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Diversity of Life-History Traits, Functional Groups and Indicator Species of Bee Communities from Farmlands of Central Uganda

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Abstract

Bees are keystone organisms that sustain human life on earth through their pollination services. However, very little is known about functional groups and indicator species of bee communities from agricultural landscapes in Sub-Saharan Africa and Uganda. Responses to anthropogenic disturbances and the quality of pollination services delivered by different bee species are often associated with bee life-history traits. Diverse bee functional groups have different pollination service impacts on wild and cultivated plants. Efficient bee species are often good indicator species belonging to the same functional group. To provide baseline data on functional traits of bee communities in agricultural landscapes, a study was conducted in 2006 at 26 sites with varying local and landscape characteristics in central Uganda. Bees were sampled using colored pantraps, hand nets and line transect counts. The farmland bee community was characterized by a diversity of functional traits and guilds. Most of the bee species and individuals collected by the three sampling methods applied were solitary (37-70%), ground-nesting (41-65%), polylectic (74-91%), long-tongued (73-87%) and of small (<5.5mm) to medium (5.5-10.5mm) body sizes (18-37%). Using Indicator value method (*IndVal*), more than 17 species were recorded as indicator species or "species characteristics of the farmland habitats". Indicator species are generally ubiquitous species potentially delivering pollination services of high quality to cultivated and wild plants in farmland environments. They were recommended by to monitoring programs aiming at detecting the status and trends in Apoidea communities in central Uganda. To prevent future decline in the functional diversity, it is important to develop strategies to conserve landscape and habitats as reservoirs of different functional groups of bees. This will greatly contribute to the spatio-temporal stability of yield of pollinator-dependent crops that are pollinated by different pollinator groups. Monitoring programs aiming at detecting changes in bee faunas in farmlands of central Uganda may focus on the 17 indicator species identified by this study.

keywords: Functional groups, life history-traits, coffee-banana agroforests, functional group conservation, crop yield stability, Central Uganda.

1. Introduction

Pioneering works have highlighted the fact that bees are by far the most important providers of biotic pollination services in the world (Roubik, 1995; Dag, 2009). As the world's primary pollinators, bees constitute a critically important functional group. Although other taxa including butterflies, flies, beetles, wasps, bats, birds, lizards, and mammals may be important pollinators in certain habitats, none achieves high functional role of fidelity to particular plant species as key pollinators as bees (Munyuli, 2010 ; Ollerton *et al.*, 2011).The likely reason for this is that unlike other taxa, bees are obligate florivores throughout their life cycle, with both adults and larvae dependent on floral products, primarily pollen and nectar (Ollerton *et al.*, 2011).

With an estimated 20,000-30,000 species worldwide; bees are also a useful group for the study of biodiversity in farmland habitats. Currently, it is well established that bees are the world's dominant and vital pollinator taxa (Liow*et al.,* 2001; Fontaine *et al*., 2006) of most crop species: bees are essential for the productivity of many agricultural crops worldwide. Scientific studies state that a great proportion of the economic value of crop production is attributed to the free services ("public good") of pollinating services provided by a diversity of functional groups of bees (Klein *et al.*, 2007; Morandin *et al*., 2007). Bees are therefore keystone organisms that sustain human life on earth, through their irreplaceable pollination services (Munyuli, 2011a). Overall, bees play key roles in the maintenance of a diversity of wild plant communities (Bawa, 1990).They also provide pollination services of high economic values (Steffan-Dewenter and Westphal, 2008; Olschewski *et al.*, 2007). Some calculations are available for European Union and North America. For example, honey bees (*Apis mellifera* L.) are the main crop pollinator in the USA, contributing annually to estimate $1.6- $14.6 billion in pollination services (Southwick and

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Southwick, 1992; Chamberlain and Schlisin, 2008). The economics gains from non-managed wild bees are estimated to be of US$3.07 billion per annum in USA alone (Slaa, *et al.*, 2006; Losey and Vaughan, 2006). The ecological, agricultural and economic importance of pollinators is immense (Munyuli, 2011b). The value of pollination to agricultural production worldwide is currently estimated to be worth US$226 billion (€153 billion) (Klein *et al.*, 2007; Morandin *et al.*, 2007; Gallai *et al*., 2009). In Uganda, the economic value of pollinating services delivered to the crop production sector is on average of 0.52 billion per annum (Munyuli, 2010). Despite the vital role played by bee communities in traditional agro-ecosystems, the ecological and economic importance of these organisms (Martins, 2004), the diversity of functional groups remain largely unknown and un-surveyed (Eardley *et al.*, 2009; Gordon *et al.*, 2004) in Uganda.

There is a need to understand functional groups and indicator species of farmland bee faunas since these two types of bio-indicators can be used to monitor trends, predict and prevent degradation of bee biodiversity pollination services needed by a high number of pollinator-dependent wild and cultivated plants found in rural landscapes of Uganda and in East Africa. Also, the knowledge of the diversity of functional groups is important to acquire since responses to anthropogenic disturbances and the quality of pollination services delivered by different bee species are often associated with life-history traits. Diverse functional groups of bees have different pollination service impacts on wild and cultivated plants. Efficient bee species often belong to the same functional group. Some wild and cultivated plant species are pollinated by bee species in the same functional group such as sharp decline in these bee groups may lead to the disappearance of plant species in strong linkage and interactions with them. For example, in Uganda, efficient pollinators of cucurbits are often solitary bees in the Halictini (Halictidae). Thus, cultivation of cucurbits in environments that have low Halictid bee diversity may lead to high pollination limitation and to drastic reduction of the yield (Munyuli, 2010).

The diversity and incidence of different bee traits varies from a region to another. Within a region, bee species may also differ in their degree of voltinism (number of generations produced within a single growing) and in their flight season (period when the bee start being active and period when it ends or cease being actively seen foraging on different flowering plant species). Generally dietary specialization is associated with a higher extinction rate and/or with sensitivity to disturbance for a variety of bee taxa and functional groups. Oligolectic bee species are species known to gather pollen from a small number of related flower species, whereas polylectic bees are pollen generalists. Oligolectic species probably account for a large fraction of global bee diversity, since they constitute about 30% of species in temperate communities and up to 60% of species in the more species-rich desert (Moretti *et al*., 2008). Cleptoparasitic bees are morphologically highly divergent from other bees. They are often heavily armored and lack pollen-collecting structures. Cleptoparasitic species are widespread in Apidae, Megachilidae, and Halictidae (Michener, 2007). There are only a few species of cleptoparasitic Colletidae. Overall, no cleptoparasites have been reported from Melittidae, Andrenidae and Stenotritidae bee families worldwide. Cleptoparasitism in Uganda remains largely un-documented. It is important to know how many bee species are parasitic in a given habitat to better plan the conservation of pollination services delivered by non-parasitic bee species.

In most European countries, 89% of bee species are known to be bees with narrow habitat range; whereas 10% have wide habitat range. More than 65% of European bees are univoltine (one generations per year) and less than 35% of them are multivoltine (species having several generations per year) (Bismeijer *et al*., 2006 a and b). The degree of voltinism for afrotropical bees remains largely undescribed. Approximately, 80% of the European bee species are oligolectic bees and less than 20 % are polylectic. In the European countries, 70% of bee species are long-tongued bees and only 30% are short-tongued bees (Biesmeijer *et al*., 2006; Westphal *et al.*, 2008). The proportion of long-tongued bees in Uganda remains undescribed.

The ability to predict the responses of ecological communities and individual species to human-induced environmental change remains a key issue for ecologists and conservation managers alike (Williams *et al.*, 2010). Apoidea fauna with shared life-history and ecological traits can present variability in responses to different drivers within the same environment (Williams et *al.*, 2010). Therefore, knowledge of range of life-history and ecological traits of bee community can be used to predict bee responses to a change in a variety of disturbance types (agriculture intensification, cropping intensification, proportion of semi-natural habitats, distance to natural habitats, pesticide use, distance to natural habitats or habitat isolation, climatic factors, farm management system, grazing intensity, fire regimes, deforestation rate, etc) or drivers from a given farm-landscape. Understanding the diversity of functional traits can also help in better planning conservation strategies for the protection of bees to guarantee spatio-temporal delivery of pollination services to crops and wild plants. Ensuring spatio-temporal delivery of pollination services to crops is very critical for Ugandan agriculture since 78% of crop species and varieties grown by farmers in Uganda are pollinator-dependent (Munyuli, 2011a and b).

Monitoring changes in bee populations and functional traits in relationship to changes in drivers is another important step to build good conservation strategies that can reduce or prevent decline in bee species and populations. Overall, understanding how diverse bee traits are can also help in predicting the vulnerability of plant-pollinator interactions to global environmental change, to land-use (land-cover) changes and to various other drivers and pressures including climate change. Such knowledge can also help in better managing pollination services to meet the requirements of different plant /crop species. Some plant species can only be pollinated by certain group of bee species. The absence of such functional groups of bees may lead to decline or disappearance of such specialized plant / crop species.

Functional traits can be used to predict the response of bee species to various drivers. Functional traits can

therefore be used in monitoring programs to detect trends and status of species and population in a habitat subjected to pressures of different types. Indicator species can also be used to monitor the status of a bee community in a given habitat / landscape. If time and resource allow, monitoring the overall status of bee communities in a landscape / habitat can simultaneously be conducted using functional traits and or indicator species information. Policy measures and conservation strategies based on both indicator species and bee functional traits are likely to be useful in the protection of most species in a landscape/habitat (Munyuli, 2010). In addition, indicator species can help in developing effective monitoring plans of bee communities in farmland habitats of central Uganda. Challenges related to monitoring and conservation of bee faunas in farmlands of Uganda include the absence of basic knowledge about their natural history, abundance, diversity, functional traits and in their spatio-temporal distribution in agricultural landscapes.

The objective of this study was to identify the different functional groups and indicator species of bee communities found in agricultural landscapes in central Uganda.

In this study, it was hypothesized that farmland bee communities are not diverse in functional groups and indicator species because agriculture intensification may cause the disappearance of specialized bee groups and may tend to favor few common groups. In general farmland habitats are expected to support poor rich bee communities because they subjected to constant disturbances (e g crop production intensification) although studies from Kakamega Forest in Kenya (Gikungu 2006) showed that bees can be more diverse in farmland matrix habitats.

2. Material and Methods

2.1. Study area

This study was conducted in the banana-coffee system of Lake Victoria Arc zone, central Uganda (Fig. 1). The study zone belongs to the Lake Victoria phytochorion with shrubs of *Acacia* spp, legume trees, melliferous plant species, *Papyrus* and palms ranging from 2 to 15m high dominating the remnant secondary vegetation. Several oily, food and cash crops are grown, mainly cassava (*Manihot esculentum* L.), Sweetpotato, (*Ipomoea batatus*, L.), maize (*Zea mays*), beans (*Phaseolus vulgarus* L.), groundnut (*Arachis hypogea* L.); tomato (*Lycopersicon esculentum*), watermelon (*Citrullus lanatus*), pumpkin (*Cucurbita moschata*), cucumber (*Cucumis sativus*), melon (*Cucumis melo*); chilies (*Capsicum* spp.); and several other fruits, vegetables and horticultural crops (cabbage, onion, egg plants, sim-sim, etc). The majority of these crops require different functional groups of bees to provide pollination services of high quality.

This study was conducted in 26 sites selected to represent a range of habitats types with varying degrees of anthropogenic disturbances and management intensities. Selected study sites were grouped into clusters using human population density as a surrogate measure of agricultural intensity (Bolwig *et al.*, 2006) and anthropogenic disturbances. Each cluster comprised of 2 to 4 similar sites. Detailed environmental and landscape vegetation characteristics of the 26 sites and clusters are presented in Munyuli (2011c).

2.2. Bee sampling design

In each study site (1 km^2), five linear transects of 1000 x 200 m each were set using a GPS and a tape measure. Bees were sampled on one central line transect (1000m x 20m) visited on each round of data collection to obtain replicable results. Bees were sampled using three complementary methods: transect walk-and-counts (direct observations of foraging activities on flowers: visual censuses), capture using a handnet and colored pantraps following approaches described by previous workers (Potts *et al*, 2005; Cane,2000; Wilson *et al.*, 2009; Banaszak and Manole, 1987, 1994; Roulston *et al*., 2007; Droege *et al.*, 2010; Aizen and Feinsinger, 1994)

Bee specimens were sorted out and processed at Makerere University (Zoology Department Museum). All bee species were washed in 70% alcohol solution prior to pinning activities. Specimens were pinned, mounted and sun-dried for three days. Preliminary identification of the bees to the family or genus level was conducted in the laboratory at Makerere University, using published dichotomous keys (Michener, 2007) and the online keys available through www.discoverlife.org (Ascher *et al.*, 2008). Bee identifications up to species levels were further conducted at bio-systematic division of the Plant Protection Research Institute (ARC-Pretoria, South Africa), at the Natural History Museum, London (UK) and at the University of Reading (UK). Another set of bee specimens (stingless bees) were sent to STRI (Smithsonian Tropical Research Institute) in Panama. The identification of bees at ARC-Pretoria was conducted under guidance of Dr Connal Eardley. The identification of bee specimens at Smithsonian Tropical Research Institute was done by Dr David Roubik. The identification of bees at the natural history museum- London was conducted by Dr David Notton. A reference collection of bees of Uganda was established and is currently housed at Makerere University Zoology Museum.

2.3. Data analyses.

2.3.1. Classification of bee species per functional traits and ecological characteristics

Attempts were made to group all bee species recorded in this study into different functional categories in order to examine relative importance of each functional group (Moretti *et al*., 2009). Bee species were grouped according to taxonomic affinity and body size (Biesmeijer *et al.*, 2006; Fontaine *et al.*, 2006). Functional traits were obtained from the literature (Michner, 2007; Eardley and Urban, 2010**;** Eardley *et al.*, 2010; Eardley and Urban, 2010; Roubik,1995), where available.

In case, there is no information in the literature and no particular observation was conducted in the field, the trait attributes of different species were obtained from taxonomists, with experience on African bee fauna. Species which had no information on their functional traits were put in the unknown category. Overall, dietary breadth and nesting habits were determined for most species by reference to previous studies.

