed to explore the effect of *Datura* aqueous leaf extract on the signaling pathways involved.

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Larvicidal Activity of a Neem Tree Extract (Azadirachtin) Against Mosquito Larvae in the Republic of Algeria

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Abstract

An insecticide containing azadirachtin, a tree (*Azadirachta indica*) extract, was tested against *Culex pipiens* mosquito larvae and pupae in east of the Republic of Algeria under laboratory conditions. First, after treatment of larval stage, LC_{50} and LC_{90} values for Azadirachtin were 0.35 and 1.28 mg/L in direct effect and 0.3-0.99 mg/l in indirect effect, respectively. Second, after treatment of the pupal stage, the LC_{50} and LC_{90} in direct effect were measured as0.42 -1.24mg/l and in indirect effect was 0.39mg/l-1.14mg/l respectively. Mosquito adult fecundity were markedly decreased and sterility was increased by the Azadirachtin after treatment of the fourth instar and pupal stage. The tretment also prolonged the duration of the larval stage. The results show that the Azadirachtin is promising as a larvicidal agent against *Culex pipiens*, naturally ocurring biopesticide could be an alternative for chemical pesticides.

الملخص

تناول هذا البحث دراسة تأثير مبيد نباتي Azadirachtin المستخلص من شجرة Azadirachta indica ، على يرقات وعذارى البعوض في شرق جمهورية الجزائر في شروط المختبر، حيث حسبت الجرعة المميتة للنصف (LC_{50}) والجرعة المميتة لتسعين بالمئة (LC_{90}) بالنسبة لبعوض Culex pipiens وهي أكثر العوامل نقلا لبعض الأمراض الإلتهابية على الصعيد المحلي ،حيث قدرت بعد معاملة الطور الرابع LC_{50} =0.35 ملغ /ل وLC_{90}= 1.28ملغ/ل هذا بالنسبة إلى التأثير المباشر، أما التأثير الغير مباشر فكانت 0.30 ملغ/ل و 0.99 ملغ/ل على التوالي، أما عند معاملة طور العذراء كانت النتائج بالنسبة للتأثير المباشر (LC_{50} =0.42 ملغ /ل وLC_{90} =1.24ملغ/ل) أما التأثير الغير مباشر فكانت 0.39 ملغ/ل و 1.14 ملغ/ل على التوالي. كذلك لوحظ انخفاض كبير في نسبة الخصوبة وزادة مؤشر العقم للإناث الناتجة عن معاملة الطور الرابع والعذارى للبعوض ، وزيادة في مدة الطور. حيث نقول في الأخير أنه يمكن استعمال هذا المبيد كمبيد طبيعي بدل المبيدات الكيميائية.

Keywords: Mosquito; *Culex pipiens;* Azadirachtin; Insecticide.

1. Introduction

The Meliaceae plant family is known to contain a variety of compounds that show insecticidal, antifeedant, growth-regulating, and development-modifying properties (Nugroho et al., 1999; Greger et al., 2001; D'Ambrosio and Guerriero, 2002; Nakatani et al., 2004). *Melia azedarach* L. and *Azadirachta indica* (Sapindales: Meliaceae), commonly known as Chinaberry or Persian lilac tree, are deciduous trees that are native to northwestern India; and have long been recognized for their insecticidal properties. These trees typically grow in the tropical and subtropical parts of Asia, but nowadays they are also cultivated in other warm regions of the world because of their considerable climatic tolerance. Fruit extracts of *Melia azedarach and Azadirachta indica* elicit a variety of effects in insects such as antifeedant, growth retardation, reduced fecundity, moulting disorders, morphogenetic defects, and changes of behavior (Schmidt et al., 1998; Abou Fakhr Hammad et al., 2001; Gajmer et al., 2002; Banchio et al., 2003; Wandscheer et al., 2004). The effects of the compounds extracted from *M. azedarach* on insects have been reviewed by Ascher et al., (1995) and reported by Saxena et al., (1984), Schmidt et al., (1998), Juan et al., (2000), Carpinella et al., (2003) , Senthil Nathan and Saehoon, (2005). Control of mosquito is essential as many species of mosquitoes are vectors of malaria, filariasis, and many arboviral diseases; and they constitute an intolerable biting nuisance (Youdeowei and Service, 1983; Curtis, 1994; Collins and Paskewitz, 1995). Biotechnologists and entomologists agree that mosquito control efficiency should be with selectivity for a specific target organism. New control methodologies aim at reducing mosquito breeding sites and biting activity by using a combination of chemical–biological control methods soothing several advocated biocontrol methods to reduce the population of mosquito and to reduce the man–vector contact (Service, 1983). Recently, there has been a major concern for the promotion of botanicals as environment friendly pesticides, microbial sprays, and insect growth regulators amidst other control measures such as beneficial insects and all necessitate an integration of supervised control (Ascher et al., 1995; Senthil Nathan et al., 2004, 2005b, c, and d). The development of insects' growth regulators (IGR) has received considerable attention for selective control of insect for medical and veterinary importance and has produced mortality due to their high neurotoxic effects (Wandscheer et al., 2004; Senthil Nathan et al.,

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2005a). Although, biological control has an important role to play in modern vector control programs, it lacks the provision of a complete solution by itself. Irrespective of the less harmful and eco-friendly methods, suggested and used in the control programmers, there is still a need to depend upon the chemical control methods in situations of epidemic outbreak and sudden increase of adult mosquitoes. Hence, insecticides are known for their speedy action and effective control during epidemics. Nonetheless, they are preferred as effective control agent to reduce the mosquito population irrespective of their side effects. Recent studies stimulated the investigation of insecticidal properties of plant-derived extracts; and concluded that they are environmentally safe, degradable, and target specific (Senthil Nathan and Kalaivani, 2005). Muthukrishnan and Puspalatha (2001) evaluated the larvicidal activity of extracts from *Calophyllum inophyllum* (Clusiaceae), *Rhinacanthus nasutus* (Acanthaceae), *Solanum suratense* (Solanaceae) and *Samadera indica* (Simaroubaceae), *Myriophyllum spicatum* (Haloragaceae) against *Anopheles stephensi* (Senthil Nathan et al., 2006). Several indigenous plants in India and subtropical parts of Asia, such as *Ocimum basilicum*, *Ocimum santum*, *Azadirachta indica*, *Lantana camera*, *Vitex negundo* and *Cleome viscose* (Senthil Nathan et al., 2006) were studied for their larvicidal action on the field which collected fourth instar larva of *Culex quinquefasciatus* (Kalyanasundaram and Dos, 1985). Chavan (1984), Zebitz(1984,1986), Schmutterer (1990), Murugan and Jeyabalan (1999) reported that *Leucas aspera*, *O. santum*, *Azadirachta. indica*, *Allium sativum* and *Curcuma longa* had a strong larvicidal, antiemergence, adult repellency and antireproductive activity against *A. stephensi*. In addition, *Pelargonium citrosa* (Jeyabalan et al., 2003), *Dalbergia sissoo* (Ansari et al., 2000a) and *Mentha piperita* (Ansari et al., 2000b) were shown to contain larvicidal and growth inhibitory activity against *A. stephensi.* The present investigation was conducted to study the effect of Azadirachtin, a neem tree *Azadirachta indica* extract, against larvae and pupae of *Culex pipiens* mosquitoes in east of the Republic of Algeria.

2. Materials and Methods

2.1. Mosquito Rearing

Culex pipiens eggs were collected from cellarage tribes (region sidi amar - Annaba) and readed in the ' Laboratory of Biology Animal Application' University of Annaba-Algeria. Larvae were reared in plastic and enamel trays in tap water. They were maintained at 25-27°C, 75-85% relative humidity under 14:10 light and dark photo period cycle. The larvae were fed with fresh food consisting of a mixture of Biscuit Petit Regal-dried yeast (75:25 by weight). Pupae were transferred from the trays to a cup containing tap water and placed in screened cages (20x20x20cm), where the adult emerged. After emergence, female mosquitoes obtained blood meal from caged pigeons while male mosquitoes were fed on a 10% sucrose solution. Then egg-masses were kept to continue next generation.

2.2. Bioassays and Larval Mortality

Bioassays were performed with fourth larvae stages and pupae of *C. pipiens* using concentration from 0.125; 0.250; 0.500; 0.750 and 1mg/l of the Azadirachtin. A minimum of 25 larvae/concentration were used for all the experiments. And these experiment were replicated five times. For mortality studies, 25 larvae each of fourth instar and pupae were introduced in 250 ml plastic beaker containing various concentrations of the Azadirachtin. A control was maintained. The treatments were replicated five times, and each replicate set contained one control. The percentage mortality was calculated by using the formula (1), and corrections for mortality when necessary were done using Abbot's (1925) formula (2)

$$\text{Percentage of Mortality} = \frac{\text{Number of dead larvae}}{\text{Number of larvae introduced}} \text{ X100} \quad (1)$$

$$\text{Corrected percentage of mortality} = 1 - \frac{n \text{ in T after treatment}}{n \text{ in C after treatment}} \text{ X100} \quad (2)$$

Where n = number of larvae or nymph, T = treated, C = control.

2.3. Fecundity and Sterility

The fecundity experiments were conducted by taking equal number of male and female *C. pipiens* which had emerged from the control and treated sets of each concentration. They were mated in the cages of (20 x 20 x 20) cm dimension individually to each concentration. Three days after the blood meal, eggs were collected daily from the small plastic bowls containing water kept in ovitraps in the cages. The fecundity was calculated by the number of the eggs laid in the ovitraps divided by the number of female let to mate (The death of the adult in the experiment was also considered) The Sterility Indices experiments were conducted by the formula (3) of Sexina et al ,(1993):

$$SI = 100 - \frac{\text{Total number of eggs of females treated x percentage of hatch}}{\text{Total number of eggs of control x percentage of hatch}} \quad (3)$$

2.4. Total Larval and Pupal Duration

To assay the growth factors of *C. pipiens*, test solution of concentration of Azadirachtin extract (0.125; 0.250; 0.500; 0.750 and 1mg/l) were used. A known number of eggs were made to hatch and the total larval duration (days) was calculated from hatching to pupation period, the pupa was placed in a small container closed with a transparent mesh, so that the adults were kept trapped. The pupal duration (days) was calculated from the pupal molt to the emergence of imago.

2.5. Statistical Analysis

The analysis program Probit (Finney, 1971), the lethal concentrations (µg/ml) for 50%, and 90% of the mortality, LC_{50} and LC_{90}, respectively, were at 24h after treatment.

Table 1. Larvicidal activity of Azadirachtin at various concentration applied for 24h to newly ecdysed fourth instars of *Culex pipiens*.

Effects	Concentration (mg/l)	Mortality (%)	LC_{50}(mg/l)	95%Confidancelimits (µg/ml)		LC_{90} (mg/l)	Regression equation	χ^2
				Lower	Upper			
Direct	Control	9.6						
	0.125	17.39						
	0.250	33.62	0.357	0.307	0.414	1.280	Y=2.20x-0.61	0.615
	0.500	60.17						
	0.750	76.39						
	1	85.24						
Indirect	Control	15.2						
	0.125	19.80						
	0.250	35.52	0.304	0.286	0.335	0.992	Y=2.63x-1.60	3.69
	0.500	65.40						
	0.750	81.12						
	1	93.70						

Table 2. Larvicidal activity of Azadirachtin at various concentration, applied for 24h to newly ecdysed pupae of *Culex pipiens*.

Effects	Concentration (mg/l)	Mortality (%)	LC_{50}(mg/l)	95%Confidancelimits (mg/l)		LC_{90} (mg/l)	Regression equation	χ^2
				Lower	Upper			
Direct	Control	4.33						
	0.125	7.57						
	0.250	28.78	0.426	0.379	0. 479	1.243	Y=2.76x-2.2	1.1625
	0.500	45.45						
	0.750	66.66						
	1	87.87						
Indirect	Control	4						
	0.125	7.93						
	0.250	31.74	0.398	0.355	0.445	1.141	Y=2.82x-2.3	1.485
	0.500	49.20						
	0.750	71.42						
	1	92.05						

The 95% confidence intervals, values, and degrees of freedom of the χ^2 goodness of fit tests, and regression equations, were recorded .Whenever the goodness of χ^2 was found to be significant (p <0.05) , a heterogeneity correction factor was used in the calculation of confidence limits. Data from biology, mortality, fecundity deterrence and effective concentration were subjected to analysis of variance (ANOVA of arsine square root transformed percentages).

3. Results

3.1. Insecticidal Activity

Dose-response relationship was determined for Azadirachtin applied for 24h to newly ecdysed fourth instar larvae and pupae. The mortality was scored up to adult formation.

For fourth stage, the highest concentration tested 1 mg / l in direct effect caused 85.24% mortality (Figure1). With probit, the LC_{50} was calculated as 0.35 mg/l (95% CI=0.30-0.41mg/l; n=75; Slope=2.83) and the LC_{90} was 1.28 mg/l, (Table1). For indirect effects, the highest concentration caused 93.70% mortality (Figure2), the LC_{50} was 0.32 mg/l (95% CI=0.28-0.33 mg/l; n=75; Slope=2.46) and LC_{90} was calculated as 0.99 mg/l, respectively, (Table1). After treatment, during the pupal stage and in direct effect, the highest concentration caused 87.87% mortality (Figure1), the $LC_{50}$was 0.42 mg/l (95% CI=0.37-0.47mg/l; n=75; Slope=2.83) and the LC_{90} was calculated as 1.24 mg/l, (Table 2). For indirect effects the LC_{50} was 0.39 mg/l (95% CI=0.35-0.44 mg/l; n=75; Slope=2.46) and LC_{90} was calculated as 0.99 mg/l respectively, the highest concentration tested caused 92.05% mortality (Table2, Figure2).

3.2. Effect on Fecundity, Sterility and growth

Adults fecundity also was markedly decreased by the Azadirachtin treatment the fourth instar larvae and pupal stages (Figure 3, 4). Adults' sterility indices were markedly increased by the Azadirachtin treatment, (Figure 5, 6). An adverse sub-lethal effect in pupa exposed to Azadirachtin

was evident. In addition to significantly lower survivorship and protracted development, larval duration was reduced markedly. The plant extracts (Azadirachtin) drastically reduced the fecundity of the females, and only few adults survived. The duration of larval instars and the total developmental time were prolonged. (Table3). In the present study, application of Azadirachtin greatly affected the growth of *Culex pipiens* .

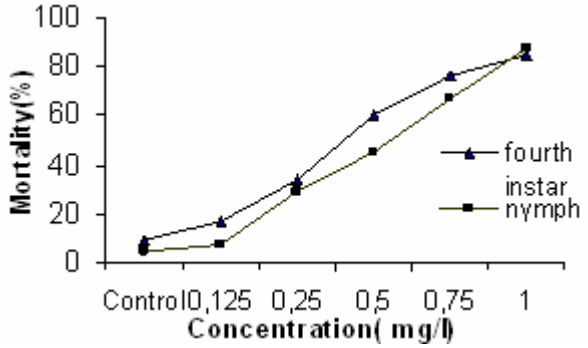

Figure1. Dose-response relationship for treatment of Azadirachtin, applied for 24h to newly ecdysed fourth instar larvae and pupae of *Culex pipiens* (*effect direct*).

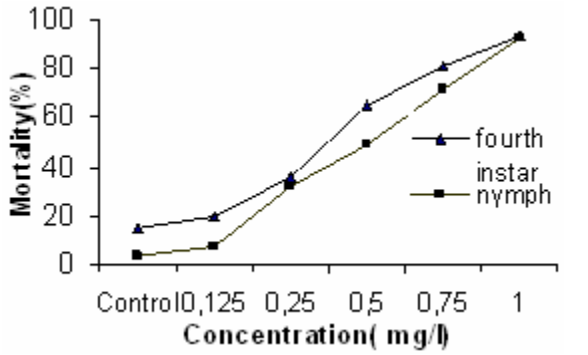

Figure 2. Dose-response relationship for treatment of Azadirachtin, applied for 24h to newly ecdysed instar larvae and pupae instars of *(Italique) Culex pipens*(effect indirect).

4. Discussion

Azadirachtin, the extract of neem tree, was tested in the present study, and is reported to be eco-friendly and not toxic to vertebrates (Al- Sharook et al., 1991). It is clearly proved that crude or partially-purified plant extracts are less expensive and highly efficacious for the control of mosquitoes rather than the purified compounds or extracts (Jang et al., 2002; Cavalcanti et al., 2004).

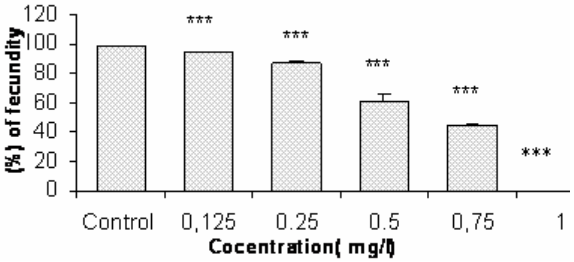

Figure 3. Fecundity of *C. pipiens* females after treated the fourth instars with Azadirachtin.(Data following by *** are significantly different from control, p<0.001)

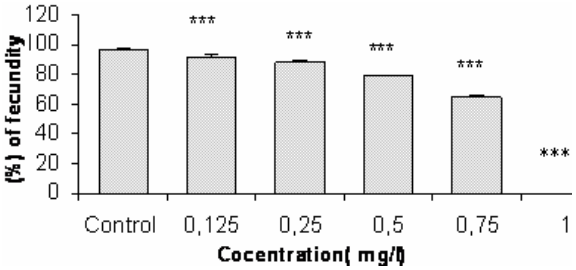

Figure 4. Fecundity of *(Italique) Culex pipens* females after treating the pupae stages with Azadirachtin.(Data following by *** are significantly different from control, p<0.001).

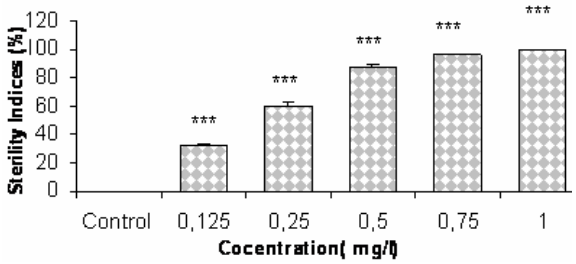

Figure5. Sterility Indices of *C. pipiens* females after treating the fourth instars with Azadirachtin.(Data following by *** are significantly different from control, p<0.001).

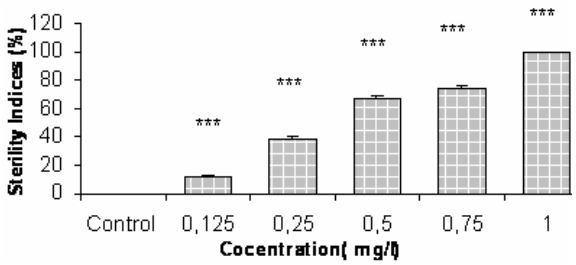

Figure 6**.** Sterility Indices of *C. pipiens* females after treating the pupae with Azadirachtin. (Data following by *** are significantly different from control, p<0.001)

Table 3. Effect of Azadirachtin applied to newly ecdysed 4th instar larvae of C. *pipiens* on the duration of development.