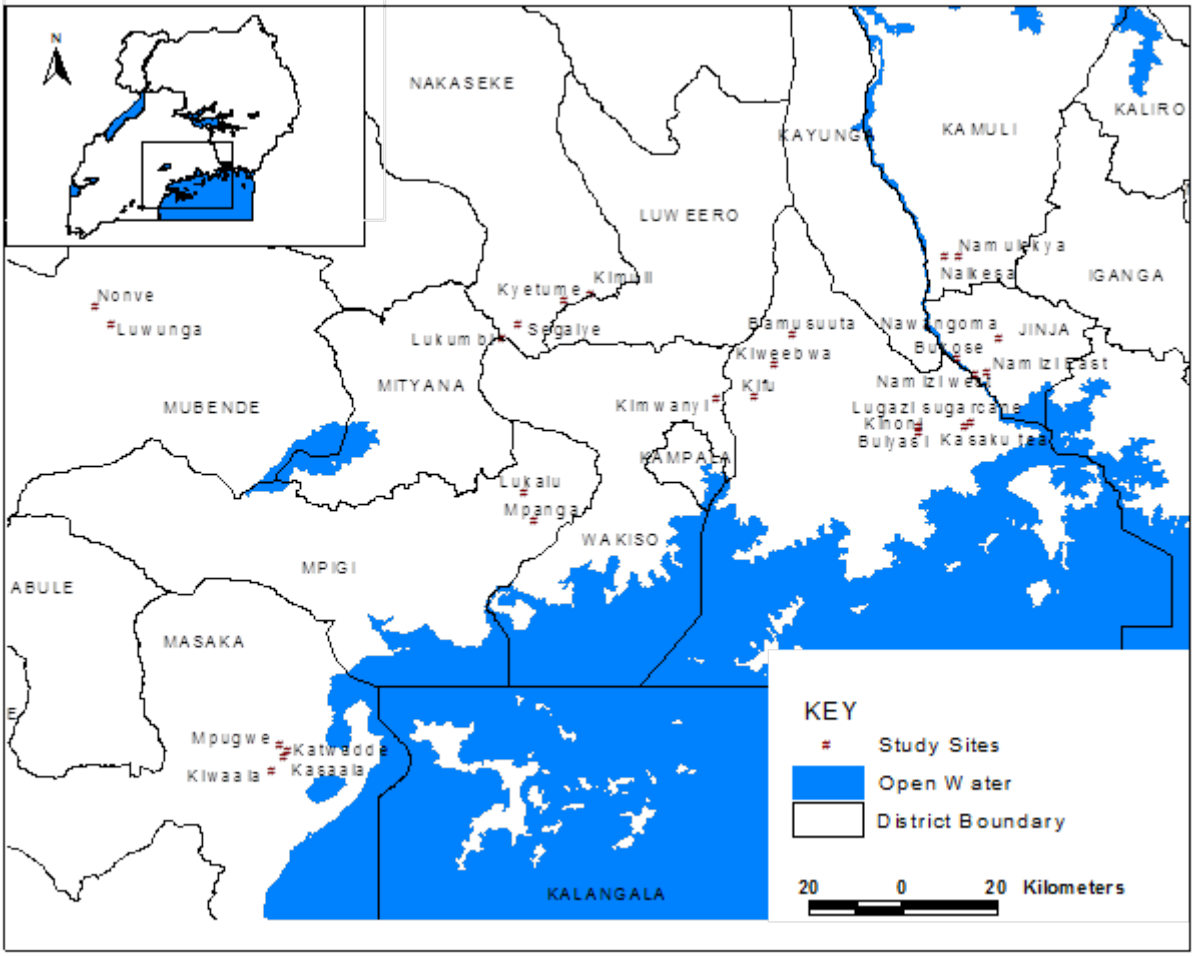

Figure1. Location of study sites in which the survey was conducted in the banana-coffee growing area around Lake Victoria in Uganda.

Nesting habits of bee species for which published records were not available were inferred if all other members of the same genus or subgenus used the same nesting substrate (e.g., all nest in the ground). Only two species represented by two individuals remained ambiguous for their nesting habits and were excluded from analyses as recommended (Cane *et al.*, 2006).

The traits to which each species was categorized included:

(i) *Feeding specialization*: (1) No lectic status (having no pollen collecting requirements; i.e. cleptoparasite), (2) Polylectic (pollen collecting on a number of species from different plant families), (3) Oligolectic (pollen collecting restricted to plants within the same plant family), (4) Monolectic (pollen collecting at a single species).

(ii) *Tongue length /length of the glossa /mouthparts length):* (1) short tongue (2) long tongue (3) medium-tongue. (1) Short tongue (<3mm); (2) intermediate or medium tongue (3-7mm); (3) long tongue (7-12mm); very long (>12-15mm) (Yanagizawa & Maimoni-Rodella, 2007).

(iii) *Nesting specializations* (*breeding strategies*)*:* (1) no nest building, (2) excavator in the ground (miner), (3) nests in termite mounds and other ground nesters, (4) carpenter: excavator in woody substrate , (5) renter of pre-existing nest and holes above the ground (cavity nesters), (6) live tree/stamp (dead wood), (7) house walls, (8) hives and (9) building nest with mud (mason bees).

(iv) *Parasitic status*: (1) cleptoparasitic (i.e. solitary parasitic bee), (2) social parasitic, (3) non parasitic;

(v)*Sociality status*/degree: (1) solitary: solitary, communal and semi-social; (2) social: colony founded by single individual on annual cycle (primitive eusocial) or a year-round colony (advanced eusocial); (3) variables: species is either solitary or social depending upon locality / environmental conditions (vi) *Bee body length* or *Bee body size* (*mm):* (1) small (<5.5mm), (2) medium (5.5-10.5mm), (3) large (15.5-2.5mm), (4), very large (>21mm).

The analysis of the different traits was limited to the determination of the importance (%) of each group using data from the count of number of species and individuals that fall under a given trait category.

2.3.2. Species constancy and indicative value

The species constancy is the proportion of sites where a given species occur permanently across sampling seasons (rounds). The species constancy provides indications about most common species in the farm-landscape habitats.

Indicator species are “ecological characteristic species” of bee communities inhabiting certain type of habitats of a given landscape. It is important to know indicator species, particularly when interested at assessing the strength and reliability of association between individual bee species and particular study sites characterized by certain land-use, habitat, biotopes and vegetation types. Indicator species are also important and effective pollinator species of many crops and wild plant species in agricultural landscapes

(Munyuli, 2010) of Uganda. The usefulness of indicator species is that these species are easy to monitor and to sample. Indicator species are also species likely to deliver pollination services of high quality with high spatio-temporal stability (Munyuli, 2010) particularly in sub-Saharan tropical environments and in Uganda.

In this study, species constancy was calculated following the approach previously described by Banaszak (2000), d´Avila & Marchini (2008) and Silva-Pereira and Santos (2006) and Lee (2002). To determine reliable and stable "indicator species" or "characteristic species" in farmland habitats of central Uganda, the indicator (*IndVal*) method of Dufrêne & Legendre (1997) was adopted and used in this study in a modified form as recommended by Banaszak (2000) and Munyuli (2010) to identify indicator species of pollinator communities.

2.4. Statistical analysis

2.4.1. Variation in abundance and species richness among bee taxonomic groups.

The number of bee species and individuals of different taxa were measured during the determination of the bee community structure. Statistical analyses were conducted to determine the importance of some taxa relative to others. To explore whether there were statistically significant differences in occurrences or proportional abundance / species richness between different taxonomic groups (genera, families, etc), chi-square tests in Minitab release version 15 were applied.

2.4.2. Indicator species identification.

Significance of the indicator value for the site with the highest indicator value was evaluated by a Chi-square test comparing the observed indicator value for a site to indicator values calculated each of the five sampling rounds conducted. Hence, Chi-square testing in Minitab 15 was applied to determine the significance of the indicative value of each species across the five rounds of data collection as recommended (Banaszak, 2000; Munyuli 2010).

3. Results

3.1. Bee biodiversity structures and composition

Overall 80,883 individuals representing 652 native bee species from 6 families, 14 sub-families, 34 tribes and 79 genera were captured (see species list in Munyuli, 2010; Munyuli 2011c). Up to 645 species were new records for Uganda from the overall bee fauna diversity. There were variations in effectiveness of different sampling methods used. There was a significant (P<0.001) difference in the number of species captured using the three sampling methods. Significantly [$\chi^2_{(2,\ n=\ 932)} = 402.414$; P<0.0001] higher number of bee species were captured using handnet (559 species) than when using transect count (59 bee species) and pantrap (314 be species) methods. Most bees were recorded through line transect counts (85% of total individuals) and very few individual bees were captured by handnet (8%) and pantraps (7%).

3.2. Functional traits characterization of bee fauna

Functional traits characterization of pantrapped bee community

A great variation in the abundances and species richness of different functional groups of bees attracted to different colors was detected (P<0.0001). Bee families differed significantly in species richness (χ^2 5df = 56.1, P<0.001) and in abundance (χ^2 5df = 75.87, P<0.0001). Apidae was the most species-rich (41.38% of species recorded in pantraps) followed by Halictidae (30.09%), Megachilidae (15.99%) and Colletidae (6.02%). Similarly, the bee family with the highest number of individuals was Apidae (46.5% of total bee individuals recorded) followed by Halictidae (38.52%), Andrenidae (5.82%) and Colletidae (4.13%).The most species-rich bee genus captured by pantraps was Halictus followed by Lipotriches and Patellapis, Ceratina, Allodapula and Braunsapis.

The species richness (χ^2 1df =69.886, P<0.001) and the abundance (χ^2 1df = 79.92, P<0.001) of non-parasitic bees were significantly higher compared to the abundance and species richness of parasitic bees captured in pan traps. In fact, 91.8% of 314 species registered and 94.7% of 5672 individuals recorded in pantraps belonged to non-parasitic bee species category compared to parasitic (cleptoparasitic) bees that accounted for only 5.3% of the total bee individuals recorded in five rounds of data collection across 26 study sites.

Bees of different breeding strategies occurred in pantraps; but overall, the bee fauna was composed of a significantly (χ^2 2df = 99.9397, P<0.0001) greater proportion of solitary bee species (96.25% of total number of bee species recorded) than of social bee species richness (3.75%).

3.3. Functional traits of transect-recorded bee fauna

Social status and breeding strategy

There was a significant difference among the different sociality (breeding strategy) categories in species richness (χ^2 3df =114.92, P<0.0001) and proportion of bee individuals (χ^2 3df =151.7351, P<0.0001). Solitary bees were the most species rich bee category (Fig.3), although social-bees were more abundant than other bee categories (Fig.3).Very few bee species and individuals were parasitic (cleptoparasitic). Except for bees in the cleptoparasitic genera (e.g., Thyreus, Cleptotrigona), most bee species recorded are potential efficient and good pollinator species of many entomophilous crop species.

3.4. Diversity of nesting specialization habits

There were significant differences in species richness (χ^2 5df = 189.86, P<0.0001) and abundance (χ^2 5df =74.081, P<0.0001) among the 6 different nesting-guild categories found in the bee fauna inhabiting farmlands of central Uganda. Ground-nesters were the most species-rich followed by wood/tree/pith nesters and cavity-nesters (Fig.4). House-wall nesters and beehives were the least species rich nesting categories recorded (Fig.4). Ground nesters (soil nesters, termite mound nesters) that were the most species-rich were also the most abundant bees. Wood or pith nesters were the second most species-rich and abundant, followed by hive, house wall and cavity nesters (Fig.4).

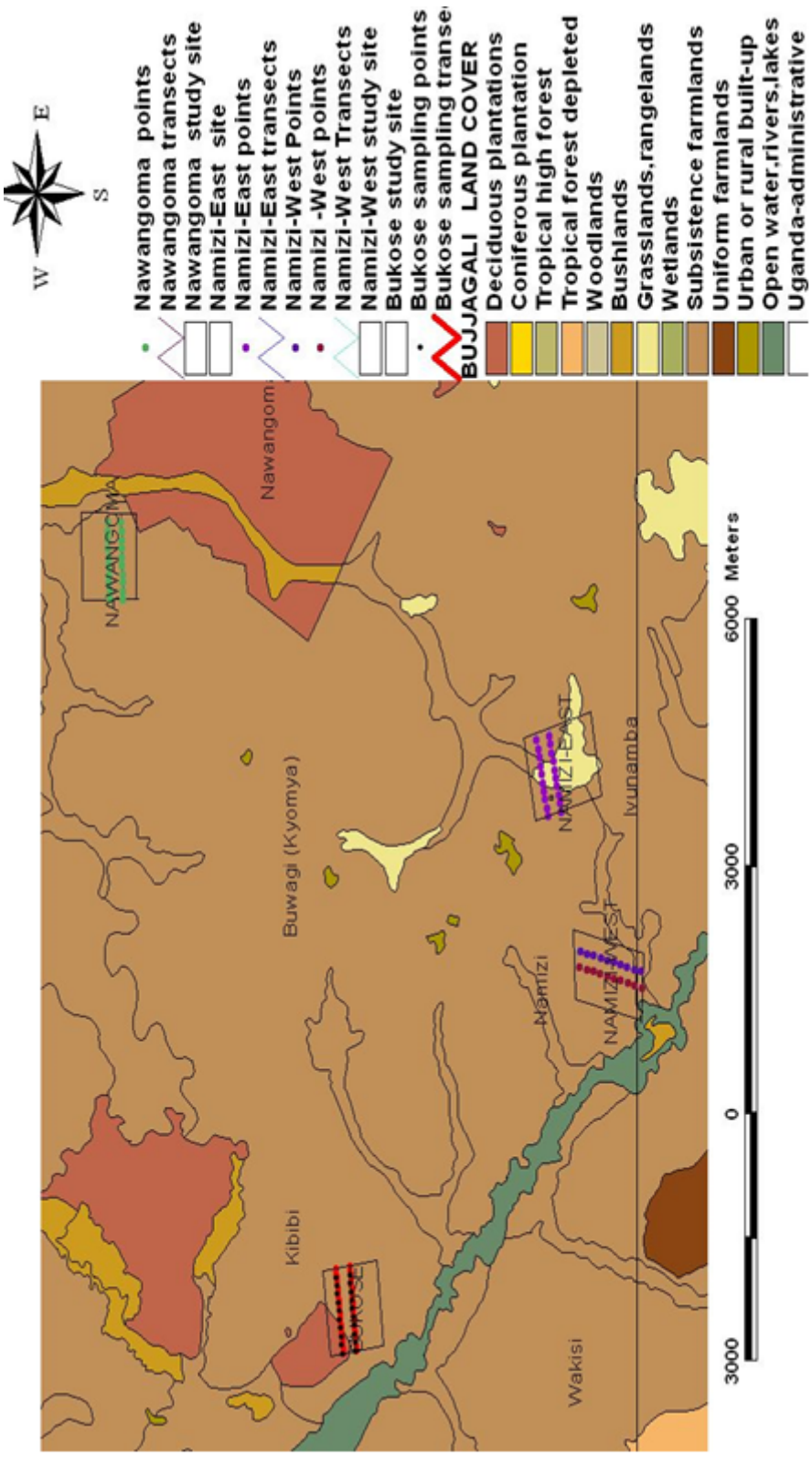

Figure 2. Location of Bukose, Namizi-East, Namizi-West and Nawangona study sites in Jinja district