Concentration (mg/l)	Duration (days)Mean±SE	
	4th larval instar	Pupal stage
0	8.75±2.99	2.61±0.65
0.125	15.24±1.28	3.81±0.84
0.250	16.00±2.82	3.54±0.55
0.500	17.94±1.54	3.42±0.87
0.750	18.45±2.80	3.33±0.28
1	19.75±2.22	3.33±0.46

The effect of these crude plant extract on the biology, reproduction, and adult emergence of the mosquitoes are remarkably greater than those reported for other plant extracts in the literature. For example 50% inhibition of the emergence of the adult mosquitoes was observed by the use of *C. inophyllum*, *S. suratense*, *S. indica* and *Rhinocanthus nasutus* leaf extracts (Muthukrishnan and Puspalatha, 2001). Similarly 88% of the adult mortality was observed by the use of *P. citrosa* leaf extracts at 2% concentration (Jeyabalan et al., 2003). The Meliaceae plant family is used as growth regulator against many insect pests (Saxena et al., 1984; Jacobson, 1987; Schmutterer,

1990; Hammad et al., 2001; Gajmer et al., 2002; Banchio et al., 2003; Wandscheer et al., 2004). The growth regulatory effect is the most important physiological effect of *M. azedarach* on insects. It is because of this property that family Meliaceae has emerged as a potent source of insecticides. Exposure of *A. stephensi* larvae to sub-lethal doses of neem leaves extract in the laboratory prolonged larval development, reduced pupal weight and ovipostion (Murugan et al., 1996; Su and Mulla, 1999). In the field, delayed phenology of surviving larvae and reduced pupal weight are common occurrence after treatment with neem (Zebitz, 1984, 1986). The direct and indirect contribution of such effects to treatment efficacy through reduced larval feeding and fitness need to be properly understood in order to improve the use of *M. azedarach* for management of *A. stephensi*. The results of this study indicate the plant-based compounds such as Azadirachtin (compounds present in the Meliaceae plant family seed) may be an effective alternative to conventional synthetic insecticides for the control of *Culex pipiens*. Undoubtedly, plant derived toxicants are valuable sources of potential insecticides. These and other naturally occurring insecticides may play a more prominent role in mosquito control programs in the future (Mordue and Blackwell, 1993). The results of this study will contribute to a great reduction in the application of synthetic insecticides, which in turn will increase the opportunity for natural control of various medicinally important pests by botanical pesticides. Since these are often active against a limited number of species including specific target insects, less expensive, easily biodegradable to non-toxic products, and potentially suitable for use in mosquito control programme (Alkofahi et al., 1989), they could lead to development of new classes of possible safer insect control agents. Plant allelochemicals may be quite useful in increasing the efficacy of biological control agents because plants produce a large variety of compounds that increase their resistance to insect attack (Berenbaum, 1988; Murugan et al., 1996; Senthil Nathan et al., 2005a). The intensive use of pesticides produces side effects on many beneficial insects and also poses both acute and chronic effects to the milieu (Abudulai et al., 2001). Recently, bio-pesticides with plant origins are given for use against several insect species especially disease-transmitted vectors, based on the fact that compounds of plant origin are safer in usage, without phytotoxic properties; also leave no scum in the environment (Schmutterer, 1990; Senthil Nathan et al., 2004, 2005a, d). Large alterations in the fecundity and sterility of insects exposed to neem have been extensively reported, such as those in the fly, *Ceratatis capitata* (Steffens and Schmutterer 1982); banana root borer, *Cosmopolites sordidus* (Germar) (Musabyimana et al., 2001); and mosquitoes, *A. stephensi* and *A. culicifacies* (Dhar et al., 1996). The work published by Khan et al., (2007) microscopically demonstrated that the decrease in fecundity of *Bactocera cucurbitae* and *Bactocera dorsalis* exposed to neem compound was due to the block of ovarian development. Likewise, mixing of a commercial formulation of neem in the adult diet caused reduction in the fecundity of *C. capitata* by interfering with oogenesis (Di Ilio et al., 1999). The block in the ovarian activity of *C. capitata,* resulting from neem compound, was verified by histological observation (Di Ilio et al., 1999). Results from the study of Lucantoni et al., (2006) clearly indicated that the neem treated female mosquito, *A. stephensi*, displayed a delay in oocyte development in the vitellogenesis. As discussed by Weathersbee III and Tang (2002), the disruption of reproductive capability could lead to substantial population decline over time. Furthermore, Dhar et al., (1996) revealed that exposure to neem extract suppressed rather than inhibited oviposition in mosquitoes. The present study clearly proved the efficacy of Azadirachtin on larvae, pupae, and adult of *Culex pipiens*. Further studies such as mode of action and synergism with the biocides under field condition are needed.

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Organochlorine Pesticides and Polychlorinated Biphenyls Carcinogens Residual in some Fish and Shell Fish of Yemen

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Abstract

The concentrations of Dichloro-Diphenyl Trichloroethane (DDTs) as organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) in seventeen important commercial species Fish and Shell Fish from the Gulf of Adan and Red Sea of Yemen Coast were investigated in this study. Relation between weight and length of the organisms and the values of DDTs and PCBs were also considered. The concentrations of DDTs and PCBs were measured, using GC chromatography and using electron capture dtector. The values of DDTs in the Red Sea ranged from 0.9-7.8 ng g^{-1} and the OCPs ranged from 0.1-1.0 ng g^{-1}. As for the fishes and shell fishes of the Gulf of Aden, the concentration ranges were 0.3-6.4 ng g^{-1} and 0.1-0.9 ng g^{-1} for DDTs and PCBs respectively. Although PCBs are not manufactured in Yemen, their presence can be attributed to the industrial usage and the possible dumping of some products which contain PCBs. The study of relationships between DDTs and PCBs and weights or lengths indicated that these compounds are not concentrated in the tissues of fish and shell fish species.

الملخص

في هذا العمل تم دراسة تراكيز المبيدات العضوية المكلورة والبوليمرات ثنائية الفنيل في سبعة عشر نوع من الأسماك الاقتصادية المهمة من مياه خليج عدن وساحل البحر الأحمر اليمني. كما درست العلاقة بين طول وزن كل سمكة الى هذه التراكيز.

تم قياس هذه التراكيز بواسطة جهاز الجاز كروماتوجرافي، فيم المبيدات العضوية المكلورة في اسماك البحر الأحمر كانت من 0.9 ألى 7.8 نانو جرام / جرام ، قيم ثنائية الفنيل عديدة الكلور من 0.1 الى 1.0 نانو جرام / جرام.

فيم المبيدات العضوية المكلورة في اسماك خليج عدن كانت من 0.3 الى 6.4 نانو جرام / جرام ، قيم ثنائية الفنيل عديدة الكلور من 0.1 الى 0.9 نانو جرام / جرام. وبالرغم من أن ثنائية الفنيل عديدة الكلور لا ينتج في اليمن لكنه يتواجد في الاستعمالات الصناعية. هذه الدراسة أوضحت أن تراكيز هذه المركبات ليست بالكبيرة في أنسجة هذه الاسماك

Keywords: Organochlorine Pesticide; DDTs; PCBs; Fish; Shell fish; Gas Chromatography; Red Sea; Gulf of Aden

1. Introduction

Organochlorine pesticides are a class of toxic compounds characterized by their relative chemical and biological stability, and hence persistence in the environments. Consequently, organochlorine pesticides have been placed on the top of the list of potential environmental hazards. The persistent and widespread occurrences of organochlorine pesticides have stimulated research into the nature, behavior, fate of pesticides in addition to their metabolites in the environment. The widespread occurrences of these compounds as environmental pollutants have been reported for all major terrestrial, fresh water, and marine environments. Damage to marine environment, by organochlorine pesticides, is well documented. However, very few data are available for the concentration of chlorinated pesticides in the seas around India (Jackson *et al.,* 1994; McCain and Varanasi, 1991; Nicholson, 1980; Tanbe and Tatsukawa, 1991; Mansour, 2004; Pfeuffer and Rand, 2004; Chou and Lee, 2005). As India is predominantly an agricultural country, large quantities of pesticides (55,000 t) have been used in agriculture for many years (McCain *et al.*, 1992).

At present, almost nothing is known about the existence of these pesticides in the Red Sea and Gulf of Aden. Organochlorine pesticides (OCPs) including Dichloro-Diphenyl-Trichloroethane (DDTs) have been used in Yemen and neighboring countries for more than four decades. According to a survey conducted in March 1990, there were a total of 80 pesticides brands of which twenty four are not recommended or even outlawed (Al-Ghashm, 1991; Bidleman and Leonard, 1982). Shipboard measurements in the Indian Ocean were made in the northern Arabian Sea, Arabian Gulf and the Red Sea. Average DDTs levels in the Arabian Sea -Arabian Gulf - Red Sea were 25-40 times as much of what is found in North Atlantic background value. They have attributed these higher levels to the continued use of DDTs in countries bordering these areas (Farrington *et al.,* 1983).

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Red Sea is an extremely isolated body of water. Water losses through evaporation far exceed rainfall, run-off from rivers is negligible; and apart from the Suez Canal, the only other mean of water exchange with other oceans is through the straits of Bab-el- Mandab. The Yemen coast is characterized by a narrow coastal plain between the Gulf of Aden and the mountain range that parallels the shoreline. This range averages 1.070 m in height and influences the local weather, especially the wind. The Gulf of Aden, which connects the Red Sea with the Arabian Sea (Indian Ocean), is approximately 900 km long, and large rivers flowed from the interior to the sea. Today, small wadi channels have been eroded into older and, larger river channel (DouAbul and Al-Shwafi, 2000; Al-Shwafi, 2008; Abdallah, 1996; Edwards and Head, 1987).

The need to identify organic and inorganic pollutants in the Red Sea has become a major concern for all countries in the region within the past few years because of the need for baseline data (or background levels), the chronic pollution from industrial and anthropogenic sources and the continual pollution of the areas fishing ground. In addition, and because of the potential impact on marine life and fisheries, it is also important to know the extent of the pollution and its impact on marine life, and the period of the pollution impact. Hence, the first necessary step is the determination of the seriousness of pesticides in the marine environment including the magnitude of their residues. However, such data are not available for the Red Sea and Gulf of Aden. Therefore, the present study is aimed mainly at determining DDTs and PCBs residues in fish and shell fish, and to attempt to identify their major sources and to perform a baseline study on the pollution in the muscle tissues of the commercial fish species, which were surveying in the Red Sea of Yemen and Gulf of Aden. The main objectives of the present study are to determine the residue levels of potential pollutants mainly the DDTs and PCBs in the flesh of the seventeen fish and shell fish species collected from the Red Sea of Yemen and The Gulf of Aden, and to identify the most important sources of contamination to the region. Also this study will focus on the sub-lethal effects of the most prominent contaminants, as well as their possible implications with humans who consume them. In addition, to generate baseline data for further follow- up study in the region.

2. Material and Method

2.1. Sampling

Fish and shell fish samples were collected from several locations along the Red Sea and Gulf of Aden Coast of Yemen during summer 1998. The collection of samples depending on the feasibility and importance of the site itself. Seventeen species of fish and shell fish were collected from the local commercial fishermen of Aden City and Hodiedah City. Samples were collected by fishermen, and then they were frozen upon return to the city center. Samples of fish and shell fish, having similar size (length and weight) were chosen for each species. Sub-samples (5gm each) of 17 species were dissected according to the procedure described by (ROPME Manual, 1983). The edible portions (muscles) were only taken for the determinations of DDTs and PCBs analysis.

2.2. Chemical Analysis

All solvents were redistilled, using distillation apparatus with a 150 cm vacuum jacketed fractionation column filled with 3 mm diameter glass helices. DDTs and PCBs were determined using a Hewlett Pachard HP5890-GC with split/splitless injector and a 25 m x 0.3 mm fused silica capillary with a chemically bonded gum phase SE54 sodium chloride and sodium sulfate that were kiln fired at 450^{o} C overnight and cooled in a greaseless desiccator. Silica gel, used for column chromatography, was solvent extracted with n-hexane in a flass cartridge inserted into an extraction apparatus, as described by Ehrhardt (1987). After extraction, the silica gel was first dried in the same cartridge by passing ultra-pure nitrogen through; and was then activated by heating the cartridge in an electric tube oven to 200^{o} C for 6 h with nitrogen stream reduced to a few ml per minute.

The extraction method was based upon that of Wade *et al.* (1988). A total 5g of dried tissues was Soxhlet-extracted with methyl chloride and concentrated in Kuderna-Danish tubes. The extracts were fractionated by alumina: silic gel (80-100 mesh) chromatography. The extracts were sequentially eluted from the column with 50 ml of pentane (aliphatic fraction) and 200 ml of 1:1 pentane-dichloromethane (PCBs/DDTs fraction) and concentrated for GC analysis. The recovery rate exceeded 80% for all the measured samples.

DDTs and PCBs were separated by gas chromatography in the split/splitless mode, using an electron capture detector (ECD). A 30 m x 0.32 mm i.d. fused-silica capillary column was used for this purpose with a chemically bonded gum phase SE54 (J&W Scientific, Inc.) provided component separations. Four-calibration solutions were used to generate a nonlinear calibration curve. A sample, containing only PCBs, was used to confirm the identification of each PCBs congener. The surrogates DBOFB (dibromooctafluorobiphenyl), PCB-103 and PCB-198 were added during the extraction during the extraction of DDTs and PCBs. The internal standard, TCMX (tetrachloro-*m*-xylene), was added prior to GC/ECD analysis. The chromatographic programm for DDTs and PCBs analysis was 100^{o}C for 1 min, then 5^{o}C min $^{-1}$ until 140^{o} C, hold for 1 min, then 1.5^{o}C min^{-1} to 250^{o}C, hold for 1 min, and then 10^{o}C min^{-1} to a final temperature of 300^{o} C, which was held for 5 min. The detection limit for the method is 0.001 ng g^{-1}.

3. Result

Table 1 shows the average concentrations of DDTs and PCBs in different fish and shell fish species collected from the Red Sea of Yemen. The concentrations were averaged across at least three measurements. The results of DDTs revealed that the concentrations ranged from 0.9-7.8 ng g^{-1} dry weight with an average of about 3.5 ng g^{-1}. DDTs values showed no relation either with weight or with length. The PCBs ranged from 0.1 to 1.0 ng g^{-1} dry weight with an average of about 0.5 ng g^{-1}. Also here, no

Table 1. Number, weight, and length of the collected fish and shell fish species in addition to concentrations of DDTs (ng g^{-1}) and PCBs (ng g^{-1}) in the muscles of the fishes and shell fishes collected from the Red Sea of Yemen. The errors are collected from standard deviations.

Fish and shell fish species	No	Total weight (g)			
Scomberomorus commerson	5	250-500	80-100	5.3±0.03	0.3± 0.04
Crenidens crenidens	15	500-600	30-50	4.2±0.04	0.5±0.02
Rastrelliger kanagurta	20	50-65	22-30	7.8±0.05	0.7±0.03
Thunnus albacares	7	500-700	70-90	3.9±0.07	0.4±0.02
Carchaias palasrras	10	200-500	55-62	2.4±0.03	0.1±0.01
Himantura uarnak	10	255-450	62-70	6.5±0.06	0.9±0.05
Caranx sem	10	105-320	50-55	6.7±0.09	0.4±0.02
Scomberoides commersonianus	10	562-765	72-75	5.1±0.03	0.3±0.03
Chanos chanos	10	50-108	35-40	4.8±0.07	0.5±0.02
Lutjanus sanguineus	10	120-142	34-47	1.2±0.07	0.8±0.06
Rachycentron canadus	8	152-256	60-63	2.0±0.03	0.9±0.05
Euthynnus affinis	10	320-450	44-50	2.8±0.02	0.7±0.07
Epinephelus areolatus	10	280-320	35-37	1.9±0.01	1.0±0.01
Panutirus homarun	10	320-460	40-42	2.3±0.03	0.2±0.05
Sepia pharnais	15	268-357	33-40	1.8±0.02	0.4±0.03
Sphyraena jello	10	365-452	43-52	1.1±0.01	0.3±0.04
Penaeus semisulcatus	25	95-120	25-30	0.9±0.01	0.1±0.02

Table 2. Number, weight, and length of the collected fish and shell fish species in addition to concentrations of DDTs (ng g^{-1}) and PCBs (ng g^{-1}) in the muscles of the fishes and shell fishes collected from the Adan Gulf. The errors are collected from standard deviations.

Fish and shell fish species	No	Total weight (g)	Total length (cm)	DDTs (ng g-1)	PCBs (ng g-1)
Scomberomorus commerson	5	270-450	80-90	4.3±0.02	0.2±0.02
Crenidens crenidens	15	620-650	33-54	2.8±0.03	0.3±0.01
Rastrelliger kanagurta	20	70-80	32-73	5.3±0.04	0.4±0.02
Thunnus albacares	7	453-720	60-89	2.8±0.05	0.2±0.01
Carchaias palasrras	10	500-700	57-65	2.3±0.03	0.1±0.01
Himantura uarnak	10	259-470	65-73	5.3±0.03	0.7±0.03
Caranx sem	10	115-325	53-59	6.4±0.07	0.5±0.02
Scomberoides commersonianus	10	653-777	75-77	5.0±0.02	0.4±0.02
Chanos chanos	10	55-112	38-44	4.3±0.06	0.3±0.03
Lutjanus sanguineus	10	120-142	36-50	1.0±0.03	0.6±0.04
Rachycentron canadus	8	170-268	66-69	1.7±0.02	0.8±0.03
Euthynnus affinis	10	333-470	49-53	2.5±0.02	0.6±0.06
Epinephelus areolatus	10	295-354	38-40	1.0±0.01	0.9±0.01
Panutirus homarun	10	332-472	43-45	2.3±0.03	0.3±0.03
Sepia pharnais	15	297-380	38-45	1.5±0.02	0.4±0.02
Sphyraena jello	10	395-460	46-55	1.0±0.01	0.2±0.03
Penaeus semisulcatus	25	100-129	35-43	0.3±0.01	0.1±0.01

relationship between PCBs concentrations and weight or length was observed. The results of DDTs and PCBs in the fishes of the Gulf of Adan are shown in table 2. The ranges of DDTs extended from 0.3 to 6.3 ng g^{-1} with an average of 2.9 ng g^{-1} and the ranges of PCBs extended from 0.1 to 0.9 ng g^{-1} with an average of 0.4 ng g^{-1}. No relationship was found between weight or length and DDTs and PCBs (Fig. 1 & 2). Statistical analysis of the relations shows r^2 values less than 0.1. Generally, the concentrations of both DDTs and PCBs in the fishes and shell fishes collected from the Red Sea of Yemen were higher than those collected from the Gulf of Adan. However, statistical analysis of the data using t-test shows that the differences were not significant either in DDTs (P= 0.1331) or in the PCBs (P=0.2313).

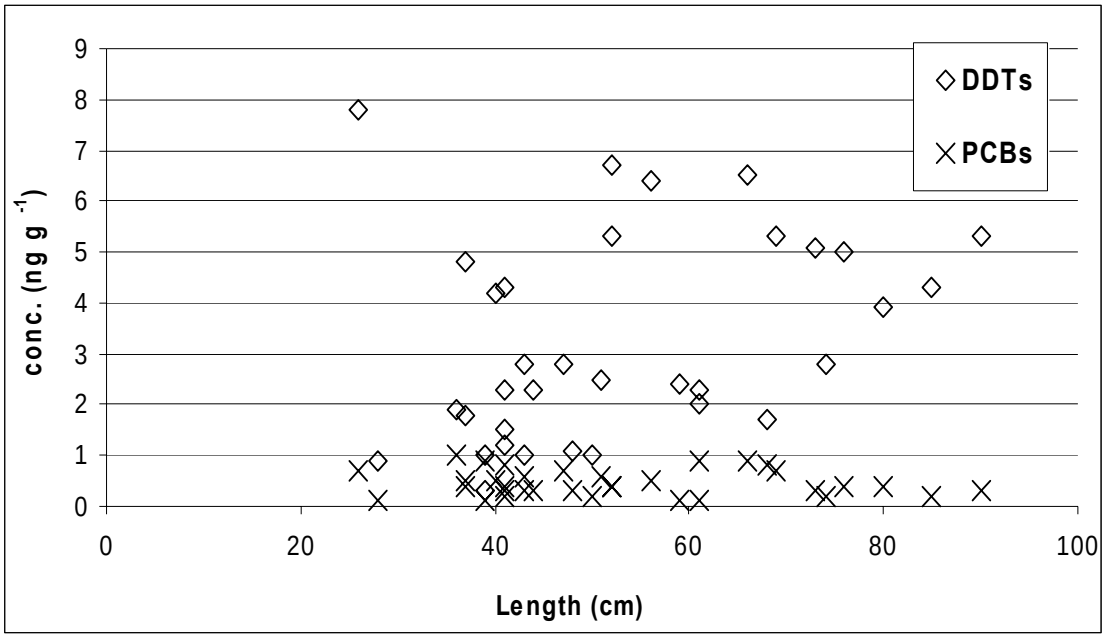

Figure 1. Relations between length (cm) and DDTs and PCBs concentrations (ng g^{-1}).

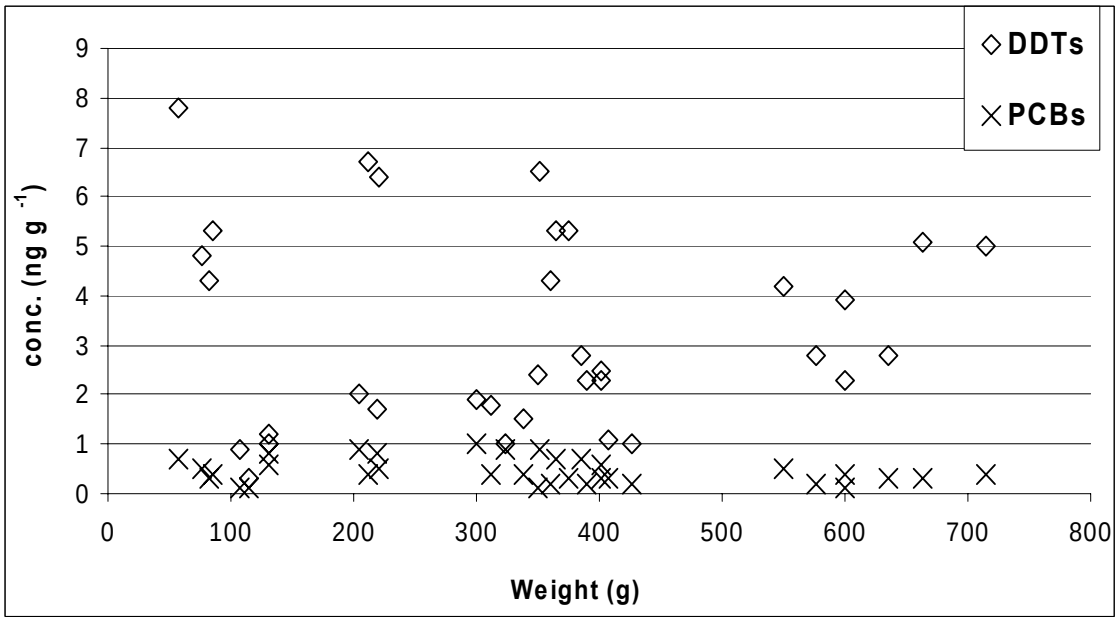

Figure 2. Relations between weight (g) and DDTs and PCBs concentrations (ng g^{-1}).

4. Discussion

It is difficult to compare the present results with other results from other studies since little information is available, especially in the Arabian context. However, DDTs in fishes and shell fishes from the Red Sea and Gulf of Aden were an order of magnitude lower than the range of values reported for fishes from the eastern Arabian Sea (Shailaja and Sen, 1989; Kishimba *et al.*, 2004). Similarly DDTs, determined in the present work, was an order of magnitude lower than the range of values reported for *Epinephelus tauvina* and *Lethrinus nebuluosus* captured from the coastal waters of Oman (Burns *et al.*, 1982). One of the primary concerns regarding DDTs is its potential for affecting fish reproduction. Early studies with trout demonstrated that DDTs concentrations in eggs or larvae in the ppm range impaired viability (Allison *et al.*, 1964; Hogan and Brauhn, 1975; Arambarri *et al.*, 2003). A number of dialed studies have also suggested that egg and larval viability or larval fitness were affected when relatively high levels of organochlorines were present in ovary tissues (Nelson *et al.*, 1991; Arambarri *et al.*, 2003; Mansourm and Sidky, 2003). Similar studies investigating DDTs and PCBs were done also in Kuwait waters and Abu Dhabi waters by DouAbul *et al.* (1987a). The authors found concentrations of 8.8-88 and 30 ng g^{-1} for DDTs and values of 5 and 7 ng g^{-1} for PCBs in Kuwait and Abu Dhabi water respectively. These values are much higher than the values reported in this study.

The higher concentrations of both DDTs and PCBs in the fishes and shell fishes collected from the Red Sea of Yemen, compared to those collected from the Gulf of Adan, may be attributed to the use of these compounds for agriculture in high mountains, where summer monsoon fall in Yemen and the water flowing mainly into the Red Sea and the Gulf of Adan. However, the amount of water flowing into the red sea is higher than the water flowing into the Gulf of Adan, which causes these higher concentrations. In addition, Red Sea is a small and semi-enclosed body when compared to the Gulf of Adan.

Polychlorinated biphenyls (PCBs) are a class of synthetic, inert, and complex mixture of many compounds originally manufactured in the USA in 1929, and until recently retained in large quantities mainly as dielectric fluids in electrical equipment (Hutzinger *et al.,* 1974; Bleatchy, 1984). Additionally, PCBs are extremely persistent in the environment, and like many other organochlorines of similar nature are widely spread among living organisms all over the world (Tanabe and Tatsudawa, 1991; Koeman and Visser, 1992).

Organochlorine compounds are generally soluble in fatty tissues, but exhibit low solubility in water. This lipophilic hydrophobic characteristic is largely responsible for their bioaccumulation and extreme persistence in marine biota (DouAbul *et al.*, 1987 a). The multiresidual extraction procedure employed in the present work followed by GC-electron capture determination should screen most of the common OCPs and PCBs in all spices. In most cases, conversion of DDT into DDE is initiated by soil micro-organisms immediately after it enters the environment. Other factors such as alkaline pH, light, or heat may also produce chemical changes in the original DDT molecule. Thus, the above observation may be due to metabolic conversion (Bridges *et al.*, 1963) and/or dehydrochlorination (Hannon *et al.*, 1970; DouAbul *et al.,* 1988; DouAbul and Heba, 1995) in the warm, rather alkaline waters of the Red Sea and Gulf of Aden. Moreover, because the volatility of DDE is several times greater than that of DDT (DouAbul *et al.*, 1987 b). It is logical to presume that DDE is more readily transferred via atmosphere to the coastal Red Sea and Gulf of Aden. Since the presence of the original compound suggests recent inputs of DDT to aquatic ecosystem (Aguillar, 1984). It may thus be concluded that there was continuing contribution of DDT to the Red Sea and Gulf of Aden. However, DDT has been officially banned in Yemen. Hence, its residues must be originated from a more remote source or more likely, from continuing illegal use. Technical DDT generally contains <25% *o.p*-DDT, an impurity, however, *o.p*-isomers are less persistent than their *p.p*-analogs (Fry and Toone, 1981; Mansour and Sidky, 2003). This phenomenon may be accounted for the relatively low percentage occurrence of both *o.p*-DDT and *o.p*-DDD, which were 10% and 0% respectively.

Chlorinated pesticide and PCBs residues in commercial fishes caused leukemia cancer, gastric carcinoma, and esophageal, (Haffer, 1983; 1985). PCBs are not manufactured in Yemen, and their presence can thus only be from industrial usage and the possible dumping of products containing PCBs. To the best of the author's knowledge, previous data for PCBs in the Red Sea and Gulf of Aden are not available. The present results are the first of their kind for the region and should serve as background information.

The major conclusions that can be drawn from the present study are; i) the concentrations of the most organochlorine residues in the commercial fish of the Red Sea and Gulf of Aden environment were either below the detection limits or in very low values ii) DDTs occurred in almost all fish samples examined. However, DDTs concentrations were relatively lower than those reported previously in the Arabian Sea. iii) traces of PCBs were also found in some samples of fishes and shell fishes from the Red Sea and Gulf of Aden.

Generally, it is recommended that a continuos monitoring programme for the Red Sea and Gulf of Aden region should be formulated and conducted to ensure that the concentration of DDTs and PCBs are within the base line levels established in the present study.

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Lethal and Sublethal Effects of Atrazine to Amphibian Larvae

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Abstract

The effects of Atrazine contamination on amphibian larval stages were assessed, using acute and chronic toxicity in the laboratory. Tadpoles of *Ptychadena bibroni* at varying post-hatch developmental stages (1, 2, 3, and 4 weeks) were exposed to environmental relevant treatment concentrations of 200, 400, 600µg/L and 3, 30, 100µg/L for acute and chronic toxicity tests respectively. The effects were assessed by comparing mortality, glycogen levels and behavioral response of a control group and a group exposed to the pesticide. The American Society for Testing and Material (ASTM) recommended semi-static renewal bioassay method to be employed, and LC_{50} was measured at 96 hours. Percentage of mortality increased with increase in concentration and exposure duration; but decreased as the tadpoles matured. Mean percentage mortality of tadpoles were significantly affected by concentrations and developmental stages . Derived 96 hours LC_{50} values decreased with increase in exposure duration but increased with each successive developmental stage. Estimated 96 hours LC_{50} ranged from 230.06 – 431.32µg/L. Glycogen levels varied negatively with concentrations, but it increased with each successive developmental stage. Mean glycogen level of tadpoles, exposed to Atrazine, were significantly different in the developmental stages but showed no significant difference with concentrations (F= 1.493, P>0.05). The above results of acute and chronic exposure to Atrazine indicate marked behavioral and physiological effect of Atrazine on *Ptychadena* tadpoles. Results obtained from this study would serve as a fundamental platform for development of Atrazine safety limits for monitoring the waters of the Niger Delta ecological zones of Nigeria.

الملخص

تم تقييم أثار التلوث بالمبيد الحشري أترازين على يرقات الضفادع باستخدام السميةالحادة والسمية المزمنة ف المختبر. حيث تم تعريض يرقات الضفدع *Ptychadena bibroni* البالغ عمرها 1 و2 و3 و4 اسابيع للتراكيز 200و 400و 600 ميكروجرام في اللتر و كذلك3و 30و 100 ميكروجرام في اللتر. وتم التقييم بمقارنة نسب الوفيات ومستوى الجلايكوجين والاستجابات السلوكية باليرقات التي لم تتعرض للتسمم. وتمت المقارنة حسب طريقة الجمعية الاميركية(ASTM) للفحوصات. وتم حساب LD_{50} بعد 96 ساعة. لقد وجد ان نسبة الوفيات تزداد بازدياد التركيز ومدة التعرض ولكنه يتناقص كلما زاد عمر اليرقة. لقد وجد ان حساب LD_{50} يتناقص بزيادة التركيز والمدة ويرتفع بزيادة عمر اليرقة وتراوحت $_{LD50}$ من 23.06 الى 431.32 مايكروجرام في اللتر. تناقصت مستويات الجلايكوجين مع التركيز وازدادت مع عمر اليرقة. وتدل النتائج السابقة على وجود تأثير سلوكي وفسيولوجي للمبيد الحشري أترازين على يرقات الضفدع *Ptychadena*. وتعتبر هذة النتائج أساسا لوضع الحدود اللازمة لتراكيز الاترازين في المناطق البيئية لمياه دلتا النيجر.

Keywords: Amphibian; Atrazine; Life-Stage; LC_{50}; Glycogen Level

1. Introduction

Using Pesticides has increased worldwide over the years to secure food supply for the teaming global population. In tropical regions, Nigeria in particular, an intensive practice has led to higher pesticides usage (Osibanjo and Jensen, 1980). Although it is undisputed that pesticides are essential in modern agriculture, there is a growing concern about possible environmental contamination from agrochemicals. The ecological effects of pesticides on amphibian populations are a growing concern (Bishop 1992, Hall and Henry 1992, Philips 1994). Human activities have led to the release of

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pollutants, for instance, pesticides into the natural environment. This often results in habitat distortion and extinction of local amphibian populations. While pesticides have the potential to affect many aquatic taxa, the impacts on amphibians are of particular concern in the past decade because of the apparent global decline of many species (Blaustein and Wake 1990, Alford and Richards 1999, Houlihan *et al*. 2001, Kiesecker *et al*. 2001). The lists of possible causes of amphibian declines are numerous, and pesticides have been implicated in at least some of these declines.

The decline of world populations of amphibians is a major environmental issue (Vertucci and Corn 1996). Amphibians are an integral part of their ecosystems; affecting nutrient cycling and also serving as high quality prey for many species (deMaynadier and Hunter 1995). In the last 15 years, scientists have accumulated evidence supporting a global decline in amphibians. As the quantitative evidence grows, it is difficult to deny the validity of this global trend (Houlahan *et al.* 2000, Stuart *et al,* 2004). Amphibians are especially at risk from agricultural contamination because they have permeable skin and eggs that readily absorb chemicals from the environment. Many species are vulnerable to aquatic contamination because they experience aquatic and terrestrial stressors, and play vital roles in communities and are sensitive to contaminants. Most amphibians complete their life cycles near fields, where pesticides are applied and have vulnerable embryo and larval stages whose development coincides with pesticides application (Blaustein and Kiesecker 2002, Hayes *et al*. 2003).

The larvae of frog species, *Ptychadena bibroni*, was chosen as test organism for this study because it is the most dominant and widely spread in the Niger Delta regions of Southern Nigeria (Akani and Luiselli 2003) where Atrazine use is substantial.

Atrazine (2 – Chloro – 4 ethylamino -6-isopropylamino-S-trazine) is a selective, pre and post–emergence herbicide used on a variety of terrestrial food crops, non-food crops, forests, residential turf, golf course turf, recreational areas, and rangeland. In Nigeria, it is commonly used for the control of weeds in most farms. Although used to control broadleaf and many other weeds on a range of agricultural and non-agricultural sites, the herbicide's largest use is on corn, sorghum, and sugarcane (Solomon *et al*. 1996). Despite its widespread, intensive use of Atrazine is considered safe because of its short half-life and negligible bioaccumulation and biomagnifications (Solomon *et al*. 1996). The present study investigates the hypothesis that Atrazine may interfere with survival of tadpoles (*P .bibroni*) at ecologically relevant low doses.

Until recently the adverse effects of pesticides on non-target organisms have not seriously been considered when compared with research in the parent fields of experimental Ecology and Toxicology (Sparling *et al. 2000*). Pesticide use is known to cause serious environmental problems, especially in the dry season when the dilution capacity of water systems is low; thus increasing the risk of high concentration of toxic chemicals. Studies on the effectiveness of many commonly used pesticides on target organisms have been carried out extensively in virtually all agro – ecozones globally. However, the side effects of these pesticides on non-target organisms remain largely unknown.

This study simultaneously evaluates the lethal (survival) and sublethal (glycogen level and behaviour) effects of Atrazine on larval stages of the dominant amphibian species: *Ptychadena bibroni* of the Niger Delta of Nigeria. The results of this study would provide a fundamental platform for establishing regulatory limits for Atrazine load in Nigerian Niger Delta waters.

2. Materials and Methods

2.1. Collection of Test Organisms

Eggs of the amphibian species were collected from an inlet of Ikpoba River, an inland River in Southern Nigeria. Egg clutches of the frog were identified in the field by a dichotomous field guide (Amphibian Web 2003, Gosner 1960, and Roedel 2000).

Hatching of eggs, rearing of tadpoles, and testing were done in the post-graduate ecotoxicological research laboratory at the Department of Animal and Environmental Biology, University of Benin, Nigeria. After hatching, emerging larval tadpoles were distributed into six (2.2 x 2.2cm) plastic tanks each containing 1 liter of dechlorinated tap water. They were allowed to acclimatize for seven days in the holding tanks prior to the bioassay (ASTM, 1985). Tadpoles were fed with ad–libititum daily with ground maize powder. Larvae were reared on a 10:14h light: dark cycle (dark from 6 p.m. to 8 a.m.) to minic natural , condition, and room temperature were maintained at 30± 2^0C throughout the duration of the experiment. The water in each holding tank was change every three days.

2.2. Test Chemicals

The pesticide, used for the 96-hour acute toxicity and chronic toxicity tests, was the organochlorine, Atrazine (Atraforce, 80% Top Atazine). The pesticide is commonly used on farms in Nigeria for the control of weeds.

2.2.1. Test Water

Water for toxicity testes was dechlorinated tap water. The water was dechlorinaated by allowing it to stand exposed for 36 hours (Ezemonye and Enuneku, 2005). This water was used for acclimatization, control tests, and for making the various concentrations of the test chemical.

2.2.2. Test Solutions

Stock solutions of the required concentrations were prepared for both pesticides. 1g of 80% pure commercially available Atrazine was dissolved in 1 litre of dechlorinated tap water. The solution was mixed thoroughly until all granules dissolved. One milliliter of this solution was added to 999 ml of dechlorinataed tap water to make a stock solution of 1mg/L. The stock was than diluted into environmental relevant treatment concentrations of 0, 200, 400, 600 µg/L and 0, 3, 30, 100µg/L for acute and chronic tests respectively (Freeman and Rayburn, 2004, Storrs and Kiesecker, 2004).

2.2.3. Acute Toxicity Tests

Acute toxicity tests were conducted according to standard procedures (ASTM, 1996). Fourty amphibian

larvae (two replicates of 20 each) were exposed for 96 hours to each selected concentration of pesticide solution. The semi-static renewal bioassay procedure started with a range finding test (ASTM, 1985, ASTM, 1996). This was used to determine the range that would produce the desired LC_{50} effect for the different life stages. Amphibian larvae of Gosner stages 20, 27, 35 and 43 (Gosner, 1960), which were 1, 2, 3, and 4 weeks old respectively, were used for the test. Exposures lasted for approximately 28 days. Twenty (20) tadpoles were assigned to individual experimental units containing one of the treatments of Atrazine (0, 200, 400 and 600µg/L).

A new stock solution for Atrazine was made up every 3 days immediately before each water change since it has a minimum half-life of 48hours in water (Solomon *et al*. 1996).

2.2.4. Mortality

Mortality was recorded at an interval of 24 hours over a period of 4 days (96-hours) for each post-hatch maturation stage. Tadpoles were taken dead when they turned upside down and sank to the bottom of the tank or when their tail showed no form of movement even when prodded with a glass rod (Mgbaeruhu, 2002).

2.2.5. Chronic Toxicity Tests

The Chronic toxicity test was carried out in a similar manner as the acute test, however, for chronic toxicity tests; very low, and sublethal concentrations of the pesticides were used. Amphibian larvae were exposed to concentrations of 0, 3, 30, and 100µg/L. The lowest concentration 3µg/L of Atrazine was based on the drinking water standard of Atrazine, as set by the U.S. Environmental Protection Agency, (U.S. EPA, 2002). Exposures lasted for approximately 28 days, and every week tadpoles were collected to assess their glycogen levels.