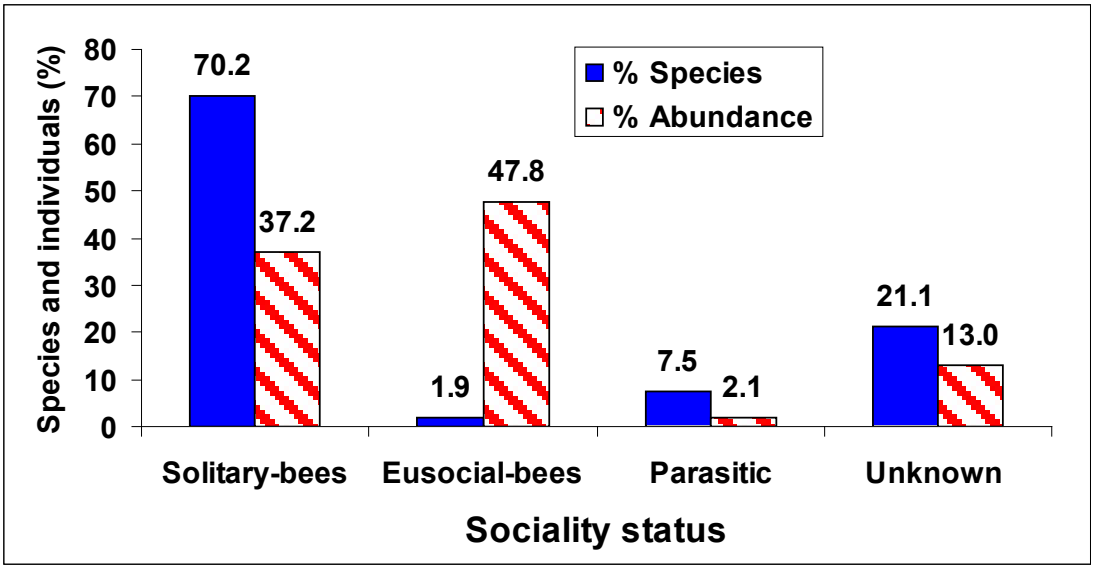

Figure 3. Percent (%) of species and individuals with respect to sociality/breeding strategy of different bee species collected from agricultural landscapes of central Uganda in 2006. *(N for bee species=602; N for bee individuals= 75221)*

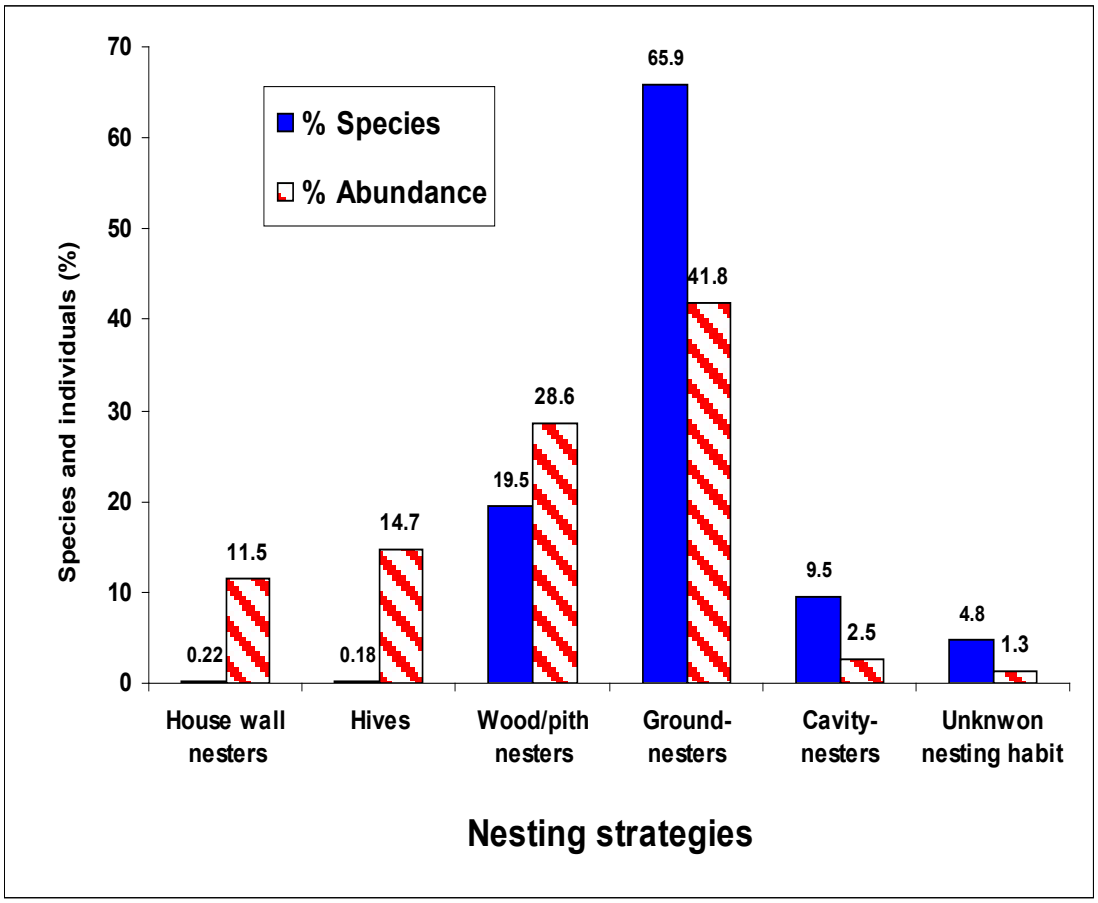

Figure 4. Proportion (%) of species and individuals with respect to nesting strategies of different bee species collected from agricultural landscapes of central Uganda in 2006. *(N for bee species=602; N for bee individuals= 75221)*

avity nesters were third most species-rich compared to house-wall nesters and hive users (honeybees) but the abundance of hive users and that of house wall nesters were greater than that of the cavity nesters (Fig.4). The nesting strategies of 4.8% of the bee species and 1.3% of total individuals encountered were unknown.

3.5. Feeding specialization and tongue length

Bee individuals recorded in farmlands of central Uganda included both oligolectic (characterized by flower constancy) and polylectic bees. Fewer specialist bees (oligolectic) occurred in farmlands of central Uganda

compared to generalist bees (polylectic bees) (Fig.5A). Monolectic species were not recorded in this study.

Four tongue-length categories characterized the farmland bee community: long-tongue, short-tongue, and medium-tongue and uncertain tongue length class. There were significant differences among the tongue categories in species-rich (χ^2 3df = 130.559, P<0.0001) and abundance (χ^2 3df = 209.943, P<0.0001). The most species-rich and abundant category was long-tongued bees. Medium-tongued- bee group was the least species rich comprised only 1.9% of total bee individuals recorded (Fig.5B).

3.6. Body size (length)

Significant differences in bee species richness (χ^2-test: χ^2 5df = 45.06, P<0.0001) and in bee abundances (χ^2 5df = 217.7, P<0.0001) among the 6 bee-body length classes were detected. Medium sized bees formed the richest class followed by small, very small and large bees (Fig-6). Contrastingly, very small-bodied bees (34.3% of individuals) were the most abundant category followed by medium sized and small-sized classes (Fig. 6).

3.7. Species constancy and Indicator species of bee assemblages

The results of the analysis of the species constancy indicated that 16 species could be classified as highly constant species (C>70-100%).Fewer species (14 species) were found to be constant species (C>50-70%).The rest of the species encountered (622 species) were considered as occasional or accidental species (species with constancy values of C=0-49%) visiting or inhabiting coffee-banana systems of central Uganda (Table-1). *Apis mellifera adansonii* had maximum species constancy values (100%), indicating that the species occurred with maximum prevalence in all farmland locations/sites (Table.1).

Overall 17 bee species scored significantly (P<0.05, df=4, χ^2-test) high indicative values (*IndVal*>12%; Table.1). The "indicator species" were considered as the most ecologically important species and characteristic of bee assemblages inhabiting coffee-banana system(a system characterized by high level of habitat disturbance due to farming activities) (Table.1).These characteristic species had also high constancy value (>50%).

These 17 species are also the most common bee species among wild bees; they are also very plastic, both in respect of their range forage plants (polylectic), and in selection of nesting sites (they can nest in various substrates (in wooden materials, human buildings, underground soils, hollow dry stems, etc). However, these species are characterized by unstable abundances over different seasons of the year (occurring with high abundance in rainy seasons and represented by few individuals in dry seasons).These few species that dominated the bee fauna and that were well-represented in different study sites were also the most frequent species characterized by a high constancy. Thus they seem having lower environmental requirements (they are not specialized) because they were also found on more than 50% of studied sites; so they can be regarded as characteristic species to be considered for further monitoring program in farmlands of central Uganda.

4. Discussion

4.1. Patterns of indicator species

In this study, 17 species were identified as indicator species of farmland bee communities. Compared to the large number of bee species recorded in this study, only few species could occur as "characteristics" of farmlands habitats in Uganda for years 2006-2008 when the study was carried out. Although few species occurred as indicator species, this information may be of relevance to long-term ecological monitoring programs of species, populations and activities particularly when interested at detecting and predicting changes in the composition and structure of the entire bee community from the landscape/habitat. Based on predictions of the entire bee community, it is believed that sound measures for the protection of pollinators can be outlined in relationship to mitigation of potential drivers of changes in species occurrence.

A high diversity of bees was recorded in farmlands of central Uganda. Similarly, Gikungu (2006) highlighted a high diversity of bees in an agricultural matrix in Kakamega region (western of Kenya). In both cases (Uganda and Kenya), a few numbers of species (<20 species) are common species were recorded. These can be used as indicator species of the entire bee communities when interested at monitoring the status of bee assemblages in rural landscapes in East Africa.

For the case of central Uganda, monitoring 17 species may help in the future to provide an indication on of trends and patterns in the most common and constant species. The 17 species appeared to be relevant in the coffee-banana farming system of central Uganda. However, it is possible that a high number of species may be recorded in other sub-Sahara African countries.

In the future, a simple guide of identification of these indicator species should be produced and distributed to bee biodiversity planners in order to facilitate their work and alert regularly other stakeholders on probable future decline of bees while highlighting the drivers and mitigation measures.

4.2. Patterns of functional traits and ecological groups

The relevance of identifying bee functional traits composition (dominant traits and functional diversity) is that traits can be used to predict the functional responses of bee communities to local, regional and global environmental changes in habitats of conservation importance in different regions with distinct disturbance regimes (Moretti *et al.*, 2009). Functional traits can be used as indicators of pollinator biodiversity response to land-use changes across ecosystems and climatic regions.

The exploration of different functional traits of bees can be also conducted to identify functional groups that are probably delivering pollination services of high quality to crops and wild plants in farmland habitats such as the banana-coffee system in Uganda.

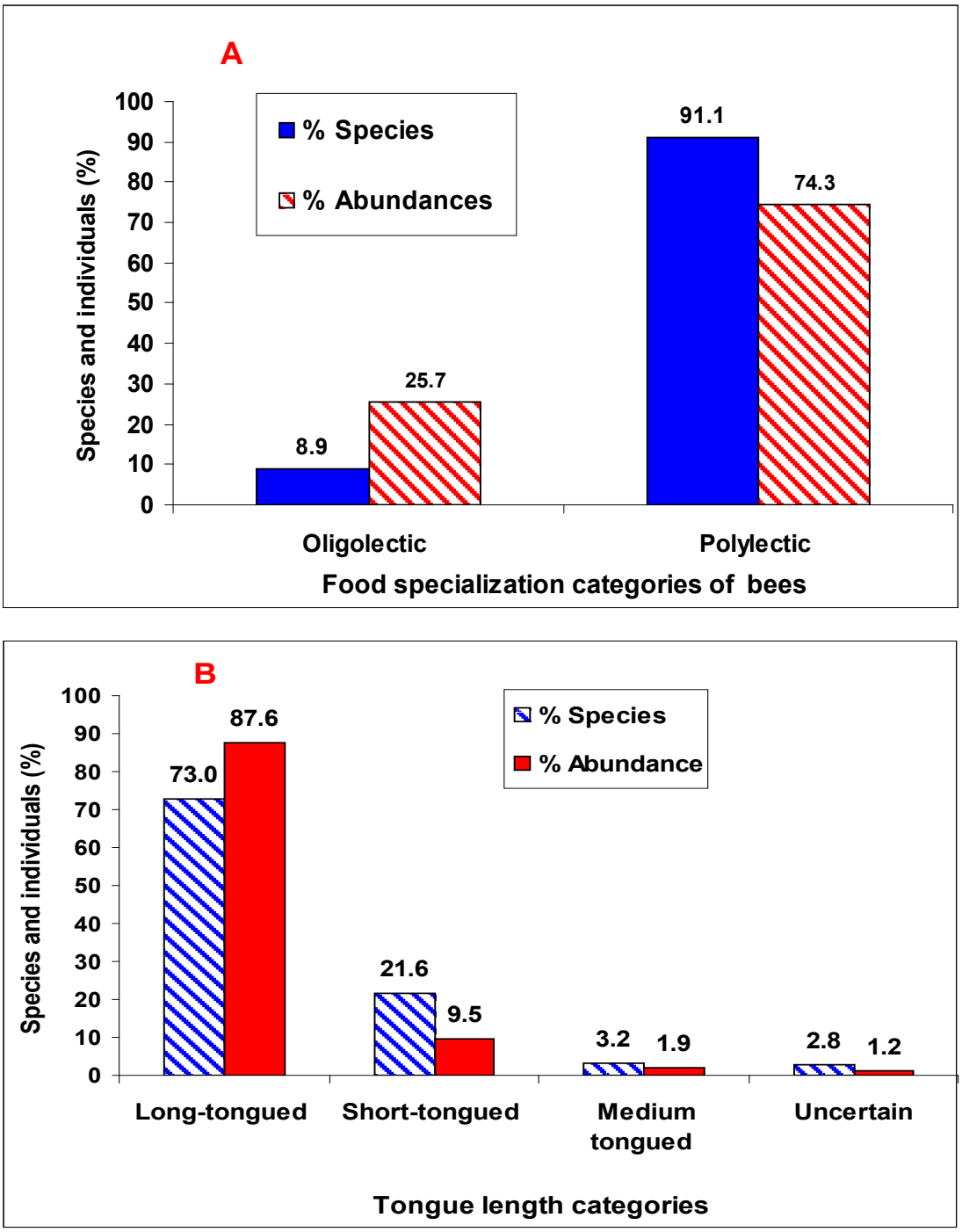

Figure 5. Percentage (%) of bee species and individuals captured in agricultural landscapes of central Uganda with respect to (A) floral specificity (oligolectic: pollen-gather specialists, polylectic: pollen-gather generalists) and (B) proboscis or tongue length (feeding strategy= feeding ability index) of bees collected in farmlands of central Uganda in 2006. N for bee species=602; N for bee individuals= 75221.

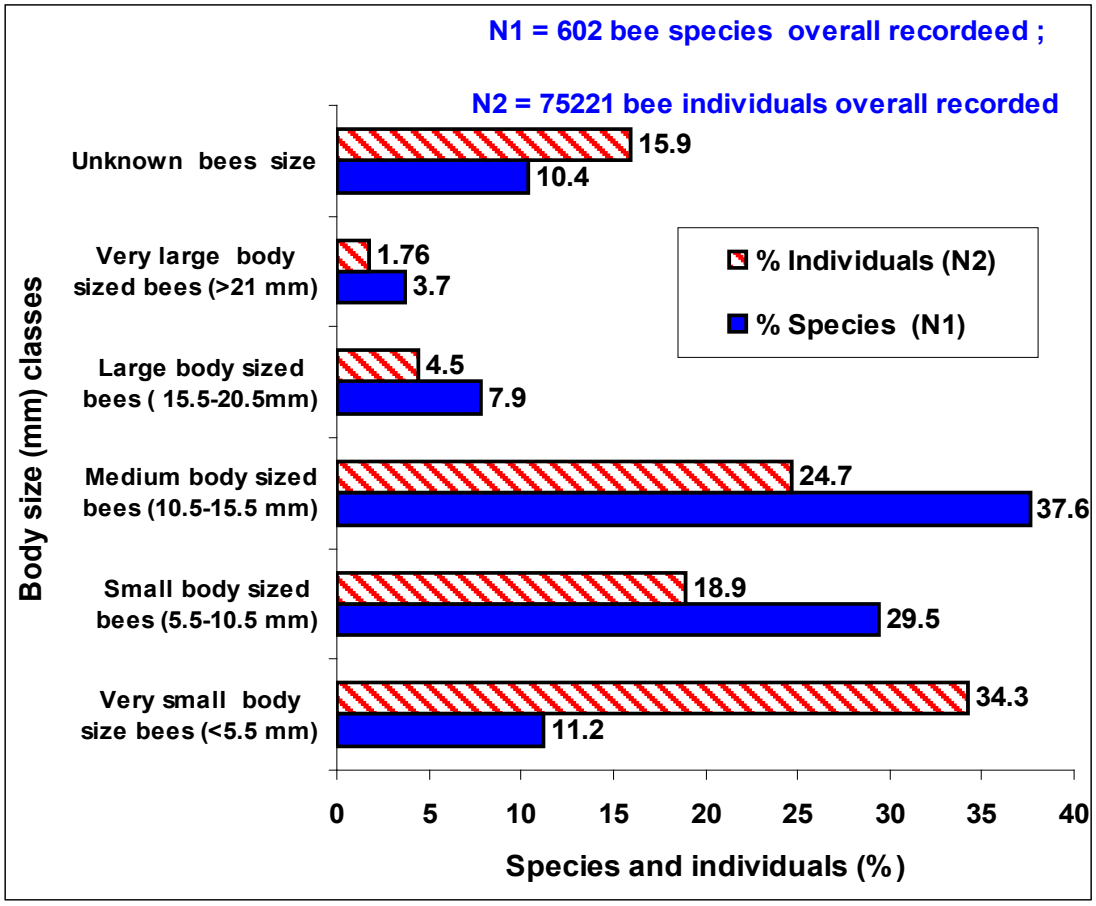

Figure 6. Percentage of bee species and individuals in different body size classes captured on agricultural landscapes of central Uganda in 2006.

Ecological grouping of different bee species can also generally be conducted to identify functional groups of bees from farmlands that are likely to be more susceptible (or resist, tolerate or adapt) to various climatic, pressures and anthropogenic changes; as the degree of susceptibility vary from a group to another one.

Overall, it has been shown that knowledge of range of life-history and ecological traits of bee communities can be used to predict bee responses to a change in a variety of environmental disturbance types (agriculture intensification, cropping intensification, proportion of semi-natural habitats, distance to natural habitats, pesticide use, distance to natural habitats or habitat isolation, climatic factors, farm management system, grazing intensity, fire regimes, deforestation rate, etc) or drivers from a given farm-landscape (Williams et *al.*, 2010).For example, species that nest above ground, and species that use previously established nest cavities may be more sensitive to various disturbance regimes than species that nest in the ground or excavate their own nests.

In this study, it was found that the majority of bee species and individuals recorded were solitary, long-tongued, polylectic and of small to medium body sizes. From 181 bee species recorded in total during a faunistic survey conducted in "Cerradão" area in Brazil, 30.8% and 69.2% of them were classified as sociable and as solitary respectively (d´Avila and Marchini, 2008). Similarly to observation made in farmland of central Uganda where it was found that farmland bee fauna was dominated by long-tongued bees followed by short tongued bees species; in coastal sand dune of northeastern of Brazil, very long to long-tongued (>7-12mm) solitary bee species (best represented by Anthophorinae) were the dominant bee categories followed by bees of intermediate glossa (3-6.9mm) and by the category of short tongued bees or bees with short bilobate (<3mm) from Apinae, Halictidae, Megachilidae and Colletidae bees (Viana and Kleinert,2005).

Worldwide, relatively small proportions (~6%) of all bees are eusocial bees (Michener, 2007). Even in the coffee-banana farming system of central Uganda, only 3.25% of bee species occur as eusocial bee species. Globally, an estimated 20% of all bee species are cleptoparasites (cuckoo parasites) that lay their eggs in the nests of other bees (Michener, 2007). Parasitic bee species comprised <10 % (64 species) of the total bee species that visit flowers of various crop/plant species but do not collect pollen in central Uganda. Cleptoparasitic species are among the poorly known bees in the world and in Uganda. Less than 10% of species were identified as cleptoparastic bees in farmlands of central Uganda.

Among non-parasitic species recorded from central Uganda, more than 65% of them were found to be ground-nesting and approximately 20% were wood-nesting bee species. Nesting-sites preference of different farmland bee species differed between the study sites, and the results suggested that differentiation in the distribution of farmland bee fauna from central Uganda is mostly related to food resources availability and to differences in farming practices and regimes (Munyuli, 2010).

Table-1: Sites of permanent occurrence (SPO), species constancy (SC) and species indicative value (IDV) of bee community, from the coffee-banana-farmlands of central Uganda during year **2006.**

Bee species	SPO	SC(%)	MIDV(%)	Bee species	SPO	SC(%)	MIDV(%)	Bee species	SPO	SC(%)	MIDV(%)
Apis mellifera adansonii	26	100	96.25 *	*Meliponula bocandei*	4	15.4	0.17 ns	*Pachymelus sp.*	2	7.9	0.034 ns
Hypotrigona gribodoi	22	84.6	81.42 **	*Nomia scutellaris*	7	26.9	0.28 ns	*Pasites jenseni*	2	7.7	0.033 ns
Axestotrigona ferruginea	22	84.6	70.92 **	*Patellapis albofasciata*	8	30.8	0.03 ns	*Liotrigona sp.*	4	15.4	0.028 ns
Lasioglossum sp.1	17	65.4	44.51 **	*Ceratina rufigastra*	15	57.7	0.10 ns	*Ctenoplectra* sp.2	10	38.5	0.024 ns
Halictus sp.1	26	100	42.71 **	*Pachyanthidium bicolor*	9	34.6	0.03 ns	*Coelioxys torridula*	4	15.4	0.023 ns
Apis mellifera scutellata	22	84.6	40.19 **	*Ceratina lineola*	20	76.9	2.51 ns	*Cleptotrigona cubiceps*	6	23.1	0.023 ns
Lipotriches sp.1	25	96.2	23.78 *	*Patellapis (Zonalictus)* sp.1	13	50.0	0.08 ns	*Tetraloniella sp.*	3	11.5	0.020 ns
Lasioglossum kampalense	24	92.3	17.92 *	*Lasioglossum(Ctenonomia) duponti*	6	23.1	0.02 ns	*Scrapter nitidus*	8	30.8	0.020 ns
Ceratina sp.1	22	84.6	14.39 *	*Megachile gratiosa*	7	26.9	0.02 ns	*Colletes* sp.1	4	15.4	0.020 ns
Braunsapis angolensis	21	80.8	12.87 *	*Melitturga penirithorum*	12	46.2	1.29 ns	*Lithurge sp.*	3	11.5	0.019 ns
Heriades sp.1	23	88.5	12.49 *	*Hylaeus braunsi*	11	42.3	0.10 ns	*Lasioglossum* sp.3	4	15.4	0.018 ns
Allodapula sp.	22	84.6	8.19 ns	*Braunsapis* sp.	2	7.7	0.10 ns	*Lipotriches dominarum*	9	34.6	0.018 ns
Halictus jucundus	24	92.3	17.51 *	*Halictus frontalis*	8	30.8	0.09 ns	*Amegilla acraensis*	7	26.9	0.018 ns
Allodape sp.	23	88.5	14.66 *	*Compsomelissa nigrinervis*	16	61.5	1.49 ns	*Anthidium strigatum*	2	7.7	0.016 ns
Lasioglossum sp.2	23	88.5	13.85 *	*Nomia granulata*	4	15.4	0.09 ns	*Lasioglossum* sp.4	7	26.9	0.015 ns
Meliturgula sp.	22	84.6	2.57 ns	*Pachymelus* sp.1	7	26.9	1.85 ns	*Dactylurina schmidti*	6	23.1	0.015 ns
Plebeina hildebrandti	8	30.8	2.19 ns	*Halictus* sp.2	21	80.8	0.43 ns	*Melitta arrogans*	2	7.7	0.015 ns
Ctenoplectra sp.1	13	50.0	1.52 ns	*Anthophora sp.*	11	42.3	0.07 ns	*Ctenoplectra politula*	4	15.4	0.015 ns
Apotrigona nebulata	20	76.9	15.54 *	*Scrapter flavostictus*	2	7.7	0.07 ns	*Megachile* sp.2	7	26.9	0.014 ns
Ceratina sp.2	15	57.7	1.42 ns	*Othinosmia globicola*	12	46.2	0.06 ns	*Xylocopa inconstans*	18	69.2	0.012 ns
Lipotriches sp.1	12	46.2	1.12 ns	*Halterpis nigrinervis*	11	42.3	0.06 ns	*Andrena* sp.	4	15.4	0.012 ns
Plebeilla lendliana	9	34.6	1.12 ns	*Patellapis* sp.1	3	11.5	0.05 ns	*Hylaeus ugandicus*	5	19.2	0.011 ns
Nomia atripes	24	92.3	13.13 *	*Patellapis dispostia*	2	7.7	0.05 ns	*Megachile fimbriata*	2	7.7	0.011 ns
Ceratina tanganyicensis	15	57.7	12.99 *	*Braunsapis fascialis*	7	26.9	0.05 ns	*Braunsapis bouyssoui*	5	19.2	0.011 ns
Allodapula acutigera	18	69.0	16.89 *	*Ctenoplectra polita*	9	34.6	0.04 ns	*Thyreus* sp.	2	7.7	0.006 ns
Xylocopa caffra	17	65.4	0.35 ns	*Coelioxys natalensis*	6	23.1	0.03 ns	*Hoplitis sp.*	3	11.5	0.006 ns
Lipotriches sp.3	14	53.8	0.23 ns	*Pseudanthidium sp.*	10	38.5	0.03 ns	*Tetralonia (Eucara) sp.*	2	7.7	0.007 ns

Significant:* =P<0.05; ** =P<0.001; ns=not significant, χ2-test (n=5). Chi-square test done based on IDV values of each species in the five rounds of data collection.SPO= represent the number of sites where the species was frequently recorded each round of data collection.SC (%) = represent the proportional number of sites where a species occur permanently in 26 farmland sites studied. MIDV(%) = is the mean species indicative value=Relative abundance X Relative frequency X 100 of the species in sites where it occurs permanently.

The response of different functional groups of bees to farming practices, land-use and environmental variables are presented in a different manuscript submitted elsewhere.

Few oligolectic species were recorded in this study and this was expected since the study was conducted in disturbed habitats (farmlands) compared to less disturbed habitats (where oligolectic foraging strategies are expected to dominate given higher floral diversity) (Schlindwein, 1998).

In central Uganda, polylectic bee species (generalist foragers :> 90% of species recorded) dominated in farmlands of central Uganda. Similarly, the dominance of polylectic bee species was recently reported in USA (Norden, 2008) and in Poland (Moroń *et al.*, 2008). However, findings from this study do not concur with the results of Minckley (2008) who stated that areas where bee species richness is greatest have a greater proportion of oligolectic bee species compared to polylectic bee species. On the contrast, farmlands of central Uganda were found to be species-rich with dominance of polylectic bee species. Oligolectic bees are rarer than diet generalists (Grundel *et al.*, 2010); they are generally well associated with linear and non linear semi-natural habitat and natural habitat features; hence their populations may be especially affected by degradation of natural and semi-natural habitats in the farm landscape.