2.2.6. Behavioural Response

Larval behavioral response was monitored in this Behavioral response was assessed in-situ by observing the swimming activity of tadpoles. This was achieved by gently prodding all individual larvae and gauging their response as normal when larvae swim away immediately or as abnormal when there is a delay, or no response, or impaired swimming ability.

2.2.7. Glycogen level Bioassay

The glycogen levels of tadpoles were estimated using digestion, based on glucose oxidase method of Trinder (1969). Reagents used for the glucose oxidase method were Reagent 1(R_1), which contain phosphate buffer (PH7), phenol, and sodium azide. Reagent 2(R_2) contains glucose oxidase, peroxide, 4-aminophenozone, a standard glucose solution, and a color reagent. 1m l of the color reagent was pipetted into dry test tube and, 0.01 ml of sample was added. After color development, 2.0 ml of distilled water was added. The test tubes were thoroughly shaken and incubated at 37^{o}C for 10 minutes. A standard glucose solution was also similarly treated. They were subsequently analyzed, using spectrophotometer; and absorbance was read at 500ml against the reagent blank. Glycogen levels in the samples were extrapolated from a graph of glucose concentration vs. absorbance (Cicik and Engin, 2005).

2.2.8. Statistical Analysis

The susceptibility of the tadpoles to both pesticides was determined by using the Probit (Probit software) method of analysis (Finney, 1971), for median lethal concentration at 96 hours. Safe concentrations at 96 hours for each developmental stage were obtained by multiplying the lethal concentration by a factor of 0.1 (EIFAC, 1998). Computation of confidence internal of mortality rate was also obtained from the Probit analysis used to determine the LC_{50}. The two-factor ANOVA (analysis of variance) in Microsoft Excel was used to test the variable at $P< 0.05$ level of significance. Multiple bar graphs and line graphs were also generated in this study for the pictorial representation of assessment endpoints.

3. Results

The results of the acute and chronic toxicity of tadpoles of *P. bibroni,* exposed to varying concentrations of Atrazine pesticides, are presented in Tables 1 and 2 and further illustrated in Figures 1-3.

3.1. Control

No mortality or morphological changes were observed in the controls for the 96-hour acute toxicity test at the different developmental stages. Tadpoles in the control experiment for both acute and chronic toxicity tests appeared active and healthy throughout the test period. The proportion of abnormal behavioral response in the control was less than 10%.

3.2. Acute Toxicity

The tadpoles of *P.bibroni,* exposed to varying Atrazine concentrations, recorded mortality in all the concentrations. The mean percentage mortality was increased with increase in concentration and exposure duration for each developmental stage (Table I). This indicated that mortality was concentration-dependent. However, mortality was decreased with increase in the developmental stages (Table 1). One hundred percent (100%) mortality was observed in one (1) week old tadpoles at 96 hours. Successive developmental stages of two (2), three (3), and four (4) weeks showed a decrease in percentage mortality at 96 hours of 90%, 80% and 75% respectively (Figure).

Derived 96-hour LC_{50} values for the different developmental stages ranged between 230.058 – 431.323µg/L. Estimated 96-hours LC_{50} values were increased with increase in developmental stages (Table II), which is indicative of a decrease in mortality as the tadpoles mature (Figures I). The Probit analysis also showed that 96-hours LC_{50} values were also decreased with increase in concentration. This indicates an increase in toxicity with increase in concentrations and exposure duration.

LC_{50} values for 96-hours toxicity test at one (1) post hatch could not be determined by using the Probit analysis since the maximum allowable difference of four (4)

Table I. Mean Percentage Mortality of Tadpoles Exposed To Different Concentrations of Atrazine Pesticide at Successive Developmental Stages.

Treatment Time (Hours)	Conc. (µg/L)	Percentage (%) Mortality			
		1 week	2 weeks	3 weeks	4 weeks
24	0	0	0	0	0
	200	18	10	5	5
	400	20	15	13	8
	600	45	30	25	18
48	0	0	0	0	0
	200	38	35	23	15
	400	65	55	33	23
	600	95	68	45	38
72	0	0	0	0	0
	200	65	40	30	20
	400	98	70	50	35
	600	100	83	63	58
96	0	0	0	0	0
	200	95	43	38	28
	400	100	80	63	45
	600	100	90	80	75

Table 2. Relative Acute Toxicity of Atrazine Pesticides to *P. Bibroni* Tadpoles at 96 Hours.

Pesticide	Developmental stages (Weeks)	LC_{50}(95% CL) µg/L	Safe concentrations at 96 hours(µg/L)	Probit line equation
Atrazine	1	-	-	-
	2	230.058 (129.296-297.353)	23.0058	-2.878+3.336XLog(Conc.)
	3	305.897(115.277-461.692)	30.5897	-2.37+2.07XLog(Conc.)
	4	431.323(329.763-633.268)	43.1323	-3.241+3.128XLog(Conc.)

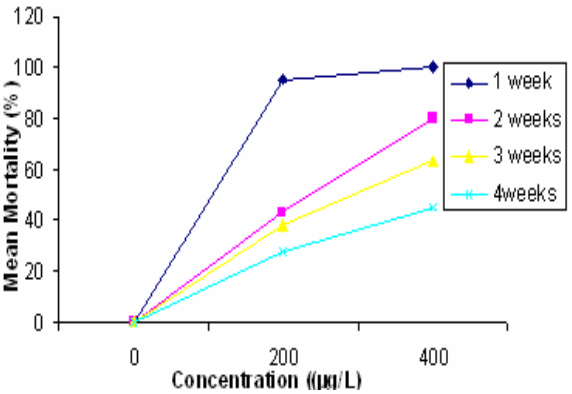

Figure 1. Mean Percentage Mortality of Tadpoles Exposed to Different Concentrations of Atrazine Pesticide at 96 Hours for the Different Developmental Stages.

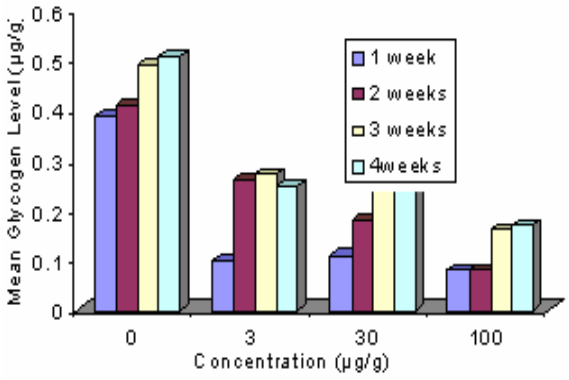

Figure 2. Mean Glycogen Level (µg/g) Of Tadpoles Exposed to Different Sublethal Concentrations of Atrazine Pesticide at Successive Developmental Stages.

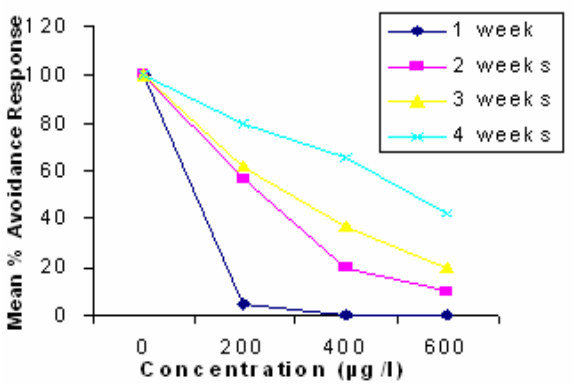

Figure 3. Mean Percentage Behavioural Response of Tadpoles Exposed to Different Concentrations of Atrazine Pesticide at 96 Hours for the Different Developmental Stages.

between successive mortalities was exceeded, and the very high mortality was observed.

Safe concentrations at 96 hours for 2, 3, and 4 weeks old tadpoles ranged between 23.0058 - 43.1323µg/L (Table II).

Mean % mortality of tadpoles was significantly affected by concentrations ($F= 5.120$, $df = 9$) at $P<0.05$ and developmental stages ($F= 29.407$, $df = 9$) at $P<0.05$.

3.3. Chronic Toxicity

Glycogen levels were used as an assessment endpoint in the chronic toxicity test. The result showed that glycogen levels of the amphibian tadpoles varied with concentrations of the test chemical and with successive developmental stages. The values obtained decreased with increase in concentration (Figure 2). However, glycogen values were observed to increase with each successive developmental stage. The levels of glycogen observed in the control experiments were higher than the test experiments. This is indicative of the possible effect of the pesticide on the glycogen levels of the tadpoles.

Mean glycogen level of tadpoles for Atrazine pesticides was significantly different in the developmental ($F= 13.460$), but showed no significant difference in concentrations ($F= 1.493$).

The concentrations also had varying degrees of behavioral alternations in surviving tadpoles as observed in the behavioral response for the pesticide. The behavioral response was also concentration dependent (Figure 3). In the highest treatment concentration for the different developmental stages of the test organism, tadpoles displayed abnormal avoidance response at approximately three (3) hours post-treatment and some died in subsequent days.

4. Discussion

Until recently, the adverse effects of pesticides on non-target organisms have not been seriously considered in Nigeria, and toxicological studies with amphibians are relatively limited in number (Sparling *et al.,* 2000, Ezemonye and Enunekwu 2005). Consequently, only limited data on the toxicity of Atrazine to amphibian larvae of *P. bibroni* are available for comparison with the results of this study. The results of the present study further demonstrated that Atrazine could have adverse direct (mortality) and indirect (physiological and biochemical) effects on amphibian tadpoles with special reference to *P.bibroni*.

4.1. Acute Toxicity

4.1.1. Variations in Percentage Mortality of Tadpoles with Concentrations

In this study, no observable mortality was reported in all the control tests while varying degrees of mortality were reported in the tests concentrations. This is also a clear expression of the effects of the pesticides as possible source of death of test organisms. The results from this study clearly indicate that Atrazine varied greatly in their effects on survival of *P. bibroni.* The highest mortality was found at the highest concentrations, suggesting dose-dependent survival and concentration graded lethality.

Atrazine did not affect larval survival in gray frogs (Diana *et al.* 2000), Northern leopard frogs (Allran and Karasov 2000), and American toads (Berrill *et al.* 1994). However, significant lower survival was reported in streamside salamander (*Amoystoma barbouri*) and spring peepers (*Pseudacros crucifer, Bufo americanus, Rana clamitans* and *Rana sylvatica*) at low concentrations of 3ppb and 4ppb respectively (Storrs and Kiesecker 2004, Rohr *et al.* 2003). Atrazine produced mortality on *Xenopus laevis* as reported by Freeman and Rayburn 2004; and is consistent with this study.

The mortality of larvae could be explained by bioconcentration of this agrochemical or by the vulnerability of amphibian larval stages. Atrazine has been reported to bioconcentrate in amphibian tadpoles (Allarn and Karasov 2004, Naqvi and Vaishnqvi 1993, Saglio and Trijasse 1998). This is an issue of serious ecological consequence because this pesticide is retained in the amphibian's body tissues, which when fed on by a predator can lead to concentration of the chemical from one trophic level to the next (ASTM, 1998, Suter, 1993). Larval mortality occurred most rapidly in the higher concentrations of Atrazine than the controls, suggesting that death may have been influenced by pesticide concentrations.

The data from the present study suggests that exposure of early developmental stages of tadpoles of *P. bibroni* to Atrazine may have permanent effects on these amphibians. They may not have any recovery from their exposure as earlier reported by Rohr *et al.* (2003).

4.2. Stage Dependent Variation in Percentage Mortality of Tadpoles of P. Bibroni

Pesticide toxicity and accumulation studies with freshwater organisms of different trophic levels indicated that uptake and toxicity of pesticides were stage-dependent (Harris *et al.,* 1998). Hall and Henry (1992), Holcomb *et al.* (1987), also stated that many effects of pesticide toxicity seem to be species or life-stage specific. Berrill *et al.* (1998) describing the lethal and sublethal effects of endosulphan pesticide, an organochlorine on the development of embryos and tadpoles of *R. sylvaltica, R. clamitans* and *B.. americanus* reported that different developmental stages of these amphibians displayed obvious differences in susceptibility. Two weeks-old tadpoles of all species tested were sensitive, displaying paralysis as the primary effects. This observation is consistent with the results of this study, the percentage

mortality values decreased with increase in developmental stages for the amphibian species of *P. bibroni.* The varying degree of mortality reported in this study is consistent with the report of Sparling *et al.* (2001), who reported that differences in an organism's biological adjustment and behavioral responses to changes in water chemistry and osmotic conditions depend on the stages of development. The implication of this observation is that the high level contamination of the aquatic environment with pesticides would adversely affect early developmental larval stages of amphibian species.

The 4 weeks old larval stage was found to be a better experimental material for ecotoxicological studies. This is attributable to the observed higher survival rates of 4 weeks old tadpoles of *P. bibroni,* exposed to Atrazine, and make them possible sentinel species for the pesticide. On the other hand, the sensitivity of early-stage amphibian larvae may be a more appropriate bioindicator for these pesticides. It is therefore imperative that a test organism's stage of development should be clearly specified if valid toxicologic comparisons are to be made.

4.3. Chronic and Sublethal Effects.

Sublethal exposure of amphibians to pesticides may be valuable in assessing sensitivity to contaminants than lethal effects (Little *et al.* 1990). This can have important impacts on amphibian communities, and can be more detrimental to amphibians than direct mortality.

4.4. Behavioural Response

Abnormal behavioral response of tadpoles in the treatment concentrations positively correlated with the concentration gradient, many tadpoles displaying abnormal behavioral responses died in subsequent days. However, behavioral response in the control treatment was normal. Again, behavioral response in tadpoles increased as the tadpoles mature. Alteration of normal behavioral response could increase susceptibility to predation (Brodie *et al*,.1983, Cooke 1997) and precede mortality (Kreutzweiser *et al*. 1994). This supports the view that abnormal behavioral alterations resulting from intoxication are more sensitive measures of toxicity than mortality (Brodie *et al*. 1983).

Tadpoles, displaying abnormal behavioral response, were sluggish with impaired locomotion and distorted. Similar effects could be problematic in natural environments by increasing susceptibility to larvae predation and reducing foraging capability. This could be especially detrimental given that pesticides particularly herbicides like Atrazine can also reduce or eliminate larval food supplies (Howe *et al*. 1998). deNoyelles *et al.* (1982) reported that Atrazine concentrations as low as 1- 4µg/L inhibited phytoplankton growth and reduced dissolved oxygen concentrations due to inhibition of photosynthesis. Low oxygen concentrations, which may cause additional stress and could only serve to magnify pesticide toxicity.

4.5. Glycogen Level

Glycogen level, an ecological endpoint of oxidative stress was assessed in this study. The interactions of chemicals in organisms are frequently associated with depletion in storage glycogen, which is evident in decreased energy production (Cicik and Engin, 2005). The glycogen levels of tadpoles exposed to varying concentrations of Atrazine were observed to vary negatively with concentrations. Glycogen level was highest in the control experiment. The depletion in the glycogen levels in organisms. exposed to chemicals and compared to the control experiment, is an indication of probable toxicological effect as observed in oxidative stress. The reduction in glycogen levels of tadpoles exposed to varying test concentrations could be the result of the pesticide affecting the activities of enzymes that work in glycogenolysis (Fournier *et al*. 2004). There was a general increase in glycogen levels at each successive developmental stage, indicative of reduced toxicity with development. The implication of this is that the glycogen level reserves of early larval stages of the amphibian species could be more adversely affected.

Some investigations have also showed that organic contaminants like pesticides could decrease the glycogen level of invertebrates and fish by affecting the activities of enzymes that play active role in the carbohydrate metabolism (Cicik and Engin, 2005). The loss of glycogen (a secondary stress response) could be regarded as a nonspecific response signifying stress, and this has been linked to changes in cortisol during exposures in various stressors (Wedemeyer *et al.* 1990).

5. Conclusion

The significant difference observed in the mortality between the controls and the test concentration showed that the pesticide may have impacted the death of the tadpoles. The study showed that accidental and intentional release of this pesticide into the aquatic environment could threaten amphibian survival. Chronic exposure to Atrazine resulted in reduced glycogen levels and, abnormal avoidance response in tadpoles of P. *bibroni*. The results obtained indicated the pesticide is toxic and could bioconcentrate along food chain; therefore, it is imperative that the use of Atrazine should be carefully monitored.

The amphibian assay described in this study can therefore be used to assess the toxicity of Atrazine in the course of regulatory surveillance and monitoring of the waters in the Niger Delta ecological zones of Nigeria.

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Inhibition of the *in Vitro* Growth of Human Mammary Carcinoma Cell Line (MCF-7) by Selenium and Vitamin E

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Abstract

The effects of the natural form of vitamin E; alpha - tocopherol and two forms of selenium; sodium selenite and selenomethionine on the *in vitro* growth of the human mammary cancer cell line; MCF-7 were investigated. Two experimental protocols were used. In the first, each of the test chemicals was included alone at various concentrations. In the second, one of the two selenium compounds was added at concentrations ranging from 1.0 x 10^{-8} M and 1.0 x 10^{-4} M in the presence of a fixed concentration of alpha - tocopherol (1.0 x 10^{-9} M, 1.0 x 10 M^{-8} M, or 1.0 x 10^{-7} M). In the individual treatment, high concentrations of all compounds caused statistically significant, and concentration – dependent decreases in cell viability. These depressions were in the following order: alpha - tochopherol › sodium selenite › selenomethionine. A combined dose of alpha – tocopherol and each selenium form maintained the same antiproliferative effects that were elicited by higher independent concentrations. In both protocols, a relatively higher inhibitory potency of sodium selenite over selenomethionine was obvious. Although the mechanisms of action are not well understood, several ones are discussed.

الملخص

هدفت هذه الدراسة الى البحث في تاثيرات اثنين من اشكال السيلينيوم (سيلينايت الصوديوم و السيلينو مثيونين) و الشكل الطبيعي للفيتامين (ه) و هو الفا توكوفيرول في نمو احد سلالات خلا يا الغدد اللبنية البشرية السرطانية . و لهذا الغرض، تم تنفيذ نوعين من التجارب؛ الاول زرعت فيه الخلايا لمدة ستة ايام بوجود كل مادة كيماوية بمفردها بتركيزات مختلفة. اما في الثاني، فقد احتوى الوسط الغذائي مستويات متفاوتة من احد شكلي السيلينيوم، اضافة الى تركيز محدد من التوكو فيرول. وبينت النتائج، ان التراكيز العالية من المواد الثلاث سببت تناقصا ذ ا دلالة احصائية قي نسبة الخلا يا التي بقيت حية. و يمكن ترتيب هذا الا ثر في التناقص من العالي الى المنخفض على النحو الآتي: الفا توكوفيرول، السيلينايت، السيلينو مثيونين. وعند اضافة احد مركبي السيمينيوم و الفيتا مين معا، ظهرت زيادة واضحة في مقدرة اي منهما في قتل الخلايا السرطانية. ما زالت هناك حاجة لاجراء مزيد من البحوث للكشف عن طبيعة هذه التا ثيرات و امكانية الافادة منها في مكافحة سرطان الثدي

Keywords: Mammary Carcinoma; MCF-7 Cell Line; Selenium; Vitamin E.