Oligolecty is a significant predictor of bee species' decline over time in northwestern Europe, and of sensitivity to fragmentation in desert ecosystems. For example, among European *Bombus*, all of which are polylectic, species with more specialized diets show greater population declines over time. Presumably the risk of decline is heightened by being more reliant on a smaller number of food sources. In addition, oligolectic bees have more genetically isolated populations and lower genetic diversity which further increases their susceptibility to decline (Winfree, personal communication). As part of the dietary specialization, oligolectic species are able to time

their emergence to the bloom of their host plant species better than polylectic species. Because both polylectic and oligolectic bees play distinct important roles in the maintenance of wild and cultivated plant species in Sub-Sahara Africa (Munyuli, 2010); hence they are irreplaceable by generalist bees in some countries in Sub-Sahara Africa and in neotropical regions like in Brazil. The conservation of oligolectic in landscape may therefore appear imperative (Buschini *et al.*, 2009) particularly in sites where they are the leading pollinators of some plants and crops.

In farmlands of central Uganda, two bee body size classes dominated the bee community: the class of 5-10mm followed by that of 10-15mm body size. Similarly, in an agricultural landscape in the state of São Paulo (Brazil), it was observed that 5-10mm (49%) body size and 10-15mm (35%) body size classes dominated the farmland bee communities (Souza and Campos, 2008**).** Generally, there is a strong linkage between bee body size (mm) and their foraging distance and bee foraging distance affects agricultural production (Greenleaf *et al.*, 2007). Foraging distance determines the spatial scale at which different group of wild bees can provide pollination services to crops. Animal pollination is required to produce 30% of the world foods (Klein *et al.*, 2007; Greenleaf *et al.*, 2007). It can be expected that functional groups of wild bees that pollinate crops nest in natural habitats and forage on crops within their daily travel distance that is related to their body size (Greenleaf *et al.*, 2007).

In addition larger species travel over larger distances (>2000-3000m) than do smaller species (250-1000m) for the spatial exploitation of food resources available at different scales of the landscape (Westphal *et al.*, 2008). Although small and medium sized bee species dominated central Uganda farmland bee community; unfortunately, the majority of crop species grown are pollinated by tiny, small and medium bees (Munyuli, 2011c). Hence, the need of growing pollinator-dependent crop species in the foraging range of their pollinators, or alternatively managing conservation of habitats nearby farms to attract effective pollinators to nest in the vicinity of fields.

Much as it is not always easy to predict extinction risk and sensitivity to disturbance of different bee species based on body size data; landscape-wide availability of different resources (mass flowering crops and semi-natural habitats) should be considered by farms and landscape managers to maintain a variety of functional groups of bees in the proximity of cultivated crop species.

5. Conclusion and Recommendations

In this study, it was found that farmlands support a rich bee fauna comprising of over 600 species belonging to several tribes, families, genera and functional guilds. The study highlighted species that should be currently characterize the bee fauna pollinating crops in central Uganda and could be monitored to detect potential decline in bee species in the farmland of Uganda when aiming at preventing total loss of species due to anthropogenic factors. Strategies to prevent future decline (driven by anthropogenic and climate change factors) in species richness and in different functional traits are therefore outlined below. Making farmland more suitable for different functional groups of bees can benefits both agriculture and nature conservation (Carvalheiro *et al.*, 2010). In Sub-Sahara and in East Africa, there exist few studies of bee communities in rural landscapes (Gikungu, 2006; Kajobe, 2008).

This study focused on the characterization of agricultural bee fauna (functional groups) in central Uganda. This is the first solid contribution to the study of bee faunas from farmlands in Uganda. There has been no previous extensive study highlighting the diversity of bee functional traits occurring in the region. It is therefore believed that this study will form a basis for further studies in other countries of Sub-Sahara Africa with similar environmental characteristics in order to develop and set proper monitoring and conservation strategies of pollination services delivered by functionally diverse bee communities.

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References

Aizen M A and Feinsinger P.1994. Habitat fragmentation, native insect pollinators, and feral honey-bees in Argentine Chaco Serrano. *Ecological Applications*, **4(2):** 378–392.

Ascher J, Eardley C, Griswold T, Melo G, Polaszek A, Ruggiero M, Williams P, Walker K and WarritN.2008. World Bee Checklist Project – update 2008-09, manuscript (version 10/09/2008), [online] Integrated Taxonomic Information System. http://www.itis.gov/ beechecklist.html (accessed on: 29 August 2009).

Banaszak J and Manole T. 1994. Diversity and density of pollinating insects(Apoidea) in the agricultural landscape of Rumania. *Polskie Pismo Entomologiczne*, **57**:747-766.

Banaszak J. 2000.*Pollinating insects (Apoidea) from "Puszcza Zielonka" Landscape Park, Poland*. In: Banaszak (editor), **Ecology of Forest Islands**. p:108-134. Bydgoszcz University press 2000.

Bawa R.1990. Plant-pollinator interactions in tropical rain forests. *Annu. Rev. Ecol. Syst,* **21**:399-422.

Biesmeijer JC and Slaa EJ 2006b.The structure of Eusocial bee assemblages in Brazil. *Apidologie*, **37**: 240–258.

Biesmeijer JC, Roberts SPM, Reemer M, Ohlemuller, R, Edwards M, Peeters T, Schaffer AP, Potts SG, Kleukers R, Thomas CD, Settele J and Kunin WE. 2006a. Parallel declines in Pollinators and insect-pollinated plants In Britain and the Netherlands. *Science,* **313**: 351-354.

Brosi JB, Daily GC and Ehrlich PR. 2007. Bee community shifts with landscape context in a tropical countryside. *Ecological Applications*, **17(2):** 418–430.

Buschini MLT, Rigon J and Cordeiro J. 2009. Plants used by Megachile (Moureapis) sp. (Hymenoptera: Megachilidae) in the provisioning of their nests. *Brazilian J of Biol.*, **69(4):** 1187-1194.

Byarugaba D. 2004.Stingless bees (Hymenoptera: Apidae) of Bwindi impenetrable forest, Uganda and Abayanda indigenous knowledge. *Inter J of Tropical Insect Science*, **24(1):**117–121.

Cane JH, Minckley RL. and Kervin L.2000. Sampling bees (Hymenoptera: Apiformes) for pollinator community studies: pitfalls of pan-trapping. *J of the Kansas Entomol Society,* **73**:225–231.

Cane JH, Minckley RL, Kervin LJ, Roulston TH. and Williams NM. 2006. Complex responses within a desert bee guild (Hymenoptera: Apiformes) to urban habitat fragmentation. *Ecological Applications*, **16 (2):** 632–644.

Carvalheiro LG, Veldtman R, Shenkute RG, Tesfay GB, Walter C, Pirk W, Donaldson JS and Nicolson, SW. 2011. Natural and within-farmland biodiversity enhances crop productivity. *Ecology Letters*, **14**: 251–259.

Chamberlain SA and Schlising RA. 2008. Role of Honey Bees (Hymenoptera: Apidae) in the Pollination Biology of a California Native Plant, *Triteleialaxa* (Asparagales: Themidaceae).*Environ Entomol.*, **37(3):** 808-816.

d´Avila M and Marchini LC 2008.Faunistic analysis of flower-visiting hymenoptera in Cerradãofragment .*Ciência Florestal Santa Maria*,**18 (2):** 271-279.

Dag A. 2009. Interaction between pollinators and crop plants: The Israeli experience. *Israel J of Plant Sciences*, **57(3):** 231–242.

De Albuquerque P MC, de Camargo JMF. and Mendonça JMC.2007. Bee community of a beach dune ecosystem on Maranhão Island, Brazil. *Brazilian Archives of Biol and Technol.*,**50 (6):**1005-1018.

Droege S, Tepedino VJ, Lebuhn G, Link W, Minckley RL, Chen Q. and Conrad C. 2010. Spatial patterns of bee captures in North American bowl trapping surveys. *Insect Conservation and Diversity*, **3**: 15–23.

Eardley C. and Urban R. 2010. Catalogue of Afrotropical bees (Hymenoptera: Apoidea: Apiformes).*Zootaxa*, **2455**: 1–548.

Eardley C, Kuhlmann M. and Pauly A 2010. The Bee Genera and Subgenera of sub-Saharan Africa, *AbcTaza*, **7**: 1-145.

Eardley CD, Gikungu M. and Schwarz MP 2009. Bee conservation in Sub-Saharan Africa and Madagascar: diversity, status and threats. *Apidologie,* **40**: 355–366.

Fontaine C, Dajoz I, Meriguet J. and Loreau M. 2006.Functional diversity of plant–pollinator interaction webs enhances the persistence of plant communities. *PLoS Biology*, **4(1)**: e1. (DOI: 10.1371/journal.pbio.0040001).

Gallai N, Salles JM., Settele J and Vaissière B.E 2009. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecological Economics* **68**: 810-821.

Gikungu MW.2006. Bee diversity and some aspects of their ecological interactions with plants in a successional tropical community. Dissertation, University of Bonn, 201p.

Gordon JR, Balkwill K. and Gemmill B. 2004. African pollination studies: where are the gaps? *Inter J of Tropical Insect Science*, **24 (1):** 5–28.

Greenleaf SS, Williams NM, Winfree R and Kremen C.2007.Bee foraging ranges and their relationship to body size. *Oecologia*, **153**:589–596.

Grundel R, Jean RP, Frohnapple KJ, Glowacki GA, Scott PE. and Pavlovic NB. 2010. Floral and nesting resources, habitat structure, and fire influence bee distribution across an open-forest gradient. *Ecological Applications*, **20(6):**1678–1692.

Kajobe R. 2008. Foraging behaviour of equatorial Afro-tropical stingless bees: Habitat selection and competition for resources. PhD thesis, University of Utrecht, Netherlands, 128pp.

Klein A-M, Vaissiere BE, Cane, JH, Steffan-Dewenter I, Cunningham SA, Kremen C. and Tscharntke T. 2007. Importance of pollinators in changing landscapes for world crops. *Proceedings of Royal Society of London. B.*,**274**: 303–313.

Lee RA.2002. Interactive design of farm conversion. Linking agricultural research and farmer learning for sustainable small scale horticulture production in Colombia. PhD Thesis, Wageningen University, Wageningen, The Netherlands , 294pp; ISBN: 90-5808-7492.

Liow LH, Sodhi NS. and Elmqvist T. 2001.Bee diversity along a disturbance gradient in tropical lowland forests of south-east Asia. *J of Applied Ecol.*, **38**: 180-192.

Losey J E. and Vaughan M. 2006. The economic value of ecological services provided by insects. *BioScience*,**56**: 311–323.

Martins DJ.2004. Foraging patterns of managed honeybees and wild bee species in an arid African environment: ecology, biodiversity and competition. *Inter J of Tropical Insect Science*, **24(1):**105–115.

Meléndez-Ramirez V, Magaña-Rueda S, Parra-Tabla V, Ayala R. and Navarro J. 2002. Diversity of native bee visitors of cucurbit crops (Cucurbitaceae) in Yucatán, México. *J of Insect Conservation*, **6**: 135–147.

Michener CD.2007. **The Bees of the World**, the John Hopkins University Press, Baltimore and London, pp 913.

Minckley R. 2008. Faunal composition and species richness differences of bees (Hymenoptera: Apiformes) from two north American regions. *Apidologie*, **39**: 176–188.

Morandin LA, Winston ML, Abbott VA and Franklin MT. 2007. Can pastureland increase wild bee abundance in agriculturally intense areas? *Basic and Applied Ecol.*, **8**: 117—124.

Moretti M, de Bello F, Roberts SPM and Potts SG. 2009. Taxonomical vs. functional responses of bee communities to fire in two contrasting climatic regions. *J of Animal Ecol.*, **78**: 98–108.

Moroń D, Szentgyörgyi H, Wantuch M, Celary W, Westphal C, Settele J and Woyciechowski M. 2008. Diversity of wild bees in wet meadows: implications for conservation. *Wetlands*, **28 (4):** 975–983.

Munyuli TMB. 2010. Pollinator biodiversity and economic value of pollination services in Uganda (PhD Dissertation), Kampala, Uganda, 431 pages: Makerere University.

Munyuli TMB. 2011a. Farmers' perception of pollinators in coffee production in Uganda. *Agricultural Sciences,* **2 (3):** 318-333.

Munyuli TMB. 2011b. Factors governing flower visitation patterns and quality of pollination services delivered by social and solitary bee species to coffee in central Uganda. *African J of Ecol.,* (In press) (doi: 10.1111/j.1365-2028.2011.01284.x).

Munyuli TMB. 2011c. Pollinator biodiversity in Uganda and in Sub-Sahara Africa: Landscape and habitat management strategies for its conservation. *Inter J of Biodiversity and Conservation* (Inpress).

Norden BB. 2008. A Checklist of the bees (Insecta: Hymenoptera) and their floral hosts at Plummers Island, Maryland. *Bulletin of the Biol Society of Washington*, **15**:168-172.

Olschewski R, Tscharntke T, Benítez BC, Schwarze S and Klein A-M. 2007. Economic evaluation of ecosystem services as a basis for stabilizing rainforest margins? In: T. Tscharntke *et al*.(Eds), **The Stability of Tropical Rainforest Margins: Linking Ecological, Economic and Social Constraints of Land Use and Conservation**. Edited by T. Tscharntke *et al*. Environmental Science Series. Berlin: Springer. 265–278.

Ollerton J, Winfree R and Tarrant S. 2011. How many flowering plants are pollinated by animals? *Oikos*, **120**: 321–326.

Potts SG, KevanPG and Boone JW.2005.*Conservation in Pollination: Collecting, surveying and monitoring.* In: Dafni A and Kevan P (Eds.), **Pollination Ecology: A Practical Approach,** Enviroquest, Cambridge, Canada.

Roubik D W .1995.**Pollination of Cultivated Plants in the Tropics**. FAO, Rome.,198pages.

Roulston T H, Smith SA and Brewster AL2007. A comparison of pan trap and intensive net sampling techniques for documenting a bee (Hymenoptera: Apiformes) fauna. *J of the Kansas Entomol Society*, **80**:179-181.

Schlindwein C. 1998. Frequent oligolecty characterizing a diverse bee–plant community in a xerophytic bushland of subtropical Brazil. *Studies on Neotropical Fauna and Environ.*, **33**: 46–59.

Silva-PereiraVD and Santos GMM. 2006. Diversity in Bee (Hymenoptera: Apoidea) and Social Wasp (Hymenoptera: Vespidae, Polistinae) Community in "Campos Rupestres", Bahia, Brazil. *Neotropical Entomol.*, **35(2):**165-174.