1. Introduction

Specific nutrients and dietary constituents are known to be important players in cancer prevention and treatment (Go et al., 2003; Kritchevsky, 2003; Bingham and Riboli, 2004; Menach et al., 2004). It is becoming increasingly clear that treatment of aggressive cancers that have metastasized to distant secondary sites is a daunting task, and expectations that a single agent will eliminate such cancers are not realistic. Furthermore, a chemopreventive agent should ideally be synthetic or natural component of the diet, and must be non-toxic to the host. Breast cancer has become the second cause of death in women, after lung cancer, and the leading cause of death for women between 35 and 54 (Kamangar et al., 2006). Worldwide, every year approximately one million women are newly diagnosed with breast cancer. Selenium (Se) and vitamin E (VE), both naturally occurring, are antioxidants. They are capable of neutralizing toxins known as free radicals that otherwise damage the genetic material of the cell and impair the immune system and possibly lead to cancer (Ambrosone, 1999; Thomson et al., 2007). Free radicals, specifically hydroxyl radicals, have been implicated in spread (metastasis) of breast cancer (Brown and Arthur, 2001). Researchers have determined that women with metastasized breast cancer exhibit twice as much as free radical damage to the breast tissue DNA than women with localized cancer do (Malins et al., 1996).

Accumulating evidence indicates that Se compounds possess anticancer properties (Beisel, 1982; Medina, 1986; Letavayova et al., 2006). Blood levels of selenium have been reported to be low in patients with prostate cancer (Willett et al., 1983). In preliminary reports, people with the lowest blood levels of Se had between 3.8 and 5.8 times the risk of dying from cancer compared with those who had the highest selenium levels (Salonen et al., 1985;

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Fex et al., 1987). In cultured tumor cells, supplementation with Se inhibited tumor growth and stimulated apoptosis (programmed cell death) (Ip and Dong, 2001). Selenite, an inorganic Se compound, was reported to induce DNA damage, particularly DNA strand breaks and base damage (Letavayova et al., 2006).

Relatively high blood levels of VE have been associated with relatively low levels of hormones linked to prostate cancer (Hartman et al., 1999). While a relationship between higher blood levels of VE and a reduced risk of prostate cancer has been reported only inconsistently (Hartman et al., 1998; Eicholzer et al., 1999), supplemental use of VE (Chan et al., 1999) has been associated with a reduced risk of prostate cancer in smokers. In a double-blind trial studying smokers, VE supplementation (50 IU of VE per day for an average of six years) led to a 32% decrease in prostate cancer incidence and a 41% decrease in prostate cancer deaths (Heinonen et al., 1998). Both findings were statistically significant (Heinonen et al., 1998). In the latter study, however, VE, *in vitro*, has been shown to enhance the cytotoxic effect of several anticancer drugs.

The goal of this study is to test the validity of the synergistic hypothesis of VE (in the form of natural source; alpha- tocopherol; α-TOH) with inorganic (selenite) and organic (selenomethionine; SeMet) selenium, on the inhibition of the *in vitro* growth of the MCF-7 human epithelial mammary cancer cell line. The remarkable capacity of mammary epithelium to undergo development and differentiation provides a research model in which the factors that influence growth, proliferation, morphologic patterning, and differentiation can readily be explored.

2. Materials and Methods

2.1. Test Chemicals

α-TOH (from Sigma - Aldrech; Steinheim, Germany) was dissolved in ethanol (EtOH), and then taken through step-wise dilutions until the desired concentrations were reached. The final dilution using media brought the final concentration of EtOH to 0.1%, so that it does not affect the cell growth. Selenite and SeMet (both from Sigma – Aldrech) were dissolved step-wise in media until the desired concentrations were reached. Enough EtOH was added to the final Se solutions to bring the 0.1% EtOH.

2.2. Cells and Cell Culture

The human mammary epithelial cancer cell line, MCF-7, is derived from a Caucasian woman with metastatic breast cancer (no. 86012803, European Collection of Cell Culture, Salisbury, UK). This cell line is fully characterized, hormonally responsive and carries various steroid hormone receptors, including estrogen receptors (Marth et al., 1985). Cultures were made and maintained according to Maras et al. (2006). Briefly, the cells were cultured in standard growth medium (Dulbecco's minimum essential medium; DMEM, Gibco BRL, Life Technologies, Paisley, Scotland) supplied with 2 mM glutamine, 1 % nonessential amino acids, 15% heat-inactivated fetal bovine serum (FBS, Gibco BRL), phenol red as an indicator of pH, 1 ml of each antibiotic (penicillin and streptomycin, Gibco BRL) and 1 ml of antimycotics (fungizone, Squibb) per one liter of DMEM.

Untreated cells were grown in monolayer in T-75 plastic culture flasks. Treated cells were grown in monolayer in 6-well polystyrene plates (dia. 33 mm). All cultures were kept in an atmosphere of 5% CO_2 at 37 ºC. Untreated cells were fed with fresh supplemented DMEM medium on average of every three days.

2.3. Treatment

The treated cells were plated into 6-well plates with 2.5 ml of supplemented DMEM at initial density of 2.5 x 10^4 viable cells per well. One day after plating (to allow cells to attach to the surface of the well), solutions of α-TOH, or selenite or SeMet or various combinations, freshly prepared as mentioned above, were added to the wells in 2.5 ml aliquots of supplemented DMEM. α-TOH was administered alone at 1.0 x 10^{-5}, 1.0 x 10^{-6}, 1.0 x10^{7-}, 1.0 x 10^{8-}, and 1.0 x 10^{-9} M. Selenite and SeMet were administered individually at 1.0 x 10^{-4}, 1.0 x 10^{-5}, 1.0 x 10^{-6}, 1.0 x 10^{-7}, and 1.0 x 10^{-8} M. Combinations of α-TOH and Se were performed by keeping concentrations of α-TOH constant and varying those of the Se forms. α-TOH was administered to all cultures at 1.0 x $^{10-7}$ M and selenite or SeMet added in varying amounts of 1.0 x 10^{-5}, 1.0 x 10^{-6}, 1.0 x 10^{-7}, and 1.0 x 10^{-8} M. The same was repeated with α-TOH being kept at 1.0 x 10^{-8} M in all the wells and then again at 1.0 x 10^{-9} M, and the Se compounds were given at the various concentrations indicated above. The control wells were plated at the same time as the treated ones with 2.5 ml of supplemented DMEM and incubated for one day. An additional 2.5 ml of supplemented DMEM containing 0.1% EtOH were added to the control wells when the other wells were treated with 2.5 ml of treated supplemented DMEM.

2.4. Cell Harvesting

Harvesting was always done before the untreated cells become confluent or 6 days after treatment. The untreated cells were always fed the day before harvesting to reduce the trauma caused by the process. After the medium was removed, the cells were washed twice with cool Hank's-balanced salt solution (HBSS) to remove any residual medium. Then, the cells were washed once with 0.1% trypsin. After that, the cells were detached from the flasks or the wells with trypsin and EDTA (0.04%) in a 2: 1 ratio, while on a hot plate at 37 ºC. The detached cells were quickly washed into centrifuge tubes with ambient supplemented DMEM at quantities 10 times that of trypsin in the flask and centrifuged at 1000 rpm for 5 min. The cells were then re-suspended in fresh DMEM plus antibiotics. A small portion of cells was aliquoted for counting and the rest, if untreated, was re-plated at appropriate concentrations.

2.5. Cell Counting/ Viability

The cells were counted using a hemocytometer. The viability was checked by the trypan blue exclusion test.

2.6. Phase-contrast Light Microscopy

Black and white photographs of MCF-7 cells in culture were taken using a camera attached to a phase-contrast microscope.

2.7. Scanning Electron Microscopy

The scanning electron micrographs were taken while the cells were plated in Petri dishes containing circular cover slips with diameters of 12 mm. After that, the supplemented DMEM was removed very gently, and the cells were fixed by very slowly adding glutaraldehyde. After one day, the fixative was removed, and the cells were dehydrated in increasing concentrations of anhydrous EtOH (70%, 95% and 100%); two washings in each concentration for a minimum of 5 min., each. Once the cells were in 100% EtOH, EtOH was removed using CO2 Critical Point Drying. Then, the cover slips were mounted onto the scanning electron microscope (SEM) stubs with silver paints. After allowing the silver paints to dry, the cover slips were gold coated and placed into the SEM for observation and photography.

2.8. Statistics.

Results are expressed as means ± SD of three determinations. Using SPSS, values of control and treatment cells were compared by applying ANOVA on the whole treatments followed by two-sample Student's *t* test between control and each treatment group. The statistical significance of difference ($P \leq 0.05$) for the treatment groups was determined relative to the control group.

3. Results

The shape of the MCF-7 is density-dependent. When plated at low density (1.0×10^4 cells/ cm²), MCF-7 form islands of cells. The cells can migrate outward from a group in an amebiod fashion (Figure 1). Migrating cells appear thinner and more elongated producing a more three-dimensional cell than when stationary. When stationary, cells are provided with enough space and flatten out producing a clear distinction between the endoplasm and the ectoplasm (Figure 2). A degree of polyploidy is evident, and the nucleus usually contains one large dense nucleolus, but may contain as many as five and the presence of two is not uncommon. The exterior of the cells is covered with microvilli (Figure 3).

3.1. Independent Chemical Administration

The number of control MCF-7 cancer cells harvested at the end of culture period (2.66×10^5 cells /well) is used as a standard to 100 % proliferation of the cells. Figure (4) presents the degree of potency between individual treatments with α-TOH, selenite or SeMet. An increase in the concentration of α-TOH from 1.0×10^{-9} M to 1.0×10^{-5} M, as well as selenite and SeMet from 1.0×10^{-8} M to 1.0×10^{-4} M resulted in clear progressive increases in the inhibition of the proliferation of the MCF-7 cancer cells. In general, there were statistically significant differences ($p \leq 0.05$) between the higher concentrations used for each compound relative to the negative control. Also, at all concentrations used, except at 1.0×10^{-9} M (α-TOH) and the lowest two concentrations of selenium (1.0×10^{-8} M and 1.0×10^{-7} M), which showed viability values slightly above the control cell viabilities were far below those of the negative control. At a concentration of 1.0×10^{-6} M, α-TOH showed an inhibition of 37 %, selenite demonstrated an inhibition of 60 %, and SeMet produced an inhibition of 87 %. At this concentration, the viabilities of the independently treated cells (Table 1 and Figure 4) as well as in those treated in combination (data not shown in table 1) were all approximately 90 %.

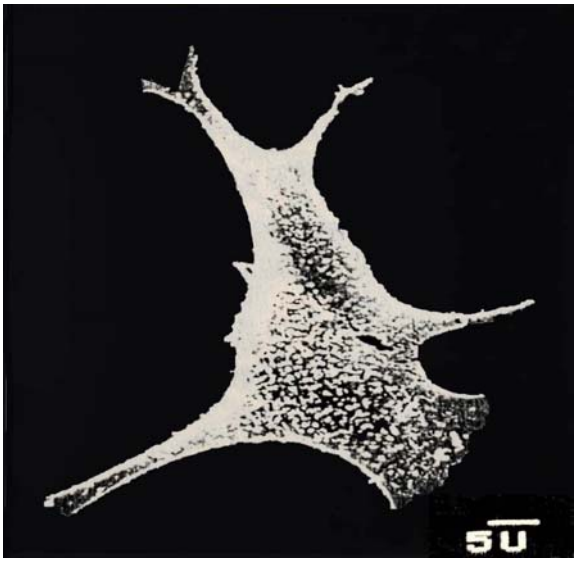

Figure1. Scanning electron micrograph of an MCF-7 human mammary epithelial cancer cell in supplemented DMEM (1000 X). The cell was in the process of extending and retracting pseudopodia to move itself across the cover slip on which it was growing. The white speckles are microvilli, which are less numerous as one goes towards the ends of pseudopodia. The dark patch and breaks in the pseudopodia are artifacts incurred during the drying process.

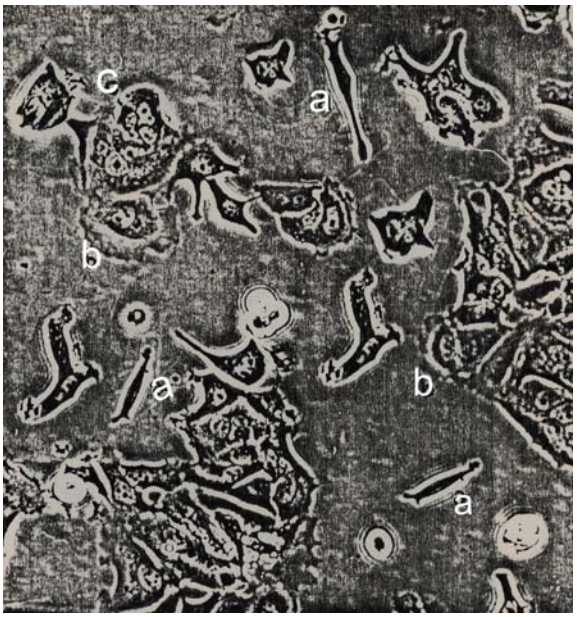

Figure2. A phase-contrast micrograph of live MCF-7 human mammary epithelial cancer cells plated in monolayer in supplemented DMEM (200 X). [a] cells moving across the flask surface. These cells appear dark due to reduced transmitted light through these thicker and more elongated cells than stationary cells [b]. The latter cells have outer, lighter and homogenous ectoplasm as opposed to the inner, darker and heterogeneous endoplasm.

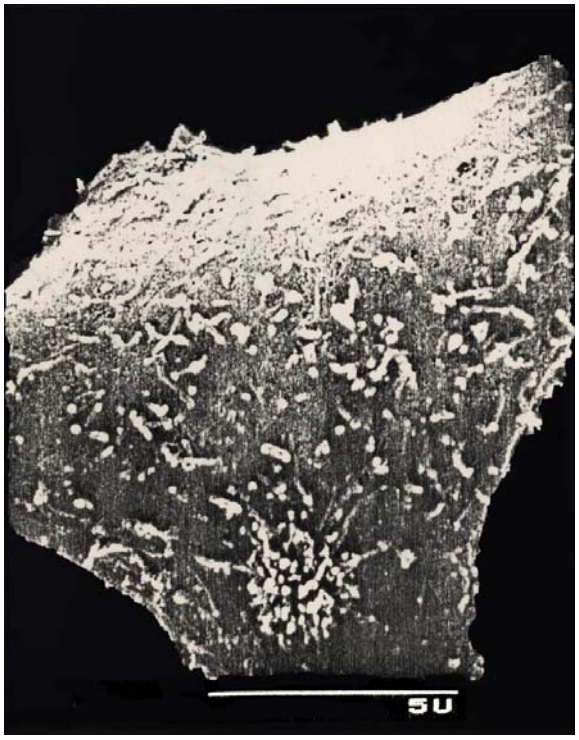

Figure3. Scanning electron micrograph of a portion of the surface of an MCF-7 human mammary epithelial cancer cell in supplemented DMEM (10000 X). Note the presence of microvilli displayed on the cell surface. The aggregation of microvilli near the bottom of the picture is obviously some form of arrested cellular-surface activity, the function of which is not clear. The small darker holes are artifacts incurred during the drying process.

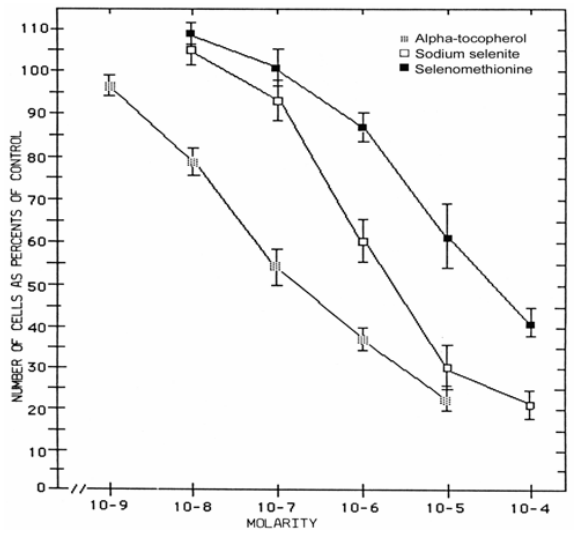

Figure 4. Dose - dependent effects on proliferation of MCF-7 cells by individual treatments. Each data point represents the mean of readings from three wells and the vertical lines are the standard deviations. The results are expressed as percents of the number of cells in the control group (100 % being the control which corresponds to 2.66 x 10^5 cells /33 mm. dia. Well). One day after plating, the cells were independently treated with the indicated concentrations for 6 days.

3.2. Combined Chemical Treatment

In the second protocol, addition of α-TOH with either selenite or SeMet produced a synergistic increase in the level of inhibition of the MCF-7 cells. This is obvious from the significant lines-shift to the left of the combined treatments towards lower concentrations for the same percent proliferation of the control. Table (1) shows the trends of the additive effects on inhibition of proliferation produced by combined treatment and the relative higher potency of selenite over SeMet.

When α-TOH was used alone at 1.0 x 10^{-7} M, the cells were inhibited to 55 % of the control. Selenite, alone, at 1.0 x 10^{-6} M inhibited the cell growth to 60 % of the control. Figure (5) shows that when α-TOH at 1.0 x 10^{-7} M and selenite at 1.0 x 10^{-6} M were used together, much lower percentages of viability relative to control were observed. Similarly, α-TOH, at the same level, in the presence of 1.0 x 10^{-6} M SeMet decreased the number of viable cells to 50 % of the control; which is again below the values recorded for either of the individual treatment; 55 % and 87 % for α-TOH and SeMet, respectively (Table 1 and Figure 6). A higher concentration of SeMet (1.0 x 10^{-5} M) was needed to lower cell viability to 35 % (close to the level [33%] produced with selenite at 1.0 x10^{-6} M). Similar differential inhibition patterns were exhibited when α-TOH was applied at 1.0 x 10^{-8} M (Table 1 and figures 7 and 8) and at 1.0 x 10^{-9} M (Table 1 and figures 9 and 10) with either forms of selenium.

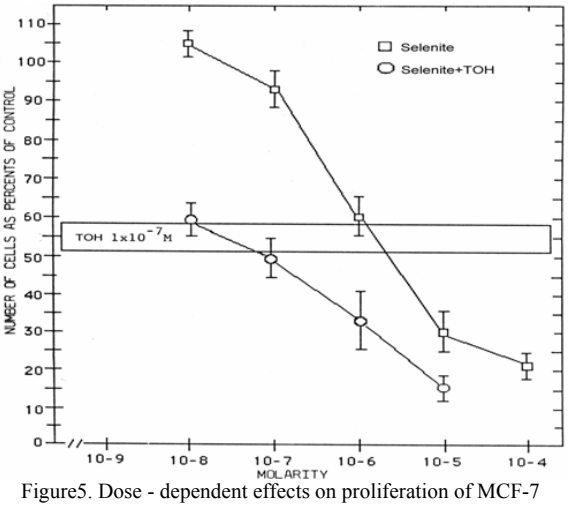

Figure5. Dose - dependent effects on proliferation of MCF-7 cells by combined treatment of alpa-tocopherol (α-TOH) at 1.0 x 10^{-7} M with varying concentrations of sodium selenite (selenite). The effects of individual treatment of selenite are plotted for comparison. The horizontal rectangle represents the effects on proliferation of the constant concentration of the α-TOH with the vertical component indicating standard deviation. Other experimental conditions are as in legend of figure. 4.

4. Discussion

This study has demonstrated that α-TOH, selenite, and SeMet caused concentration- dependent decreases in the number of cells harvested as evidenced by the reductions in the cell viability. These decreases may be due to the inhibition of cell proliferation rather than cytotoxicity of the compounds used. The antiproliferative efficacy of the three test chemicals was in the following decreasing order: α-TOH, selenite, SeMet. Furthermore, synergism between

α-TOH and both forms of selenium has been illustrated by treating MCF-7 cells with a combined regimen of α-TOH with a selenium compound. A combined dose of α-TOH and selenium maintained the same antiproliferative effects that were elicited from higher individual concentration. At a certain level of α-TOH administration, higher concentrations of SeMet were needed to produce equivalent inhibitions than those recorded under lower concentrations of selenite.

In reviewing the literature to compare our findings, very few published investigations were encountered. In the present

Table1. Inhibition of proliferation of the MCF-7 human epithelial mammary cancer cell line by α-tocopherol (TOH), sodium selenite (Selenite) and selenomethionine (SeMet) administrated individually or in combination.

Treatment	Concentration (Molar)	Percent of control in Independent Treatment*	Percent Cell Viability	Percent of Control in Combined Treatment (TOH and Selenium) ** TOH (Molar) 1.0×10^{-7}	1.0×10^{-8}	1.0×10^{-9}
TOH	1.0×10^{-5}	23	94	-----	------	-----
	1.0×10^{-6}	37	91	-----	-----	-----
	1.0×10^{-7}	55	95	-----	------	------
	1.0×10^{-8}	79	96	------	-------	------
	1.0×10^{-9}	96	95	-------	-------	-------
Selenite	1.0×10^{-4}	21	88	-------	--------	------
	1.0×10^{-5}	35	94	16	12	13
	1.0×10^{-6}	60	89	33	30	35
	1.0×10^{-7}	93	92	49	56	60
	1.0×10^{-8}	105	99	59	79	80
SeMet	1.0×10^{-4}	41	95	22	17	17
	1.0×10^{-5}	62	93	35	37	40
	1.0×10^{-6}	87	92	50	73	75
	1.0×10^{-7}	101	97	57	97	100
	1.0×10^{-8}	109	98	----	-----	------

- The percents in this column are the number of individually treated cells harvested as percentage of the number of control cells (cells untreated supplemented DMEM plus 0.1 % ethanol) harvested. The number of control cells harvested is equal to 100 %. The MCF-7 cells were either treated with α-TOH, selenite, or SeMet.
- The percents in these three columns are the number of cells harvested after combined treatments as a percentage of the number of control cells harvested. The number of harvested cells is normalized to 100 %. The MCF-7 cells were treated with various concentrations either selenite or SeMet in presence of a constant concentration of 1.0×10^{-7} M, 1.0×10^{-8} M, or 1.0×10^{-9} M.

experiments, consistent changes in the cell shape and appearance of MCF-7 cells were observed 6 days following treatment with α-TOH and/or selenium. Similar results were reported by Schwartz and Shklar (1992) who studied the cytotoxic effects of α-TOH on the in vitro growth of breast and other human tumor cell lines. Although not directly comparable, these findings may explain the observed cellular changes.

The exact molecular mechanisms underlying the development of breast cancer in general and estrogen-associated breast carcinogenesis, in particular, are not completely understood. It is generally believed that the initiation of breast cancer results from uncontrolled cell proliferation, as a consequence of cumulative genetic damages that lead to genetic alterations. In this regard, several molecular defects in the BRCA1 and BRCA2 have been associated with increased incidence in breast cancer (Bonadona et

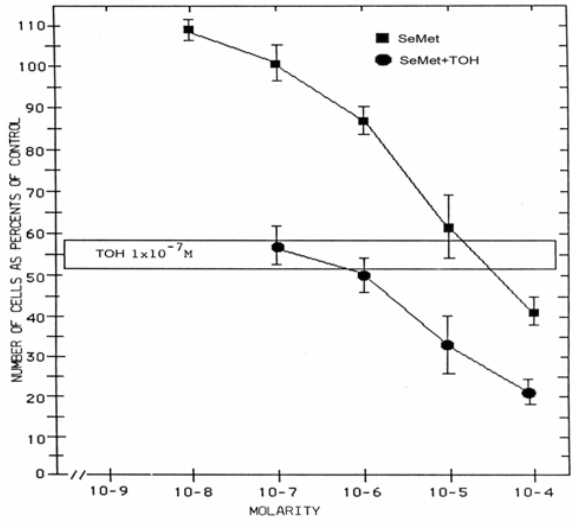

Figure 6. Dose - dependent effects on proliferation of MCF-7 cells by combined treatment of α-TOH at 1.0 x 10^{-7} M with varying concentrations of selenomethionine (SeMet). The effects of individual treatment of SeMet are plotted for comparison. Other experimental conditions are as in legends of figs. 4 and 5.

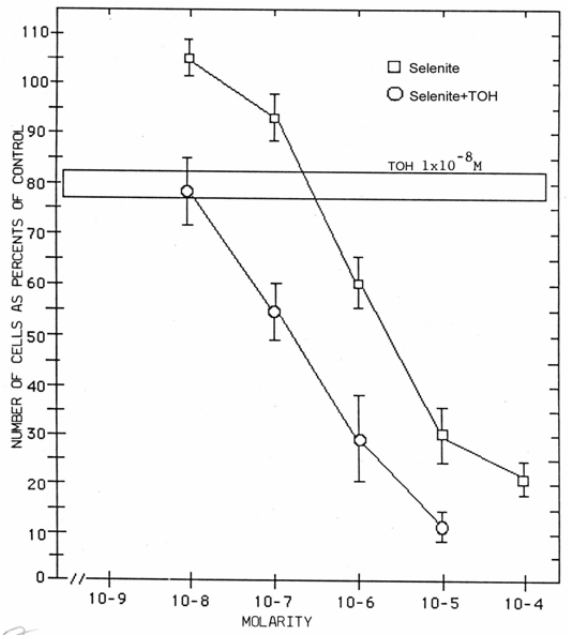

Figure 7. Dose - dependent effects on proliferation of MCF-7 cells by combined treatment of α-TOH at 1.0 x 10^{-8} M with varying concentrations of selenite. For details see legend of figure.5.

al., 2005; Vasickova et al., 2007; Krajc et al., 2008). The possibility that has been raised is that decreased lipid peroxidation may be a mechanism responsible, at least in part, for the increased risk associated with several hormonal and non-hormonal risk factors for breast cancer (Ambrosone *et al*., 1999; Gago-Dominguez *et al.*, 2005). Selenium has several anti-carcinogenic properties, including protection against oxidation and enhancing nucleotide excision repair. In non-dividing cultured human skin fibroblasts, selenocystine induced significant levels of DNA repair (Whiting et al., 1980). In addition, when women with BRCA1 mutation were given Se for three months**,** the number of chromosome breaks (which can lead to breast cancer) was reduced to normal level (Kowalska et al., 2005). The effects of selenium

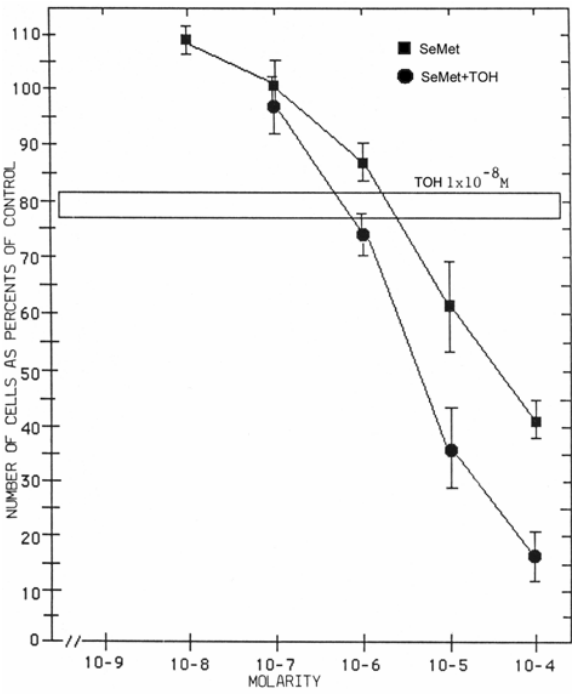

Figure 8. Dose - dependent effects on proliferation of MCF-7 cells by combined treatment of α-TOH at 1.0 x 10^{-8} M with varying concentrations of SeMet For details see legend of figure.6

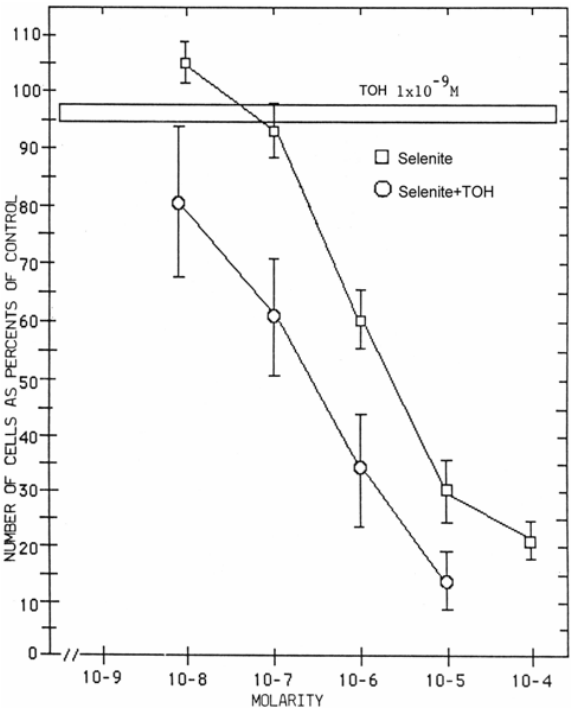

Figure 9. Dose - dependent effects on proliferation of MCF-7 cells by combined treatment of α-TOH at 1.0 x 10^{-9} M with varying concentrations of selenite. For details see legend of figure.5.

compounds on the expression levels of growth arrest and DNA damage-inducible (gadd) genes, and on selected cell death genes were examined in mouse mammary MOD cells (Kaeck et al., 1997). Selenium induced growth arrest and death of these cells. They also induced specific patterns of expression of gadd genes indicating that these genes may mediate some selenium-induced cellular responses. The findings further imply that selenium compounds may be effective chemopreventive agents for human breast carcinogenesis, in which p53 mutations are frequent.

Recently, evidence showing an association between Se, reduction of DNA damage, and oxidative stress together with data showing an effect of selenoprotein genotype on cancer risk implies that selenoproteins are indeed implicated (Rayman, 2005).
The observations of this study, as well as those of others, are in accord with previous reports indicating that Se may lead to cell death, and hence delay in cell proliferation as a result of decreased protein (Vernie, 1984) or RNA (Billard and Peets, 1974) synthesis. Another possibility is that selenium, as an important component of the antioxidant enzyme glutathione peroxidase (GPX), inhibits cell proliferation; and in animal studies protects against a variety of cancers (Ip, 1986) including rat mammary tumors (Chidambaram and Baraclarajon, 1996). In another study (Al-Jassabi and Khalil, 2007), selenium was able to protect mouse liver from microcystin-induced oxidative damage. This was evidenced from the favorable changes in the biochemical markers; alanine transaminaes (ALT), liver glycogen content, thiobarbituric acid (TBA), GPX and glutathione-s-transferase (GST).

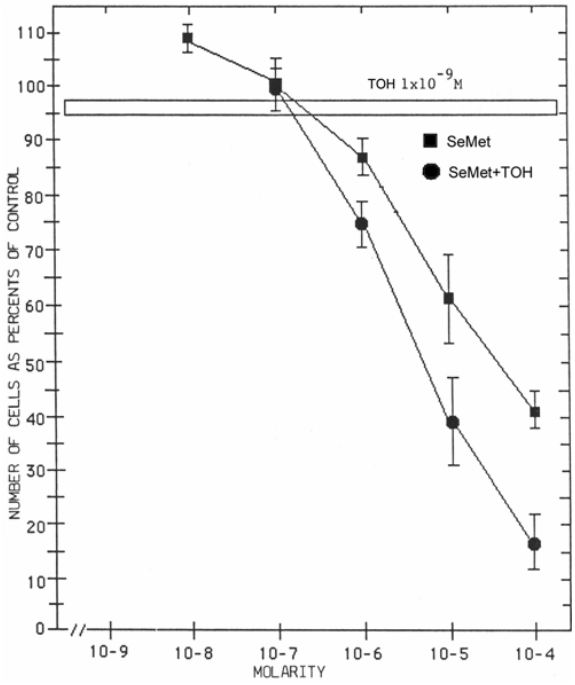

Figure 10. Dose - dependent effects on proliferation of MCF-7 cells by combined treatment of α-TOH at 1.0 x 10 $^{-9}$ M with varying concentrations of SeMet For details see legend of figure.6.

With respect to VE, it was reported that women who did not take this vitamin had a 3.8 times higher risk than did women who were supplemented with VE (Ambrosone *et al*., 1999). A form of VE called VE succinate (VES) has been shown to inhibit the proliferation of estrogen receptor- negative human breast cancer cell lines (Turley et al., 1997). This action, at least in part, is due to induction of apoptosis (or cell death to cancer cells) by VES (Zhao and Yu, 1997). In some experiments (King and McCay, 1983), VE also inhibited mammary tumors in rodents. Not only do the tocopherols quench several of the oxygen free radicals, such as peroxyl, singlet oxygen, and superoxide, but they appear to neutralize some of the nitrogen species as well (Cooney et al., 1993). Nitrogen dioxide, in biological systems, has been recognized as a possible carcinogen that can deaminate DNA bases, resulting in mutations (Christen et al., 1997). In a review of seven case-control and three prospective studies, an inverse association was found between vitamin E intake and breast cancer incidence (Kimmick et al., 1997). Significant inverse relationship between VE intake and pre-menopausal women with a family history of breast cancer was also reported (Ambrosone et al., 1999).

In vitro, VES has been shown to be a potent inhibitor of murine (Slack and Proulx, 1989) and human neuroblastoma (Helson and Parasad, 1983), rat glioma cells (Rama and Prasad, 1983) murine B-16 melanoma cells (Prasad and Edwards-Prasad, 1982), human prostate carcinoma cells (Ripoll et al., 1986), avian lymphoid cells (Kline et al., 1990a) and human promyelocytic cells (Turley et al., 1997). In a more recent study, Al-Jassabi and Khalil (2006) have shown that VE is capable of reducing microcystin-induced damage if administered prior to toxin dose. This effect was explained by VE radical scavenging potentials through inhibition of 8-hydroxydeoxyguanosine, a biomarker for oxidative damage, and generation in DNA. Vitamin E, including the tocotrienols, possesses important cellular functions outside its antioxidant activity, especially in the case of the malignant cell. It was shown that RRR-alpha-tocopherol succinate demonstrated a powerful ability to induce apoptosis in MDA-MB-435 human breast cancer cells in culture (Yu et al., 1997a). At four days following exposure, 74% of the cells were apoptotic. Utilizing antibodies to block Tumor Growth Factor (TGF)-beta, it was shown that the cytotoxic effect of VES could be completely blocked, indicating that the apoptosis was induced by stimulating TGF-beta production. In a further study, Yu and colleagues exposed murine EL-4 T-lymphocytes to VES and found a 95% apoptosis rate within 48 hours (Yu et al., 1997b). Analysis demonstrated that the cells treated with VES were locked in G_1 cell cycle phase, with decreased c-myc and increased bcl-2, c-fos, and c-jun mRNAs. There was also an increase in the transcriptional factor (activation protein1, AP-1) binding. The exact cause of the induced apoptosis remains unknown and is not entirely related to TGF-beta since VES can induce cell arrest in non-TGF-beta-responsive human prostate cells. It is important to note that cell arrest and cell growth inhibition affect only cancer cells and not normal cells which makes VES a valuable adjunct in the treatment of cancer. Another way VE affects cancer growth is by stimulating the immune system. Vitamin E has been shown to enhance both cellular and humoral immunity and to induce macrophages to produce elevated levels of interleukin-1 (IL-1) and/or down-regulate prostaglandin E2 (PGE2) synthesis (Tengerdy and Brown, 1977; Kline et al., 1990b; Meydani et al., 1990). Elevated PGE2 is known to suppress immunity. Vitamin E has been shown to inhibit the activation of phospholipase A2 and hence the initiation of the eicosanoid cascade (Douglas et al., 1986).

In conclusion, selenium at both low and high concentrations induced growth arrest and death of human breast cancer MCF-7 cells and enhanced rather than antagonized the anticancer effect of α-TOH. The mechanism(s) of this enhanced interactive effect is not clear yet, but may be related to their complementary action as antioxidants. Although these results have been generated with an *in vitro* system using a single cell line, they are encouraging and provide some scientific justification for further research using other mammary carcinoma cell lines as well as for clinical testing in both pre- and post-menopausal breast cancer patients.

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دراسة حول تغذية أفعى الأهرام *Echis* (Geoffroy Saint-Hilaire, 1827) *pyramidum* في منطقة جازان ،المملكة العربية السعودية

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الملخص

تم خلال هذه الدراسة التطرق إلى دراسة تغذية أفعى الأهرام *E. pyramidum* على مدار عام كامل خلال الفترة من شهر مارس 2003م إلى شهر فبراير 2004م في منطقة جازان من المملكة العربية السعودية والتي تمثل مكان تواجد هذا النوع .

وقد أوضحت الدراسة الحالية ومن خلال (دراسة محتويات معدة) أفعى الأهرام إلى أن الغذاء المفضل لها صيفاً هو العلاجيم (40.48%)، وشتاءاً مفصليات الارجل كذلك بينت الدراسة تغذي أفعى الأهرام *E. pyramidum* على نوعين من العقارب يشاركونها نفس البيئة هما عقرب *Nebo hierichonticus* (2.38%) و عقرب فلسطين الصفراء *Leiurus quinquestriats* (2.38%) وصرصور الحقل الأسود *Gryllus bimaculatus* (26.43%) والسحالي (2.38%).

وخلصت الدراسة في هذا الجزء إلى أن أفعى الأهرام حيوانية التغذية وأنها ذات تغذية متخصصة فبرغم من تواجد العديد من الفرائس إلا أنها ذات تغذية انتقائية عند الظروف البيئية والمناخية المناسبة . كما أشارت الدراسة النشاط اليومي للأفعى حسب فصول السنة ؛ ففي فصل الصيف يتأخر نشاط الأفعى إلى ما بعد غروب الشمس ويكون قرب المسطحات المائية وفي المناطق المفتوحة . وبعد التغذي تنتقل إلى حواف الأودية المرتفعة حيث تقل درجة الحرارة وتتعرض للتيارات الهوائية . أما في الشتاء ؛ فيكون نشاطها مع الغسق ولكن ينحصر نشاطها مع تقدم الوقت من الليل بين الأحجار وتحت الأشجار . كما لوحظ أن فترة نشاط الأفعى كان متوافقاً مع فترة نشاط فرائسها ، إضافة إلى ذلك فقد أثبتت الدراسة شرب الأفعى للمياه في البيئة ، وأشارت إلى طريقة إمساكها للفريسة والمرتبط بنوعيتها .

Abstract

An ecological study was conducted on the E. pyramidum for one year- from March 2003 to February 2004- in Jazan district in Kingdom of Saudi Arabia, the habitat of this viper.

Following an anatomy of the E. pyramidum, the present study found out that in the summer, its favorite food is toads (40.48%) and in the winter it is arthropods (14.28%) and rodents (7.14%). Water resources are available to the toads throughout the year but the different temperature during winter minimizes the viper's activity around water areas and plains, thus unable to feed on these toads. For this reason, it feeds on rodents in winter and possibly birds though there is no anatomy evidence for that. Although garra tibanica is found in these waters, the anatomy did not prove that the viper fed on this kind of fish. On the contrary, this study proved that the E. pyramidum feed on two other kinds of vipers living in the same environment which are Nebo hierichonticus (2.38%) and Leiurus quinquestriats (2.38%) in addition to Gryllus bimaculatus (26.43%) and lizards.