Slaa EJ, Chaves LAS, Malagodi-Braga KS and Hofstede FE. 2006. Stingless bees in applied pollination: practice and perspectives. *Apidologie*, **37**: 293–315.

Southwick EE and Southwick L,Jr. 1992. Estimation of the economic value of honeybees (Hymenoptera: Apidae) as agricultural pollinators in the United States. *J of Economic Entomol.*, **85(3):**621-633.

Souza LD and Campos MJDO. 2008.Composition and diversity of bees (Hymenoptera) attracted by Moericke traps in an agricultural area in Rio Claro, state of São Paulo, Brasil. *Iheringia, Sér. Zool., Porto Alegre*, **98(2):**236-243.

Viana BF and Kleinert ADMP. 2005. A community of flower-visiting bees (hymenoptera: apoidea) in the coastal sand dunes of northeastern Brazil. *Biota Neotropica*, **5(2):** 1-13.

Westphal C, Bommarco R, Carré G, Lamborn E, Morison N, Petanidou T, Potts SG, Roberts SPM, Szentgyörgyi H, Tscheulin T, Vaissière BE, Woyciechowski M, Biesmeijer JC, Kunin WE , Settele J and Steffan-Dewenter I. 2008. Measuring bee biodiversity in different habitats and biographic regions. *Ecol Monographs*,**78(4):** 653–671.

Williams NM, Crone E, Roulston TH, Minckley RL, Packer L and Potts SG. 2010. Ecological and life-history traits predict bee species responses to environmental disturbances. *Biol Conservation,* **143**: 2280–2291.

Wilson JS, Messinger OJ and Griswold T. 2009. Variation between bee communities on a sand dune complex in the Great Basin Desert, North America: Implications for sand dune conservation. *J of Arid Environ.*, **73**: 666–671.

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Cytotoxic and Cytogenetics Effects of Aqueous, Methanolic and Secondary Metabolites Extracts of *Capparis spinosa* on Tumor Cell Lines *in vitro*

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Abstract

Capparis spinosa is one of the most aromatic plants growing in wild in the dry regions around the west or central Asia and the Mediterranean basin, and reports to contain a wide range of phytochemical constituents. The present study evaluates the cytotoxic effects of aqueous, methanolic crude extracts and secondary metabolites extracts polyphenolic, rutin, and alkaloids of mature fruit of *C. spinosa* on Human larynx carcinoma (Hep-2) and Human cervix adenocarcinoma (HeLa) tumor cell lines *in vitro*. The present study also includes the investigation of the effect of polyphenol mature fruit extracts on mitotic index (M.I.) of HeLa tumor cell line. The effect of (aqueous and methanol) crude extracts and secondary metabolites extracts (polyphenol, rutin, and alkaloids) of mature fruits of *C. spinosa* on Hep-2 and HeLa tumor cell lines have been showed highly significant difference ($P \leq 0.0001$) or ($P \leq 0.01$) among all types of extracts, and among all concentrations for each extract in two periods 24 and 48 hrs of the treatment. However the study reveals that the effective extracts against the proliferation of tested cell line were polyphenol extracts with concentration 10000 µg/ml in Hep-2 cells after 24 and 48 hrs. and with concentrations 10000 and 5000 µg/ml in HeLa cell line after 48 hrs. Polyphenolic extract showed a cytotoxicity concentration 50% (CC50%) 6400 and 6800 µg/ml on Hep-2 tumor cell line after 24 and 48 hrs. of treatment, respectively. The CC50% of HeLa cells was 7100µg/ml after 48 hrs. Other extracts; aqueous, methanolic crude extracts and secondary metabolites extracts (rutin and alkaloids) of mature fruit of *C. spinosa* caused less inhibition activity on the growth of Hep-2 and HeLa tumor cell lines. The CC50% for all these extracts were more than 10000 µg/ml. The result of present study shows that non significant difference of polyphenol mature fruit extracts effect on the type of tumor cell line either HeLa or Hep-2 cell lines. The cytogenetic study on HeLa cell line shows that polyphenol mature fruit extract has antimitotic index against tested cell line. Some of structural and numerical chromosomal aberrations were observed in both treated and untreated groups. Structural chromosomal aberrations include: ring chromosome (R. Ch.), dicentric chromosome (D. C.Ch.), chromatid gap and symmetrical interchange of chromosome as well as pulverization in treated group with higher concentrations 3550and 1775 µg/ml of extract. Numerical chromosomal aberrations include: octoploidy, euploidy and aneuploidy.

keywords: Cytotoxic, cytogenetics, extracts, Capparis spinosa, tumor cells.

1. Introduction

Cervical cancer is the second most common form of cancer among women worldwide about 274,000 deaths in 2002, and accounted for 15% of all female cancers (Fayed, 2008). In Iraq the cancer of the cervix is 2.1% (IARC, 2008). Laryngeal cancer is the most common non cutaneous malignancy. Cancers of the mouth, pharynx, and larynx, together, are the seventh most commonly occurring types of cancer worldwide. These cancers are three times more common in men than in women (AICR, 2007), especially those older than 60 years (Parkin *et al.*, 1990). Over 550000 cases were recorded in 2002, accounting for around 5 % of cancer cases overall (AICR, 2007).

Larynx carcinoma can develop in any part of the larynx. Most of the cancers of the larynx begin in cells that line the inner walls of the larynx (Parkin *et al.*, 1990). In the last three decades, cancer has been transformed from a fatal disease to one in which the majority of people diagnosed with cancer receive highly effective treatments that result in either cure or long-term survivorship (Angela *et al*., 2007).

Medicinal plants possess an important position in the drug discovery (Newman *et al*., 2000). According to the estimates of the WHO, more than 80% of people in developing countries depend on traditional medicine for their primary health needs (Sivalokanathan *et al*., 2005; Pandey and Madhuri, 2006). A major reason for the

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increase seems related to consumers' perceptions that these products are environmentally pure and without side effects (Montbriand, 1997 and 2000). Plant extracts have longer been a fertile source of cure for cancer (Mukherjee *et al*., 2001).

Several studies were carried out during the last years in Iraq to evaluate the cytotoxic activity of several local plants *in vitro* and *in vivo*. Al-Atby (2001) has found that the ethanolic extract of *Withaniasomnifera* had a growth inhibitory action against plasmacytoma cell line. In addition, the study of AL- Dabhawi (2005) revealed the great growth inhibition of aqueous and ethanolic extracts of *Artemisia herba alba* on Hep-2 and AMN-3 cell lines. Aqueous root extract of *Echium sericeum*posses inhibitory affect on the growth of Hep-2 and RD cell lines (Al-Habbib and Al-Asady, 2009). Seeds of *C.spinosa* contain glucocapparin, glucocleomin (Bhargara and Soni, 1980; Mathur, 1986) palmitic, oleic acid and linoleic acid (Attila and Ozcan, 1999). A dimeric 62-KDa lectin exhibiting a novel N-terminal amino acid sequence was purified from *C. spinosa* seeds (Lam *et al.*, 2009). Glucosinolates like sinigrin, glucoiberin and glucocleomin were isolated from the seeds and leaves of *C. spinosa* (Romeo *et al*., 2007). *Capparis spinosa* fruits contain alkaloids, glucosides, reducing sugar, fats, resins, ascorbic acid (Rastogi and Mehrotra, 1995) and isothyiocyanate (Mitchell, 1974). Alkaloids have been isolated and identified from *C. spinosa* fruits (Fu *et al*., 2008). Yang *et al*.,(2010) found that fruit of *C.spinosa* contain a significant amount of compounds with many health benefit, Three new alkaloids, (1) Capparisine A, (2) Capparisine B, (3) Capparisine C and (4) two known alkaloids, (4)2-(5-hydroxymethyl-2-formylpyrrol-1-yl) propionic acid lactone and (5)N-(3'-maleimidy1)-5-hydroxymethyl-2-pyrrole formaldehyde were isolated from the fruit of *C.spinosa* . Indole-3 acetonitrile glycosides and (6S)-Hydroxy-3-oxo-α-ionolglucosides were isolated from *C. spinosa* mature fruits in Turkey (Çalis*et al*., 1999; Çalis*et al*., 2002). Triterpenoids like α-amyrin, sterols, β-carotene, saponins were found in the preliminary phytochemical screening (Satyanarayana*et al*., 2008). Recently study of A. Al-Sqeer (2011) revealed that the total antioxidants in hot-water extracts of *C.spinoso* were 115.66µmol per 100mL.

The total alkaloids of *C. spinosa* can inhibit the growth of human gastric adenoma cells SGC-7901(Yu *et al*., 2008). Aqueous and methanolic root extract of *C. spinosa* possess considerable inhibition of AMN3 cells ,whereas Hep-2 tumor cell line is sensitive to aqueous root extract as well as aqueous leave extract *in vitro*. Aqueous and methanolic root extract of *C.spinosa* has ability to reduce the tumor volume *in vivo* (Al-Asady, 2007). The lectin potently that isolated from seeds of *C.spinosa* inhibited the proliferation of both hepatoma HepG2 and MCF-7 cell lines. (Lam *et al.*, 2009).

According to the medical properties of *C. spinosa* fruits in the world those not detected in Iraq, the present study was designed to evaluate the cytotoxic effects of aqueous , methanolic crude extracts and secondary metabolites; polyphenol, Rutin, and Alkaloids of mature fruits of *C. spinosa* on Human larynx carcinoma (Hep-2) and Human cervix adenocarcinoma (HeLa) tumor cell lines *in vitro*.

2. Material and Methods

2.1. Plant Collection

Capparis spinosa was collected from Duhok governorate in September 2008. The whole plant was deposited to be identified, the identification done by Dr. Salem Shahbaaz, Department of Forestry, College of Agriculture, University of Duhok, Duhok, Iraq. Then whole mature fruits and seeds from mature fruits were dried at room temperature. According to (Harborne,1984), each part was ground into powder by electrical grinder (mesh No. 0.5mm), and the powdered parts were kept in plastic tubes in deep freeze -20°C until the time of use. Crude aqueous extract and crude methanolic extract, from whole mature fruit, alkaloid from seeds of mature fruit of *C.spinosa* were prepared according to (Harborne, 1984). Extraction of Secondary Metabolites Polyphenol from mature fruit as described by Yu and Dahlgren, (2000), whereas rutin was extracted from mature fruit of *C. spinosa* according to Kim *et al.* (2005).

Chemical test for plant extracts both Wagner's Reagent and Hager's Reagent was used to test the presence of alkaloids in extracts ,whereas Ferric Chloride Solution according to Gayon (1972) and lead acetate solution according to Harborne (1984) was used to test the presence of polyphenol (tannins). The presence of flavonoids in extracts was tested according to (AL-Shahaat, 1986). The identification of rutin according to Harborne (1984). Liebermann-Burchard test was used to test the presence of triterpenoids, whereas Peptides and Free Amino Group Test used to test the presence of peptides, primary or secondary amino groups (Harborne, 1984). To test the presence of carbohydrate compounds Molish reagent was used (Hawk *et al*., 1954).The presence of glycosides was detected according to AL- Shahaat (1986). Saponins were identified according to Harborne (1984).

2.2. Cell Line

Human Larynx Epidermoid Carcinoma (Hep-2) tumor cell line Passages 220-223 in RPMI-1640 medium (Sigma, USA) and Human Cervix Adenocarcinoma (HeLa) passage 240-243 tumor cell line in Eagles MEM (Sigma, USA) supplemented with L-glutamine, non-essential amino acids and 10% FBS. was kindly supplied by Tissue Culture Unit/Iraqi Center for Cancer and Medical Genetic Researches (ICCMGR)/Baghdad, Iraq.

To determine the viability of tumor cell lines, confluent monolayer were treated with trypsin-versene and cells were further dispensed by pipetting in growth medium then 0.2 ml of cells suspension was mixed with 0.2 ml of trypan blue solution and 1.6 ml phosphate buffer saline (PBS), and a sample of cells counted by using an Improved Double Naubauer Ruling Counting Chamber. Magnification powers of 100X and 400X were used to count the cells, viable cells do not stain, but dead cells stain blue.

The following formula was then used to calculate the number of cells per unit volume (cells/ml) (Freshney, 1994):

$$C = N \times D \times 104 \quad (1)$$

Where **C** is the number of viable cells per milliliter, **N** is the number of viable cells counted, and **D** is the dilution factor **(D= 10).**

About 200 µl of cells suspended (55000 cells/ml) in growth medium was seeded in to each well of a sterile 96-well micro-titration plate. The plates were sealed with a self-adhesive film, lid placed on and incubated at 37°C. When the cells are in exponential growth (approximately 70-80% confluent monolayer), the medium was removed and serial dilutions of each aqueous, methanolic crude extracts and secondary metabolites extracts (polyphenolic , rutin ,and alkaloids) of mature fruit, separately in maintenance medium (10000, 5000, 2500, 1250, 625, 312.5, 156.25, 78.125, and 0 µg/ml) were added to the wells. Three replicates were used for each concentration of either extract, and the plates were re-incubated at 37°C for the selected exposure times (24 or 48 hrs).

Cytotoxic effect of each extract on both tumor cell lines using neutral red dye assay according to Freshney (1994). The optical density (O .D) of each well after treatment was read using Enzyme Linked Immunosorbent Assay (ELISA) reader at a transmitting wavelength of 492 nm (Mahoney *et al*., 1989; Freshney, 1994). The percentage of cytotoxicity was calculated as (A-B)/A X100, where A was the mean O.D of untreated wells and B is the O.D of wells with plant extracts (Betancur-Galvis*et al*.,1999).The cytotoxic concentration 50% (CC50%) for each extract was calculated from concentration-effect-curves after linear regression analysis (Hayslett and Patrick, 1981).

Cytogenetic Study on HeLa Tumor Cell Line before and after Treatment with Polyphenol mature fruit extracts .Three replicates were used for each concentration (3550, 1775 and 887.5 µg /ml) dependent on CC50%. Another three culture flasks were used as a control group treated with maintenance media + phosphate buffer (PBS). All flasks were incubated at 37°C for 48 hrs., the chromosomes was prepared according to Modi (1987). One thousand cells were examined to calculate the M.I. In these cases the slides were examined fewer than 10 X magnifications, and observed chromosomes or chromatides aberrations under 100X magnification. The M.I % was determined as a ratio of the mitotic cells to the cells in interphase in 1000 calculated cells. M.I. % = (No. of dividing cells / No. of dividing cells + No. of non-dividing cells) X 100 (Babu *et al*., 2005, Kleinsmith, 2006).