In this respect, the study concluded that E. pyramidum is carnivorous and is selective in its feeding during suitable environmental and climatic conditions. It was also found out that its daily activity differs according to the seasons; during the summer, the viper delays its activity until sunset which is often near the water area and plains. After feeding, it moves to the edges of high valleys where there are lower temperature and air currents. In winter, the viper is active with nightfall where its activity decreases with the advent of night and is limited to trees and between the rocks where it has been noticed that its activity coincides with that of the preys. The present study also proved that the E. pyramidum drinks from the water resources and referred to the fact that the viper's manner of hunting depends on the type of prey.

الكلمات الدالة: أفعى الأهرام ، حيوانية التغذية ، العلاجيم ،جازان.

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1. مقدمة

!!تشغل المملكة العربية السعودية مساحة كبيرة من شبه الجزيرة ، وتمتد هذه المساحة جغرافياً بين المناطق المدارية والدافئة المتباينة في الظروف المناخية ، مما أدى إلى درجة فريدة من التنوع البيئي والأحيائي بين مناطق المملكة ، وانعكس ذلك على تنوع المجاميع الحيوانية (الشريف ، 2002 ؛ العبيكان وآخرون ، 2003) ، ومن هذه المجاميع مجموعة الزواحف والتي تضم بعضاً من الأنواع السامة والتي تمثل جزءاً من المنظومة الفطرية الزاحفة التي تعيش وتدب على أرض المملكة العربية السعودية ، وأشهر هذه الأنواع هي الثعابين السامة(Mazuch, 2004, 2005a, 2005b)

يوجد في الجزيرة العربية خمسة انواع لنوعين منها حدد تحت النوع من جنس *Echis* هي ؛ *Echis coloratus coloratus* ؛ *Echis pyramidum* ؛ *Echis omanensis* ؛ *Echis sochureki sochureki* ؛ *Echis kosatzki* ، (Gasperetti, 1988;Babocsay 2001,) (2003; Mazuch 2004, 2005b ،بينما في المملكة العربية السعودية يوجد تحت نوع أفعى السجاد الشرقي *Echis c. coloratus* ونوع أفعى الأهرام *Echis pyramidum* (Mattison, 1995) تعيش على افتراس حيوانات أخرى ليست ضعيفة ولا مريضة أو ذات مظهر خارجي جذاب، وذلك للحصول على حاجتها من الطاقة وليس للعبث (Russell, 1980) . ومعظم الثعابين وخاصة السامة منها تبدو نشطة في بحثها عن غذائها حتى تلك التي تستخدم طريقة الانتظار ثم الانقضاض Sit and wait predatorsأو أسلوب الكمين للفريسة Ambush (Mattison, 1995; Kardong and smith, 2002) . فمنها ما يستخدم الرؤية وبعضها يعتمد الرائحة وقسم آخر يعتمد على الخبرة البيئية المكتسبة في البحث عن الفريسة (Norrdahl and Korpimaki, 2000; Head *et al.,* 2002) ، كما تملك بعض الثعابين حواس جيدة للرائحة والطعم والاهتزازات تمكنها من التمييز بين الفريسة المناسبة والأعداء (Cundall and Greene, 2000; Shine *et al.,* 2004) ، في حين تستطيع بعض الأفاعي التمييز بين أحجام الفرائس وحقنها بكميات من السُّم يتناسب مع حجمها ، لضمان شل حركتها وسرعة قتلها (Hayes, 1995). إضافة إلى امتلاك بعض الثعابين أعضاء حساسة جداً تساعدها في تحديد وتتبع الفريسة قبل وبعد عملية العض مثل عضو جاكبسون في سقف الحلق (Chiszar *et. al.*,1986 , 1992 ; Whitaker *et. al*., 2000)

2. منطقة الدراسة

تقع منطقة جازان في أقصى الجنوب الغربي من المملكة العربية السعودية بين خطي طول ´20 □41 - ´20 □ 43 شرقاً بين دائرتي عرض ´20 □16 - ´40 □17 شمالاً ،(شكل 1). فهي تقع ضمن المنطقة المدارية ، كما وتتميز بوقوعها على جهة بحرية تمتد بطول يصل إلى نحو 270كم على ساحل البحر الأحمر ، ويضاف إلى ذلك أن امتداد سلسلة جبال عسير في القسم الشرقي من المنطقة في الاتجاه الجنوبي الشرقي والشمال الغربي كان له الأثر الكبير في استقبال الرياح الجنوبية الغربية الرطبة ومواجهتها (السرسي و عريشي ، 1995م) . كل هذه العوامل مجتمعة أثرت في مناخ منطقة جازان ، فجعلت سهول المنطقة كسائر شواطئ البحر الأحمر نزرة الأمطار شديدة الحرارة مثيرة الرطوبة ، والجبال أقل حرارة نسبيا وأخف رطوبة ، وأما القسم الجبلي المرتفع فهو معتدل بشكل عام صيفاً ويقرب للبرودة شتاءً . (الزيلعي وآخرون ، 2003م) .

إن الغطاء النباتي في منطقة جازان يتأثر بالظروف الطبيعية من أمطار وتضاريس ، ونظراً لاختلاف تضاريس المنطقة ومناخها فإن الغطاء النباتي يختلف . فعلى سفوح الجبال المرتفعة حيث تغزر الأمطار تنمو تجمعات شجرية متنوعة منها أشجار العرعر Juniperus procera ، وأشجار الزيتون البري Olea europaea ، وأشجار السَّلم Acacia ehrenbergiana والطلح Acacia gerrardii والسَّمَر Acacia tortilis ، وعند سفوح الجبال الدنيا تقل كثافة هذه الأشجار ويقل تنوعها (الزيلعي وآخرون ، 2003م) ، ويتخلل هذه الأشجار الكبيرة بعض الشجيرات الصغيرة والأعشاب ، أما في بطون الأودية ، وعلى ضفافها فتنتشر أشجار السِّدر Ziziphus spina-christi والأثل Tamarix aphylla وشجر الأراك Salvadora persica (Arak) ؛ وهي كثيفة في الداخل ، وبخاصة أشجار السِّدر ، بينما تزداد كثافة الأراك بالاتجاه صوب السهل الساحلي ، وتظهر تجمعات من شجر الدوم Hyphaene thebaica بالقرب من الساحل (الزيلعي وآخرون ، 2003م).

3. المواد والطرق

تكونت هذه الدراسة من جزأين هما الجزء الحقلي والذي يشتمل على جمع العينات من البيئة الطبيعية للحيوان ، والجزء المعملي والذي يشتمل على أخذ أطوال العينات وأوزانها ، ومن ثم تشريحها ونزع المعدة لغرض دراسة نوعية الغذاء لهذه الأفعى.

4. الدراسة الحقلية

يتضمن العمل الحقلي جمع العينات شهرياً بواقع (8 ± 1) عينات بالغة من الجنسين خلال أشهر السنة ، من الفترة مابين مارس 2003م وحتى شهر فبراير لعام 2004م من موقع الدراسة وذلك لاستخدام هذه العينات في دراسة نوعية الغذاء وطبيعة التغذية خلال العام ، بالإضافة لدراسة أسلوب هذا النوع من الأفاعي في التغذية خلال فصول السنة . هذا بالإضافة لدراسة سلوك التغذية في الحقل خلال أشهر السنة من خلال المتابعة اليومية للحيوان أثناء فترة نشاطه وتسجيل الملاحظات وأخذ الصور الفوتوغرافية أثناء التغذية .

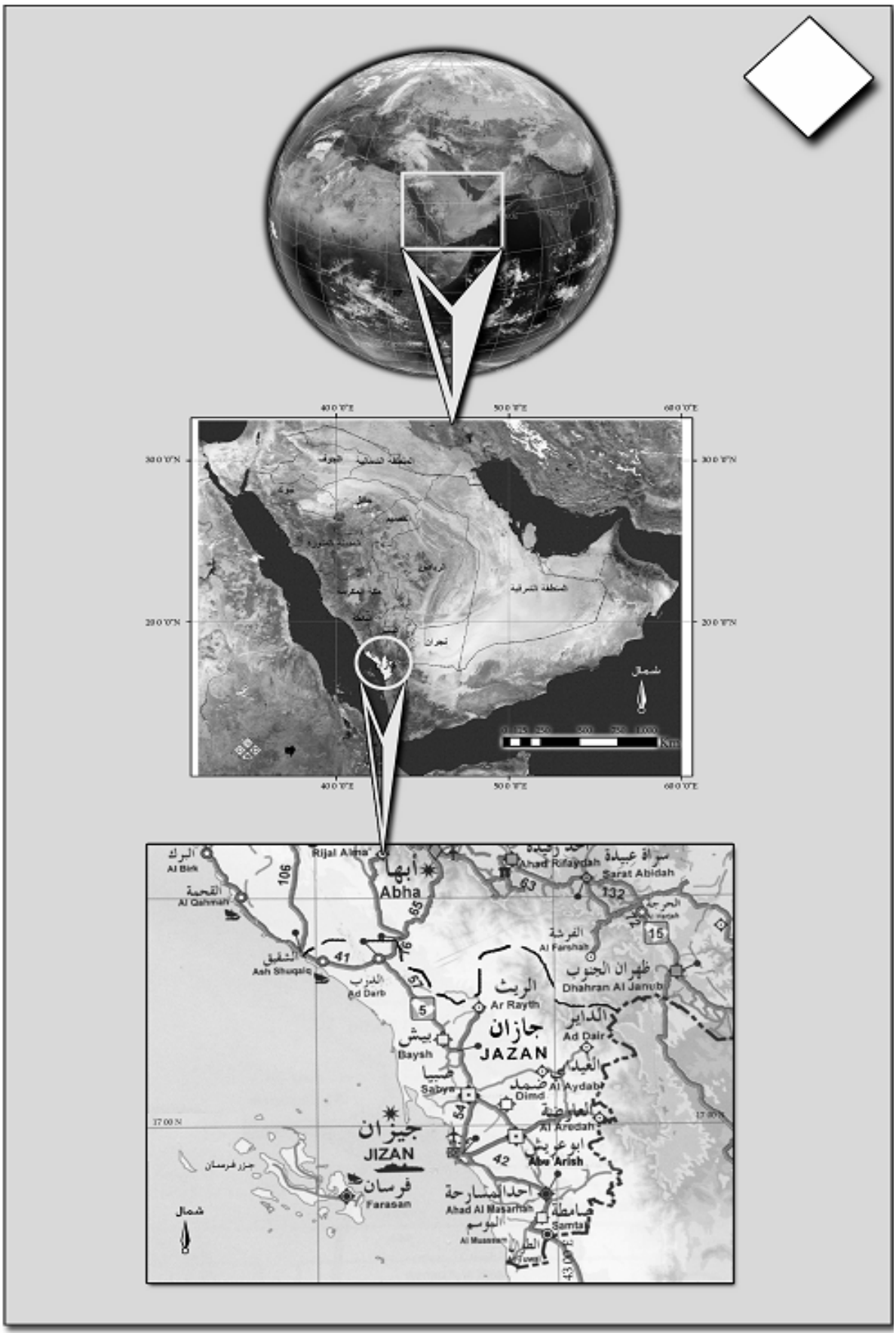
5. الدراسة المعملية

تنقل العينات التي تم جمعها في الحقل إلى المعمل ، ومن ثم تقتل بالتخدير بمادة الكلوروفورم Chloroform ، ثم تؤخذ الأوزان والأطوال اللازمة لأقرب كسر عشري ، ثم بعد ذلك تشرح العينة وتنزع المعدة وتوزن بميزان من نوع (Ainsworth, Denver instrument Co., USA) لأقرب كسر عشري ثم تفتح في طبق بتري Petri dish وتفرغ محتوياتها وتفرز ومن ثم تصنف لمعرفة نوع وطبيعة الغذاء. وبعد التشريح تحفظ العينات المشرحة في الفورمالين10٪ Formaldehyde ، أما المواد الغذائية الموجودة في المعدة فتحفظ في كحول إيثيلي تركيزه 70٪ Ethanol في درجة حرارة المعمل .

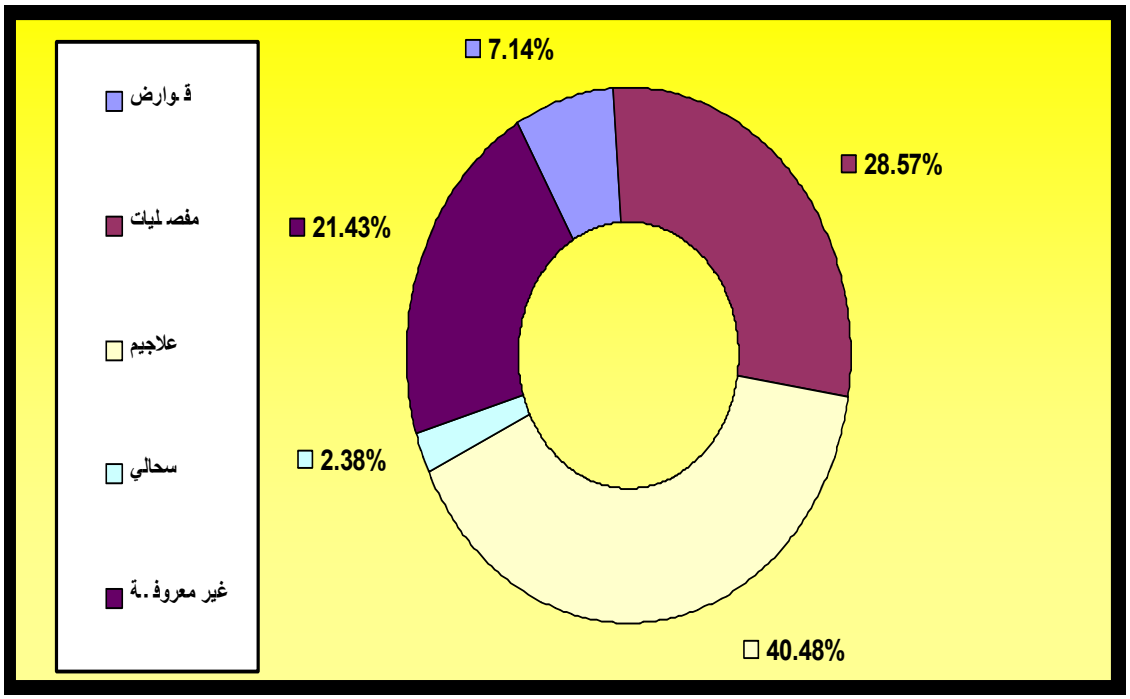
6. النتائج

لقد تمت دراسة وتشريح معدة 98 عينة من أفعي الأهرام تم جمعها من منطقة الدراسة، منها 56 عينة فارغة المعدة ، ووجد في الـ 42 عينة الأخرى حيوانات تنتمي لمجموعات حيوانية رئيسية هي: القوارض، العقارب، العلاجيم، المفصليات، والحشرات، والسحالي، و نسبة من المواد الغير معروفة (شكل 2).

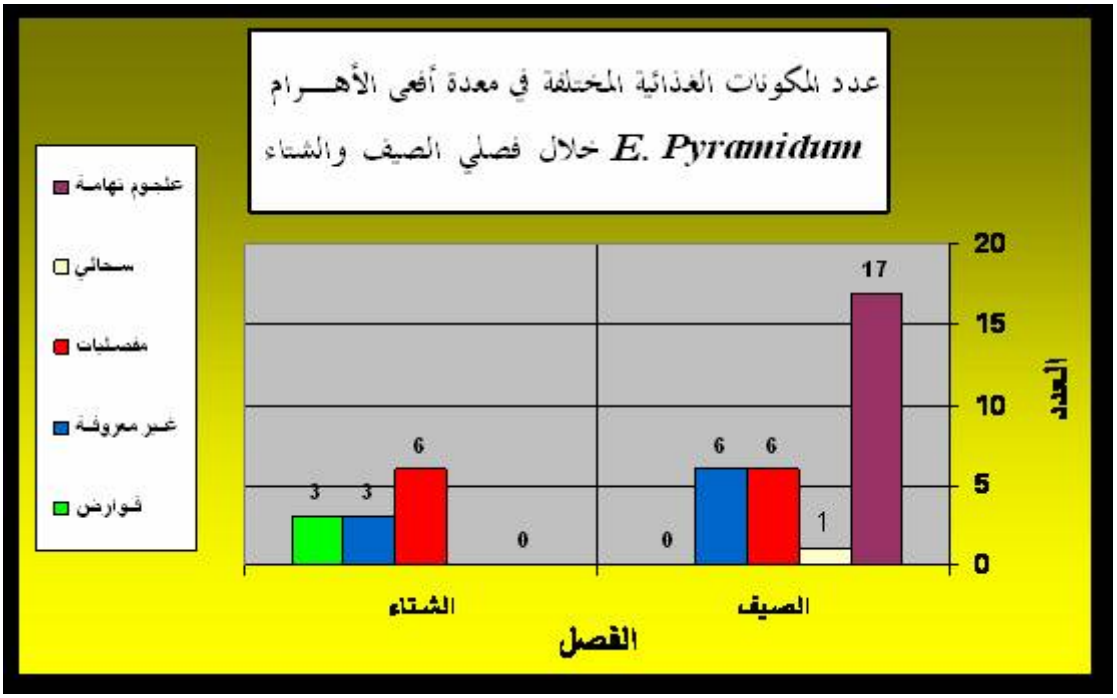
وقد وجد أن أفعى الأهرام حيوانية التغذية؛ فمن فحص محتويات المعدة شكل علجوم تهامة Bufo tihamicus التابع لعائلة العلاجيم Bufonidae نسبة 40.48٪ من الغذاء خلال فترة الدراسة ، أما المفصليات Arthropods فكانت نسبتها 28.57٪ ، شكل منها صرصور الحقل الأسود Gryllus bimaculatus من عائلة Gryllidae نسبة 50٪ ، في حين شكلت الحشرات التابعة لرتبة غمدية الأجنحة Celopeta نسبة 9.52٪ ، ثم عقرب فلسطين الصفراء Leiurus quinquestriatus في الصيف ، وعقرب نيبو Nebo hierichonticus في أشهر الشتاء التابعتين



شكل (1) . خريطة المملكة العربية السعودية والمنطقة المكبرة توضح منطقة الدراسة .



شكل (2): المكونات الغذائية لأفعى الأهرام خلال فترة الدراسة.



شكل (3) :المكونات الغذائية لأفعى الأهرام خلال فصلي الصيف والشتاء.

لعائلة Buthidae بنسبة 2.38٪ ، كتسجيل وحيد لهما خلال فترة الدراسة ، تلا ذلك القوارض Rodents بنسبة 7.14٪ فالسحالي بنسبة 2.38٪ . بالإضافة إلى مواد غير معروفة شكلت نسبة 21.43٪ من مجمل مكونات معدة الأفعى . كما وجد ضمن محتويات المعدة لحيوان الدراسة حبيبات من الرمل وأغصان صغيرة لبعض النباتات طيلة فصل الصيف (جدول 1) .