2.3. Statistical Analysis

Analysis of variance (ANOVA) and the least significant difference (LSD) were used for the statistical analysis of the results and P-values at levels ($P<0.01$) was considered to be statistically significant. These calculations were carried out according to SAS system (SAS, 2000).

3. Results

3.1. The Properties of C. spinosa Fruit Extracts, Yield of Extraction, and Qualitative Chemical Analysis

The results of *C. spinosa* aqueous , methanolic crude extracts and secondary metabolites (polyphenol., rutin. and alkaloids) extracts from Mature fruits with respect to the nature and color of the obtained extract, color of each extract solution, yield of extraction %, and qualitative chemical analysis for each extract are summarized in tables (1 and 2).

3.2. Thin Layer Chromatography (TLC) of Rutin Extract

Rutin extract from mature fruit was analyzed by TLC. The result of the analysis shows spots of standard rutin and extracts (Figure 1), the identification of rutin was done by comparison rate of flow (Rf) for extract with Rf of standard rutin as well as the color of spot under U.V. light (Table 3).

3.3. Cytotoxic effect of aqueous, methanolic crude extracts and secondary metabolites (polyphenol, rutin. and alkaloids) extracts of mature fruits of C.spinosa on Hep-2 Tumor Cell Line in vitro

The effect of (aqueous and methanol) crude extracts and secondary metabolites extracts (polyphenol, rutin, and alkaloids) of mature fruits of *C. spinosa* on Hep-2 tumor cell line have been showed highly significant difference ($P\leq 0.0001$) among all types of extracts, and among all concentrations for each extract in two periods 24 and 48 hrs of the treatment, the interaction between extracts and concentrations was highly significant ($P\leq 0.0001$) after 24 and 48 hrs. Table (4) shows that the cytotoxic effect of mature fruit extracts varied with different types of extracts and concentrations levels. Interaction between concentrations and extracts revealed that the Polyphenol extract was more effective than other extracts and its activity against the growth of Hep-2 cells was started from 1250 µg/ml as compare to control group(Figure 2a) which shows complete confluent monolayer of cohesive malignant cells and the value of O.D. was 0.248±0.005. The concentration 10000 µg/ml have higher inhibition activity 0.061±0.003 than 5000 µg/ml 0.177±0.01 (Figure 2 b and c), and this concentration is more effective than lower concentrations those exhibited the same effect. Aqueous extract was effective only in concentration 10000 µg/ml (Figure 2d) and the value of O.D. was 0.231±0.003. Methanolic extracts became effective in higher concentrations 5000 and 10000 µg/ml. The concentration 10000 µg/ml which have O.D. value 0.171± 0.002 and exhibit more inhibition activity than 5000 µg/ml with value 0.204± 0.006 (Figure 2e). The inhibition activity of rutin extract started from the concentration 2500 µg/ml up to 10000 µg/ml, these concentrations have the same effect (Figure 2f), the values of O.D. were 0.236±0.01, 0.236±0.014 and 0.226±0.01, respectively. Statistical analysis shows that both concentrations of alkaloid extracts 5000 and 10000 µg/ml has the same inhibition activity on the growth of Hep-2 cell line (Figure 2g) and the value of O.D. were 0.239±0.005 and 0.210±0.007, respectively. After 48hrs. treatment aqueous extracts became effective with concentration 5000 µg /ml as well as 10000 µg /ml both have the same effect and the values of O.D were 0.224 ± 0.001 and 0.230 ± 0.0104 respectively (Figure 3a). The effective concentrations of methanolic extracts after 48 hrs were 5000 and 10000µg/ml, the values of O.D. were 0.183±0.004 and 0.138±0.17, respectively. The higher concentration 10000 µg/ml was more effective (Figure 3b).

The effect of polyphenol extracts started from 2500µg/ml up to 10000µg/ml. The effect was concentration-dependant manner, the values of O.D. were 0.213±0.007, 0.178±0.005 and 0.076±0.0006 respectively (Figure 3c). The higher concentrations of rutin extract 5000 and 10000µg/ml were effective and both of them exhibit the same effect (Figure 3d). Alkaloid extracts started in its effect from 2500 µg/ml up to 10000 µg/ml, the effect of these concentrations have the same inhibition activity against proliferation of Hep-2 cell line (Figure 3 e)(table-5). The exposure times had a highly significant effect (P<0.0001) on the growth of Hep-2 tumor cell line treated with aqueous, methanol, and alkaloid extracts whereas no significant effect on the growth of Hep-2 cell line was noticed when subjected to other extracts Polyphenol and rutin extracts. Table (6) demonstrated that aqueous, was effective at 48 hrs more than 24 h. Methanolic extract also was more effective at 48 hrs more than 24 hrs. Similarly Alkaloid extracts was more effective after 48 hrs. Polyphenolic extract reduce the viability of Hep-2 cells down to 50% and presented CC50 value of 6400 and 6850µg/ml after 24 and 48 hrs., respectively, while those of (aqueous methanol, rutin and alkaloid) mature fruit extracts were more than 10000 µg/ml.

3.4. Cytotoxic Effect of aqueous, methanolic Crude Extracts and Secondary Metabolites (polyphenol, rutin, and alkaloids) extracts of mature fruits of C. spinosa on HeLa Tumor Cell Line in vitro

The results showed statistical differences among all types of extracts (P≤0.01) after 24 hrs of treatment and highly significant difference (P≤0.0001) after 48 hrs. The results also showed that the effect of tested extracts on HeLa cell line proliferation was highly significant (P≤0.0001) among concentrations after 24 and 48 hrs. The interaction between concentrations and extracts was not significant after 24 hrs of treatment and was highly significant (P≤0.0001) after 48 hrs.

The result revealed that each extract (aqueous, methanol, and rutin) has the same activity against proliferation of HeLa cells after 24 hrs treatment (Table 7). The activity of extracts started from concentration 78.13 µg/ml up to 10000 µg/ml. The more inhibition activity of polyphenol extract after 48 hrs. of treatment concentrated in both 10000 and 5000 µg/ml, and both have the same effect(Figure 4 a and b). Methanolic extract revealed its effect with concentrations 10000 and 5000 µg/ml both of them have the same inhibition activity (Figure 4c and d), the same result was obtained by alkaloid extracts treated cells (Figure 4e). Rutin extract revealed its activity with concentration 10000 µg/ml only (Figure 4f) (table 8).

The effect of exposure time of extracts on the proliferation of HeLa tumor cell line was highly significant (P<0.0001) after treating with each (methanol, rutin, and alkaloid.) extract and was significant (P<0.01) when treated with each (aqueous and polyphenol) extract. Table (9) appeared that crude and secondary metabolites extracts were more effective in 48 hrs than 24 hrs. Polyphenolic extract presented CC50 value of 7100 µg/ml after 48 hrs on HeLa cell line, while those of (aqueous, Methanol, rutin and alkaloid) mature fruit extracts were more than 10000 µg/ml.

3.5. Effect of mature fruit extracts. of C. spinosa on the Type of Tumor Cell Lines

Statistical analysis shows highly significant differences (P ≤ 0.0001) of mature fruit extracts effect on the types of tumor cell lines. The result reveals that HeLa tumor cell line is more affected than Hep-2 tumor cell line, the value of O.D. are 0.183±0.0022 and 0.244±0.0024, respectively (Table 10). The result of present study revealed that non significant difference of polyphenol mature fruit extracts effect on the proliferation of HeLa and Hep-2 cell lines. The value of O.D. for Hep-2 and HeLa cell lines are 0.227±0.004 and 0.172±0.003, respectively.

3.6. Cytogenetic Effect of polyphenol mature fruit extracts of C. spinosa HeLa on Tumor Cell Line in vitro

The results revealed highly statistical differences among treatments (P≤0.0001). The value of M.I in HeLa tumor cell line was decreased after treatment with concentrations 3550 and 1775 µg /ml of polyphenol mature fruit extracts only as compare with control groups 2.86±0.4, 3.4±0.26 and 6.76±0.24, respectively (table 11). Numbers of structural and numerical chromosomal aberrations were found in cells of control groups and in treated cells. The structural chromosomal aberrations in control group were: ring chromosome (R.Ch), chromosome break with fragment (Ch.B.W.F), gap chromosome (gap Ch.), dicentric chromosome (D.C.Ch.)(Figure 5a) and symmetrical interchange of chromosome (Figure 5b). The numerical chromosomal aberrations in control group were: euploidy (triploid 3n) (Figure 5c), octoploid (8n) (Figure 5d), aneuploidy (2n+2) (Figure 5 e). Pulverization of chromosomes was found in cells treated with high concentrations (3550 and 1775 µg\ml) of Polyphenol mature fruit extracts (Figure 6a, b). Other structural chromosomal aberrations in treated cells were chromatid break with fragment Cht.B.W.F., D.C.Ch and Cht. gap was found in cells treated with 1775 µg\ml Polyphenol mature fruit extracts (Figure 6c), ring chromosome, chromatid gap and D.C.Ch in cells treated with 887.5 µg\ml (Figure 6d). The numerical chromosomal aberrations were: triploid (3n) in cells treated with 887.5 µg\ml Es (Figure 6 e).

4. Discussions

4.1. The C. spinosa mature fruit extracts

The result of extraction in the present study reveals that the yield of extraction is varied according to the types of solvents those used in extraction method, and the method of extraction. This result agrees with that obtained by Henning *et al*. (2003) in which they find that the relation proportion between the amount of plant used for extraction and crude product. The result of present study revealed that the yield of extracts in mature fruit was high, may be according to the presence of high quantity of terpenoids and essential oils in mature fruits. This result is supported by Matthhaus and Ozcan (2005).

The result shows that the chemical compounds (alkaloids, tannins, flavonoids, glycosides, triterpenoids, carbohydrates, and saponins) and secondary metabolites extracts in the mature fruit under study were varied qualitatively due to the solvent of extraction. These qualitative variations can be attributed to the fact that the crude and secondary metabolites extracts from mature fruit contain different constituents that vary considerably in their relative concentrations. Study of Howard *et al*. (2000) on the *Capsicum* species, reveal that the concentration of chemical constituents such as carotenoids, flavonoids, phenolic acids and ascorbic acid increased as the *Capsicum annuum* , *C. frutescens* and *C. chinese* reached maturity.

Rutin extract is qualified as flavonoids secondary metabolites by comparison of its R_f value with that of standard. The yield of rutin extraction for mature fruit is 19.5, while Ramezani *et al*. (2008) purified rutin from different parts of *C. spinosa*, and they demonstrate that the yield of rutin extract from leaves, fruits and flowers are 18.22, 18.42 and 25.40% respectively, whereas the purity of rutin in leaves and flower extract is more than that extracted from fruits.

4.2. Cytotoxic Effect of Aqueous, Methanolic Crude Extracts and Secondary Metabolite (polyphenol, rutin, and alkaloid) extracts of mature fruit of C.spinosa on Hep-2 and HeLa Tumor Cell Lines in vitro

The cytotoxic effect of mature fruit extracts of *C. spinosa* on Hep-2 cell line varied with different types of extracts and concentrations levels. Polyphenol extract is the more effective extract in both periods of treatment (24 and 48 hrs). The activity of extracts concentrated in 10000 µg/ml against the growth of Hep-2 cells after 24 hrs and 48 hrs. The result revealed that HeLa cell line that treated with (aqueous and polyphenol) mature fruit extracts of *C. spinosa*, separately were more effective than other extracts (methanol, rutin, and alkaloid) after 24 hrs of treatment, whereas after 48 hrs, polyphenol extracts was the more effective with concentrations 10000 and 5000 µg/ml. The CC50 value for polyphenol extract on proliferation of Hep-2 was 6400 µg/ml after 24 hrs and was 6850 µg/ml after 48 hrs, whereas for HeLa cell line was 7100 µg/ml after 48 hrs. Other extracts (aqueous, methanol, rutin, and alkaloids) have CC50% more than 10000 µg/ml on proliferation of both Hep-2 and HeLa cell line. The highly inhibition activity of polyphenol mature fruit extracts against Hep-2 and HeLa cell lines can be due to its contents of active compounds such as caffeic, ellagic and ferulic acids, which have been reported to exhibit antioxidant and anticarcinogenic activities (Decker, 1995). Polyphenols were isolated from green tea, the powerful antioxidants were capable of scavenging H2O2and superoxide anions and thus preventing free radical damage to the body. This is a mechanism that has been associated with cancer (Khan *et al*., 1992). The results of present study agree with that obtained by Sa'eed, (2004) in which he found that the great growth inhibition of green and black tea extracts on Hep-2 tumor cell line. Plant extract rich in flavonoids exhibit antiproliferative effects on various cancer cell line (Adrienne *et al*., 2006). Apigenin is a widely distributed plant flavonoid that was reported as an antitumor agent, it inhibits the growth of human cervical carcinoma cells (Duthie and Crozier, 2000; Pei-Wen *et al*., 2005). Other extracts (aqueous, methanol, rutin. and alkaloid) reduce the proliferation of Hep-2 cells after 24 hrs. Simultaneously, whereas after 48 hrs methanol extracts shows its effect more than the other extracts (aqueous, rutin. and alkaloid). Methanolic extracts revealed this activity because most of the biological active compounds are extracted with methanol. Study of Betancur-Galvis *et al*. (1999) support the result that obtained in the present study. They found that seeds methanolic extract of *Annona* sp. has cytotoxic activity against Hep-2 tumour cells *in vitro*. Methanolic extracts was more effective than aqueous extracts because methnolic extracts contain the most potent antioxidant and phenolic compounds. Study of Khanavi*et al*. (2009) shows that methanolic extract from *Stachys* species contains the most potent antioxidant and phenolic compounds, whereas water extract afforded the lowest amount, therefore aqueous extracts lesser activity can be attributed to low quality of active compounds that included. Rutin extracts was also effective but the activity less than other extract, the less activity of rutin extracts may due to less purity of rutin that isolated from fruit (Ramezani*et al*., 2008). Alkaloid extracts have the same effect of methanolic extracts in HeLa cells after 48 hrs. treatment. Several studies reported that the plants have medical importance due to their alkaloids content, and these alkaloids have cytotoxic properties.