وقد لوحظ اختلاف في نسب المكونات الغذائية باختلاف الفصول ، حيث شكلت علاجيم تهامة مجمل تغذية الأفعى في فصل الصيف ، وانعدمت نسبتها خلال أشهر الشتاء. وعلى العكس من ذلك فقد انعدمت نسبة القوارض من مكونات معدة الأفعى خلال أشهر الصيف عنها في الشتاء . أما المفصليات فكانت نسبها من تغذية الحيوان متفاوتة طوال أشهر الصيف والشتاء وسجلت نسبة 14.28٪ في كلا الفصلين .

كما سجّلت الدراسة تغذية وحيدة على السحالي بالرغم من وجودها في منطقة الدراسة وبكثرة في شهر سبتمبر ، وسجلت هذه الدراسة وجود أفعى الأهرام في عش طائر الوروار Merops apiaster داخل الجحور التي يعشش فيها على حواف الأودية إلا أنه لم يوجد طائر ضمن مكونات المعدة خلال موسمي الصيف والشتاء . كما لوحظ من خلال هذه الدراسة أنه كلما كبر حجم الأفعى كلما كبر حجم فريستها ، مما يعطي دلالة على أن هناك علاقة بين حجم الأفعى وفريستها . ومن جهة أخرى لم يلاحظ أي اختلاف بين الذكور والإناث من حيث نوعية الغذاء وكميته . وقد تمت مراقبة طريقة التغذية في هذه الأفعى خلال موسم الصيف حيث كانت الأفعى تشاهد قرب

جدول (1) . النسب المئوية للحيوانات التي تتغذى عليها أفعى الأهرام *E. pyramidum* في منطقة الدراسة.

النوع	العدد	النسبة (%)
علجوم تهامة *Bufo tahamacus*	17	40.48
غير معروفة	9	21.43
صرصور الحقل الأسود *Gryllus bimaculatus*	6	14.28
حشرات من رتبة غمدية الأجنحة Cleopetra	4	9.52
قوارض Rodents	3	7.14
عقرب نيبو *Nebo hierichonticus*	1	2.38
عقرب فلسطين الصفراء *Leiurus quinquestriatus*	1	2.38
سحالي Lizards	1	2.38

المياه في بداية نشاطها اليومي خلال الليل وذلك لاقتناص فرائسها من العلاجيم ، حيث تعض الفريسة ـ العلاجيم ـ ولا تتركها حتى يبدأ مفعول السُّم ومن ثم تبتلعها (شكل 3) .

وقد استغرقت هذه العملية لإتمام عملية الابتلاع الكامل فترة زمنية تراوحت ما بين 6 – 15 دقيقة . ثم في الساعات المتأخرة من الليل وبعد عملية التغذية تذهب على حواف الأودية بعيداً عن المصادر المائية لوقوعها في بطون الأودية المرتبطة بارتفاع الحرارة في منطقة الدراسة . كما سجّلت الدراسة الأفعى وهي تشرب الماء خلال أشهر الصيف في تسجيل وحيد أثناء فترة البحث . أما في موسم الشتاء فكانت الأفعى تحت الأشجار وبين الأحجار ونادراً ما كانت بعيداً عنها مع توفر العلاجيم في المصادر المائية ، وشوهدت آثارها قبل غروب الشمس تتنقل بين الأشجار الكثيفة. ويمكن القول أن هذا النوع من الأفاعي يتغذى على مجموعة متنوعة من الحيوانات في منطقة الدراسة أو غيرها من المناطق الأخرى التي ينتشر بها .

7. المناقشة

تتغذى الثعابين بشكل عام على الحيوانات الموجودة في بيئاتها ، كالقوارض ، والضفادع ، والعلاجيم ، والأسماك ، والحشرات ، وديدان الأرض ، والطيور (Spawls et al., 2002; Gassperetti, 1988 ; Mazuch, 2005a)

ولا تختلف الأفاعي عن هذه النظرة العامة ، فبتحليل محتويات معدة أفعى الأهرام E. pyramidum تبين أن الغذاء المفضل لها صيفاً هو العلاجيم ، وشتاءاً القوارض . وربما يرجع السبب في تفضيلها للعلاجيم صيفاً توفر المصادر المائية بشكل مستمر طيلة أشهر العام وبالتالي توفر البيئة المناسبة لها للعيش بأعداد كثيرة في البيئة إلا أن اختلاف درجة حرارة البيئة شتاء يحد من نشاط الأفعى حول المسطحات المائية والمناطق المفتوحة مما يجعلها بعيدة عن أماكن وجودها وبالتالي التغذي عليها لذلك تعتمد في غذاءها على القوارض شتاءاً . وكذلك تزامن النشاط اليومي لهما خلال الليل ويدعم هذا ما أشار إليه (Balletto et. al., 1985) أن علاجيم تهامة Bufo tihamicus تكون في قمة نشاطها ليلاً وخصوصاً عند وجود كميات من المياه الناتجة من مصارف الري أو الأمطار أو أي مصادر أخرى ، أما في النهار فتكون كامنة داخل الجحور أو تحت الصخور أو أي ملجأ لها يقيها من درجات الحرارة العالية نهاراً . إضافة إلى ذلك فإن انتقائية الفرائس عند أفعى الأهرام وفقاً لظروف البيئة يتطابق مع ما ذكره (السويلم ، 2001) و (Gassperetti, 1988) في حديثهم عن تفضيل الأفعى المقرنة للقوارض مرجعين السبب في ذلك لوفرتها في البيئة والتشابه بينهما في فترة النشاط خلال الليل.

إن الحيوانات تتعرض خلال نشاطها اليومي إلى مدى واسع من الضغوط الحيوية والغير حيوية ، متضمنة تلك الضغوط تغيير الموطن (Denver,1997 ; Hoffman et. al.,1999) والتفاعلات الداخلية بين الأفراد كالتنافس والافتراس والتطفل (Lima,1998)، إلا أن أكثر هذه الأسباب تأثيراً على الحيوانات بإحداث الضرر أو الموت هو التنافس أو الافتراس . والحيوانات تقلل من احتمالية الهجوم من قبل المفترسات بالتقليل من ظهورها وزيادة الانتباه (Lima,1987) . وعليه فإن زيادة خطر الافتراس يقود الحيوانات إلى التقليل من وقت مزاولة النشاطات الحيوية كالتغذية والتكاثر (Adams,1990 ; Lima and Dill,1990). الأمر الذي فُسِّر به تغذية الأفعى على القوارض بشكل واضح خلال أشهر الشتاء ، حيث أن برودة الجو ربما تجبرها على البقاء بين الأحجار وتحت الأشجار مما يقلل من فرصة ذهابها للمصادر المائية الأمر الذي من خلاله قد تتعرض لدرجات حرارة غير مناسبة مما يجعلها عرضة للافتراس أو لظروف حرارية حرجة . يؤيد ذلك ما أورده كل من (Randall and King , 2001) و(Watson et. al., 2004) عندما أشاروا إلى أن خوف الحيوان من الإجهاد بسبب عوامل المناخ وخطر الافتراس قد يؤدي إلى قلة بحثه عن الغذاء ، ويتجلى هذا الأمر في الزواحف التي ترتبط درجة حرارة جسمها بدرجة حرارة الوسط المحيط (Wang et. al., 2002) . هذا بالإضافة لكون قوارض منطقة الدراسة لا تدخل في بيات شتوي ويقل نشاطها في ذلك الفصل ، الأمر الذي يجعلها في محيط نشاط الأفعى ، وقد اتفقت نتيجة هذه الدراسة مع ما ذكره (Spawls et al., 2002).

اتفقت نتائج هذه الدراسة مع ما ذكره (السويلم ، 2001) الذي لم يُسجّل تغذية الأفعى المقرنة على الطيور حيث أرجع السبب في ذلك لقلة تعشيش الطيور في منطقة الدراسة الرملية ، ولقلة الغطاء الخضري ، وارتفاع درجة الحرارة ، وحركة حبيبات الرمال . ولم تسجل نتائج تشريح المعدة تغذية أفعى الأهرام على الطيور بالرغم من تسجيل الدراسة الحقلية لوجودها في أعشاش طيور الورور على حواف الأودية ، الأمر الذي يدعونا إلى عدم الجزم بتغذيها على الطيور بالرغم من توفر الغطاء النباتي ومناسبة درجة حرارة الوسط المحيط . وبالرغم من تأكيد (Leviton, 1992) على تغذي أفعى الأهرام على الطيور ، إلا أن ذلك يبقى احتمالاً ممكنا وفق هذه الدراسة .

كما سجلت الدراسة تغذية الأفعى على نوعين من العقارب الموجودة في بيئتها وهما عقرب فلسطين الصفراء Leiurus quinquestriatus وعقرب نيبو Nebo hierichonticus واللتان تنشطان خلال الليل وهي نفس الفترة التي تنشط بها أفعى الأهرام ، وهذه النتيجة أيدت ما أكده (Mazuch, 2005a) الذي أشار إلى أن العقارب من عائلة Buthidae هي الغذاء المفضل لأفعى الأهرام E. p. leakeyi وأكثر هذه العقارب تفضيلاً Parabuthus granimanus .

ولأن أفعى الأهرام حيوانية التغذية فإن الأغصان والبقايا النباتية التي وجدت في معدتها لم تكن دليلاً على تغذيتها النباتية بقدر ما كانت أجزاء نباتية قد التصقت على جلد العلاجيم التي تتغذى عليها صيفاً إضافة إلى حبيبات الرمل . كما أن تلك الحشرات الصغيرة من رتبة غشائية الأجنحة Hymenoptra والخنافس الثاقبة من رتبة غمدية الأجنحة Coleoptraوالتي لم يتجاوز طولها 0.5 سم ووجدت في معدة بعض الأفاعي هي في الحقيقة مجموعة من الحشرات التي تتغذى عليها العلاجيم وفقاً لتشريح معدة مجموعة من العلاجيم من بيئة الأفعى ، إضافة لغيابها في محتويات المعدة شتاءاً. ومع ذلك فإن احتمال تغذي الأفعى عليها يبقى قائما كونها تتغذى على الحشرات .

ولاحظت الدراسة تغذية وحيدة لأفعى الأهرام على السحالي خلال شهر أغسطس ، بالرغم من توفر نوعين من الأبراص ونوعين من السحالي الحقيقية بأعداد وفيرة في بيئتها . الأمر الذي ذكره (Leviton, 1992) بأنها تتغذى على السحالي وأشار إليه (Mazuch.2005a). وهذا ما يدعونا لتفسير ما حدث بأن الحيوان ذو تغذية متخصصة عند الظروف البيئية والمناخية المناسبة . فبالرغم من توفر العديد من الفرائس المناسبة للأفعى في فصل الصيف كالسحالي والحشرات والمفصليات والقوارض إلا أن علاجيم تهامة تبقى الفريسة المفضلة لها في فصل الصيف وخصوصا عند توفر المسطحات المائية اللازمة لتلك العلاجيم .

ولأن توفر الغذاء للزواحف يعتمد على الأمطار ، والذي بدوره يؤثر على معدل النمو والتكاثر (Ballinger, 1974) من خلال توفر البيئات المناسبة للحيوانات المختلفة من حيث المصادر المائية والغطاء النباتي وبالتالي تنوع وتشابك السلاسل الغذائية ؛ فإن نشاط أفعى الأهرام يكون عادة في مواطنها الرطبة (الطبيعية والزراعية) ولم يرتبط في الأساس بكثافة الغطاء النباتي وإنما بوجود مصادر مائية مستمرة أو شبه مستمرة كفيلة بوجود مجموعة من الحيوانات في بيئتها. وعلاوة على ذلك فإن تلك الأودية ذات التربة الرملية والحواف الطينية كانت هي البيئات المفضلة لهذه الأفعى. وبالرغم من تغلغل بعض التكوينات الصخرية الخشنة لأطراف تلك الأودية إلا أننا لم نشاهدها نهائياً فيها ، بل كانت تمثل المكان المفضل لأفعى السجاد الشرقي E. coloratus والتي توجد معها في نفس البيئة مع اختلاف طبيعة التكوين السطحي لمكانهما ، الأمر الذي سبق أن أشار إليه (Gassperitti,1988).

ويبدأ نشاط الأفعى بعد غروب الشمس ـ الغسق ـ ، وقد تخرج مع الغروب في أشهر الشتاء. وتستمر طوال الليل وحتى ساعات متأخرة في الأماكن المفتوحة صيفاً ، وبين الأحجار وتحت الأشجار بعد حوالي 3-4 ساعات من غروب الشمس شتاءاً . فالأفعى تتجول بين الحواف الطينية للأودية والمغطاة بالأشجار وخاصة أشجار الأراك Salvadora persica (Arak) وبين المصادر المائية الجارية أو الراكدة في بطون الأودية صيفاً . أما في الشتاء فإن ذلك لم يلحظ بشكل يدعو لتعميمه ، بل كانت توجود تحت الأشجار وبين الأحجار ، وحالات نادرة شوهدت آثارها بين المصادر المائية وبين الحواف الطينية بعد غروب الشمس.

وفي موسم الأمطار لم تسجل الأفاعي في بطون الأودية الجارية بعد المطر مباشرة ، بل سجلت بعد ذلك بيوم على الأقل وعلى حواف الأودية في الأماكن التي يكون جريان الماء فيها بطيئاً . ولعل السبب في ذلك الخوف من جرف التيارات المائية لها من جهة ، وغياب الفريسة من جهة أخرى. وبالرغم من مشاهدة أفعى الأهرام في وضع الاستعداد على حواف المصادر المائية والغنية بأسماك Garra tibanica tibanica في شهر يوليه إلا أن الدراسة لم تسجل تغذية الأفعى عليها.

إن النشاط الحركي للثعابين والقدرة على الهرب من المفترسات غالباً ما يَقِل بعد التغذي على فريسة كبيرة ، ومن ناحية أخرى فإن الثعابين التي تتغذى على وجبة صغيرة تكون محتاجة للبحث المتواصل عن الغـذاء ، وهذا يزيد من فرصة تعرضها للأعداء (Huey and Pianka, 1981 ; Hammerson, 1989) ، وعليه فإن حواف الأودية المرتفعة كانت تمثل المكان المناسب للأفعى بعد عملية التغذية خصوصاً في الليالي الحارة والرطبة من فصل الصيف ، حيث كانت معدة تلك الأفاعي ممتلئة بالغذاء . أما تلك التي يتم الإمساك بها في وقت الغسق فغالبا كانت معدتها خالية، ولعل هذا ما يفسر لنا خروجها مبكراً بحثاً عن طعامها .

وتحصل أفعى الأهرام على غذائها بطريقتين ، الأولى: إستراتيجية الكمائن حيث تقف مُستعِدّة على حواف المصادر المائية صيفاً أو بين الصخور وتحت الأوراق المتساقطة أسفل الأشجار شتاءاً . والثانية بطريقة التجول بين الشجيرات والمصادر المائية بحثاً عن فريستها . وتتم تغذيتها بأن تعضّ الفريسة دون أن تتركها وبالتحديد العلاجيم ، وربما يعزى ذلك لإمكانية قفز العلجوم بعد العض إلى المياه وبالتالي فقدان الفريسة أما القوارض فيمكن متابعتها بعد العض . وأما في المعمل فقد كانت تغذية أفعى الأهرام على القوارض مختلفة، حيث تعضها ثم تتركها حتى يبدأ مفعول السُّم في جسدها ومن ثم تبتلعها ؛ وهذا يتوافق مع نتائج (السويلم، 2001) في دراسته على أفعى الرمال المقرنة ، والتي تعض فريستها بسرعة متناهية ثم تتركها برهة من الزمن ثم تبتلعها . إضافة إلى دعم ما ذكر (Shine et. al., 2004) من أن طريقة بحث الثعابين عن غذاءها يعتمد على أساليب المواجهة بين المفترس والفريسة ؛ فقد شوهدت الأفعى تعض القوارض أكثر من مرة دون تحديد مكان مخصص للعض بل عشوائياً لحركات المراوغة من الفريسة ليتم قتله بسرعة . وهذه الملاحظة وافقت تلك التي لاحظها (Hayes, 1992) في دراسته على الأفعى المجلجلة التي تتغذى على الفئران. كما لوحظ خلال الدراسة أن عملية إخراج الأفعى للسانها يزداد بشكل واضح بعد عملية العض للفريسة، مشابهة في ذلك نتائج (Chiszar et. al., 1977; 1982) ؛ ولعل السبب في ذلك يعود لمحاولة الأفعى الاستدلال على مكان الفريسة بسرعة .

إن معظم الثعابين تتناول فرائسها من الرأس بحيث يكون البلع باتجاه نمو الشعر والريش والحراشف (Greene, 1976) ، بالرغم من وجود بعض الدراسات التي ذكرت العكس من ذلك (Marques and Puorto,1994) . وقد جَمَعَت أفعى الأهرام في سلوك تغذيتها كلاً من الطريقتين ، فكانت لا تتناول العلاجيم من الرأس بل تعضها ممسكة بها من أي مكان ، حتى يبدأ فعل السُّموم في جسد الفريسة ، ومن ثم تبدأ ببلعها بأسرع وقت وأسهل وضع ممكن . أما في حالة التغذي على القوارض ، فكانت دائماً تحاول التعرف على منطقة الرأس بعد أن يكون السُّم قد أعطى مفعوله ، وتبدأ عملية البلع من الرأس . ولعل تفسير ذلك يبرز في أنها تعض القوارض ثم تتركها وبالتالي عندها الوقت الكافي للتمييز واختيار منطقة الرأس عن مؤخرة الجسم عند بدء عملية البلع . كما أن الفترة الزمنية اللازمة لإتمام عملية الابتلاع توافقت مع تلك التي ذكرها (Stinner and Ely, 1993) . وفيما يتعلق بحجم الفريسة فقد سَجّلت الدراسة أن الوقت اللازم لافتراس الحيوانات البالغة للفرائس الكبيرة أطول منه في حالة الفرائس الأقل حجماً ، هذا بالإضافة إلى أن الثعابين الكبيرة لا تكتفي بفريسة واحدة صغيرة بل تبتلع من 2-3 فرائس صغيرة ، في حين اكتفت بواحدة من الفرائس الكبيرة . مؤيدين بذلك ما ورد في نتائج دراسة (Canjani et. al., 2002) أن الافتراس يرتبط بحجم الفريسة طردياً ، ودراسة (Ming-Chung and Hutchison, 1995) أن الثعابين الكبيرة تتغذى على وجبات كبيرة بشكل غير مستمر.كما سَجّلت هذه الدراسة شرب الأفعى للماء من المصادر المائية ، حيث تضع فمها ملامساً لسطح الماء لمدة تتراوح بين 10 – 30 ثانية قبل أن ترفع رأسها ، ويمكن خلال ذلك رؤية حركة البلعوم أسفل الفك السفلي لها ، الأمر الذي لم يشر إليه احد من الباحثين من قبل.

إن النشاط الغذائي لأفعى الأهرام في بيئتها كان ملاحظاً في فصل الصيف عنه في فصل الشتاء ، وربما يعود السبب في ذلك لعدة أمور أهمها: توفر الفرائس النشطة في أوقات نشاطها اليومي ، ومناسبة الظروف المناخية للحدود المثلى لممارسة نشاطاتها الحيوية بكفاءة . إن تلك الأسباب من الغذاء الوفير والمناخ المناسب أتاح لإناث الأفعى خصوصاً وفي أشهر قليلة القدرة على استعادة وزنها السابق وتقلص عضلات جسمها المترهلة والبداية في دورة تكاثرية جديدة مرة أخرى ، ويوافقنا في هذا التعليل ما ورد في دراسة (Zuffi et. al. 1999) على إناث أفعى Vipera aspis.

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هيئة التحرير

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