The study of Winter (2008) indicated that the low alkaloid lupin reduce the proliferation of mouse lymphoblast of P388 cell line even at 1mg/ml, and their cytotoxicity was assessed after 48 hrs. Vinca alkaloids, vinblastine and vincristine both act specifically to block mitosis of treated tumor cells (Richard *et al.,* 2001). Aparicio-Fernandez *et al*. (2006) demonstrated that methanolic crude extract of *Phaseolus vulgaris L.* have inhibitory effect on proliferation of HeLa cells. Other extracts (aqueous, methanol, and rutin) has the same activity against proliferation of HeLa cells after 48 hrs. Aqueous , methanol ,and alkaloid mature fruit extracts were more effective against the proliferation of Hep-2 cells at 48 hrs than after 24 hrs treatment , whereas the inhibition effect of Polyphenol and rutin mature fruit extracts against Hep-2 cells was similarly at both time of treatments 24 and 48 hrs. All types of mature fruit extracts against proliferation of HeLa cells were more effective after 48 hrs, except for polyphenol mature fruit extracts that revealed their inhibition activity against the proliferation of HeLa cell line after 48 hrs more than 24 hrs. This can be attributed to chemical constituents those found in polyphenol mature fruit extracts. These compounds may possess low ability to be absorbed by HeLa cells; therefore, they show their activity after 48 hrs of treatments. Marja (2004) stated that some plant extracts have low ability to adsorption by cell membrane such as glucosinolate and isothiocyanate, these can be found in polyphenol extract. In Hep-2 cells the similarity of polyphenol mature fruit extracts activity in both times, this reflect that Hep-2 cells have a specific properties in their membrane differs from HeLa cells that facilitate the movement of polyphenol mature fruit extracts compounds across it similarly in both time of treatment.

4.3. *The Effect of mature fruit extracts of C. spinosa* on the Type of Tumor Cell Lines

The *effects of mature fruit extracts on th*e types of tumor cell lines were detected. The results revealed that HeLa tumor cell line was more affected than Hep-2 tumor cell line. Lee *et al*. (2003) demonstrated that cell membrane receptors of tumor cells vary in their response to different drugs or crude extracts during chemotherapy treatments. Elisa et *al*. (2004) found that the cytotoxic activity of tannins and phenolic compounds extracted from *Cupheaaequipetala* was different in the three cancer cell lines; Hep-2, human colon cancer (HCT_15), and human prostate carcinoma (DV-145). The whole acetone-water extract does not show cytotoxic activity on (DV-145) cells.

The effect of polyphenol mature fruit extracts on the proliferation of HeLa and Hep-2 cells was also detected, both of them affected similarly with polyphenol mature fruit extracts. This may be due to the similarity in membrane properties in both Hep-2 and HeLa cell line as a target by phenolic compounds instead of mature fruit extracts.

This may be due to the presence of some chemical compounds in mature fruit extracts of *C. spinosa* with inhibitory properties against both Hep-2 and HeLa cell lines or may be due to the differences in membrane properties between Hep-2 and HeLa cells.

4.4. Cytogenetic Effect of polyphenol mature fruit extracts of C. spinosa on HeLa Tumor Cell Line in vitro

The present study shows that both tumor cell lines Hep-2 and HeLa were affected simultaneously after treatment with polyphenol mature fruit extracts. In this experiment, we have focused on determining the effect of polyphenol mature fruit extracts on M.I. of HeLa cell line. HeLa tumor cell line shows decrease in M.I. when treated with higher concentrations3550and 1775 µg /ml of polyphenol mature fruit extracts as compared with untreated cells. On the other hand, it was noticed that the low concentration 887.5 µg /ml has non significant effect. The acceptable explanation for ability of polyphenol mature fruit extracts to reducing the M.I. can be traced to its chemical constituents that have ability to effect on cell cycle progression. This result supported by Schoene *et al*. (2005) they have found the mixture of polyphenols from aqueous *Cinnamon* extract possessed anticancer properties by blocking cell cycle progression of leukemic cell lines at the G2/M phase. In addition to the G2/M arrest with mixture of polyphenols from aqueous Cinnamon, Schoene *et al*. (2005) also demonstrated that the extract reduced total phosphatase activity in the cell lines. Duthie and Crozier (2000) and Pei-Wen *et al*. (2005) suggested that apigenin is a strong candidate for development as an anti-cervical cancer agent. Apigenin's preventive effect is shown to be mediated through induction of p53 expression, which causes cell cycle arrest and apoptosis.

Chromosomal aberrations (numerical and structural) are found in HeLa cells those treated with polyphenol mature fruit extracts as well as in untreated cells. This result is in agreement with that obtained by Rocha-Guzman *et al.* (2009). Their result shows the ability of phenolic compounds to damage the DNA of HeLa cells and transformed human cells. Duesberg and Rasnick (2000) demonstrated the notion that aneuploidy is an autocatalytic process leading to the transition from a pre-neoplastic phenotype into a neoplastic one.

Table 1. The nature and color of dried product extracts and solutions of mature crude extracts of *C. spinosa*, and the yield of extraction %:

Part of plant	Type of Extract		Nature & color of Extract	Color of Solution	Yield of extraction %
Mature fruit	Crude extracts	Methanol	Solid→ greenish black	yellowish brown	15.8
		Aqueous	Solid→ brown	Brown	18.1
	Secondary metabolite extracts	Polyphenol	Viscous → greenish black	Brown	15.3
		Rutin	Solid → brown	Brown	19.5
		Alkaloid	Crystal → brown to black	Dark brown	11.7

Table 2. The results of qualitative chemical analysis for (aqueous, methnol, polyphenol and alkaloid) extracts of *C. spinosa* mature fruits.

	Mature crude Extracts			
Compound group	Aqueous	Methanol	Polyphenol	Alkaloid from (seeds)
Alkaloids				
a- Wagner′s reagent	+	+	+	+
b- Hagers reagent	+	++	+	++
Tannins				
a-lead acetate	+	+	+	+
b-Ferric chloride	+	+	+	+
Flavonoid test	+	+	++	-
Triterpenoid	-	++	++	+
Peptides& Free amino group	+	+	+	+
Carbohydrate	+	+	+	+
Glycosides				
a-before hydrolysis	++	-	+	-
b- after hydrolysis	-	-	-	-
Saponin	+	+	+	-

+ =The extract contain the designated phytochemicals.; - =The extract does not contain the designated phytochemicals.

Table 3. The results of TLC for rutin extract (R_f and color of spot under U.V. light) and comparison with standard rutin.

Compound	Rate of flow (R_f)	Color of spot under U.V. light
Rutin standard (a)	0.58	Yellow
Mature rutin extract (c)	0.48	Yellow

Table 4. Mean ± SE for the effect of different concentrations of (aqueous., methnol, polyphenol, rutin., and alkaloid) mature fruit extracts *spinosa* on the growth of Hep-2 tumor cell line after 24 hrs treatments *in vitro*: (Observations of O.D).

	Concentration µg/ml								
Extracts	0	78.13	156.25	312.5	625	1250	2500	5000	10000
Aqueous	0.289±0.014	0.268±0.011	0.269±0.006	0.269±0.008	0.266±0.004	0.265±0.005	0.268±0.002	0.258±0.006	0.231±0.003
Methanol	0.289±0.014	0.274±0.007	0.273±0.007	0.282±0.014	0.276±0.007	0.275±0.007	0.271±0.01	0.204±0.006	0.171±0.002
Polyphenol	0.289±0.014	0.280±0.007	0.282±0.006	0.284±0.005	0.270±0.008	0.248±0.005	0.235±0.01	0.177±0.012	0.061±0.003
Rutin	0.289±0.014	0.268±0.007	0.268±0.015	0.263±0.008	0.268±0.015	0.263±0.01	0.236±0.01	0.236±0.014	0.226±0.01
Alkaloid	0.289±0.014	0.272±0.003	0.270±0.006	0.270±0.002	0.271±0.007	0.265±0.005	0.259±0.004	0.239±0.005	0.210±0.007

Effectors	Extracts	Concentrations	Extracts and Concentrations
L.S.D(0.01)	0.011	0.015	0.033

SE=Standard Error.

Table 5. Mean ± SE for the effect of different concentrations of (aqueous., methnol, polyphenol, rutin., and alkaloid) mature fruit extracts of *C. spinosa* on the growth of Hep-2 tumor cell line after 48 hrs treatments *in vitro*: (Observations of O.D).

	Concentration µg/ml								
Extracts	0	78.13	156.25	312.5	625	1250	2500	5000	10000
Aqueous	0.258±0.004	0.245±0.004	0.244±0.003	0.247±0.002	0.243±0.006	0.239±0.0006	0.234±0.004	0.224±0.001	0.230±0.01
Methanol	0.258±0.004	0.248±0.011	0.248±0.02	0.240±0.012	0.246±0.007	0.244±0.007	0.241±0.007	0.183±0.004	0.138±0.017
Polyphenol	0.258±0.004	0.245±0.007	0.251±0.014	0.247±0.009	0.246±0.004	0.245±0.004	0.213±0.007	0.178±0.005	0.076±0.001
Rutin	0.258±0.004	0.257±0.005	0.258±0.013	0.258±0.007	0.260±0.01	0.248±0.004	0.246±0.007	0.210±0.008	0.186±0.006
Alkaloid	0.258±0.004	0.248±0.009	0.245±0.002	0.241±0.004	0.242±0.003	0.232±0.008	0.229±0.007	0.221±0.004	0.218±0.004

Effectors	Extracts	Concentrations	Extracts and Concentrations
L.S.D(0.01)	0.0091	0.0121	0.0272

SE=Standard Error.

Table 6. Mean ±SE for the effect of exposure time to mature fruit extracts of *C. spinosa* on the growth of Hep-2 tumor cells *in vitro* (Observations of O.D)

	Time/hrs		
Extract	24	48	L.S.D
Aqueous	0.265±0.003	0.240±0.002	0.01
Methanol	0.257±0.008	0.227±0.008	0.012
Polyphenol	0.236±0.014	0.218±0.011	-
Rutin	0.257±0.005	0.242±0.006	-
Alkaloid	0.260±0.005	0.237±0.003	0.008

SE=standard error.

Table 7. Mean ± SE for the effect of different concentrations of (aqueous., methnol, polyphenol, rutin., and alkaloid) mature fruit extracts of *C .spinosa* on the growth of HeLa-2 tumor cell line after 24 hrs treatments *in vitro*: (Observations of O.D).

	Concentration µg/ml								
Extracts	0	78.13	156.25	312.5	625	1250	2500	5000	10000
Aqueous	0.241±0.021	0.227±0.004	0.223±0.010	0.187±0.008	0.190±0.023	0.178±0.021	0.172±0.017	0.170±0.030	0.151±0.013
Methanol	0.241±0.021	0.233±0.008	0.234±0.009	0.231±0.009	0.227±0.001	0.212±0.016	0.217±0.005	0.173±0.003	0.157±0.014
Polyphenol	0.241±0.021	0.235±0.001	0.234±0.021	0.213±0.019	0.215±0.006	0.169±0.031	0.174±0.008	0.126±0.013	0.112±0.004
Rutin	0.241±0.021	0.216±0.005	0.216±0.008	0.214±0.007	0.210±0.018	0.211±0.018	0.210±0.009	0.185±0.012	0.153±0.013
Alkaloid	0.241±0.021	0.221±0.009	0.218±0.001	0.211±0.019	0.209±0.014	0.198±0.010	0.199±0.009	0.173±0.005	0.169±0.026

Effectors	Extracts	Concentrations	Extracts and Concentrations
L.S.D(0.01)	0.0192	0.026	_

SE=Standard Error.

Table 8. Mean ± SE for the effect of different concentrations of (aqueous., methnol, polyphenol, rutin., and alkaloid) mature fruit extracts of *C .spinosa* on the growth of HeLa-2 tumor cell line after 48 hrs treatments *in vitro*: (Observations of O.D).

	Concentration µg/ml								
Extracts	0	78.13	156.25	312.5	625	1250	2500	5000	10000
Aqueous	0.190±0.007	0.176±0.002	0.173±0.006	0.169±0.006	0.168±0.006	0.162±0.004	0.162±0.008	0.161±0.0003	0.161±0.005
Methanol	0.190±0.007	0.180±0.006	0.180±0.006	0.180±0.003	0.183±0.002	0.176±0.005	0.173±0.009	0.110±0.007	0.115±0.009
Polyphenol	0.190±0.007	0.177±0.007	0.171±0.0008	0.175±0.008	0.177±0.002	0.173±0.003	0.174±0.006	0.077±0.002	0.076±0.002
Rutin	0.190±0.007	0.176±0.007	0.176±0.009	0.177±0.013	0.178±0.008	0.171±0.005	0.172±0.004	0.170±0.004	0.144±0.009
Alkaloid	0.190±0.007	0.176±0.009	0.176±0.006	0.165±0.004	0.158±0.006	0.158±0.004	0.158±0.007	0.145±0.008	0.132±0.008

Effectors	Extracts	Concentrations	Extracts and Concentrations
L.S.D(0.01)	0.008	0.01	0.0422

SE=Standard Error.

Table 9. Mean ±SE for the effect of exposure time to (aqueous., methnol, polyphenol ,rutin., and alkaloids) mature fruit extracts of *C. spinosa* on the growth of HeLa tumor cell line *in vitro*.

	Time/hrs		
Extract	24	48	L.S.D
Aqueous	0.191±0.003	0.169±0.003	0.034
Methanol	0.214±0.006	0.165±0.007	0.012
Polyphenol	0.191±0.003	0.154±0.006	0.032
Rutin	0.206±0.004	0.172±0.005	0.014
Alkaloid	0.204±0.00	0.162±0.00	0.015

SE=standard error.

Table 10. Mean ± SE for the effect of mature fruit extracts of *C. spinosa* on the types of cell lines:

Cell lines	Hep-2	HeLa	L.S.D
Mature fruit Extract	0.244±0.0024	0.183±0.0022	0.0213

SE=standard error

Table 11. Mean ±SE for M.I of HeLa tumor cells after 48 hrs. treatment with polyphenol mature fruit extract of *C. spinosain vitro*:

	Concentration µg/ml	M.I%
Control	0	6.76±0.24
Treatment with Polyphenol mature fruit extracts	3550	2.8±0.4
	1775	3.4±0.26
	887.5	5.2±0.23
L.S.D		1.397

SE=standard error

Figure 1. TLC of rutin extract after treatment with ammonium hydroxide (NH_4OH) solution. a-Standard rutin b- Mature fruit

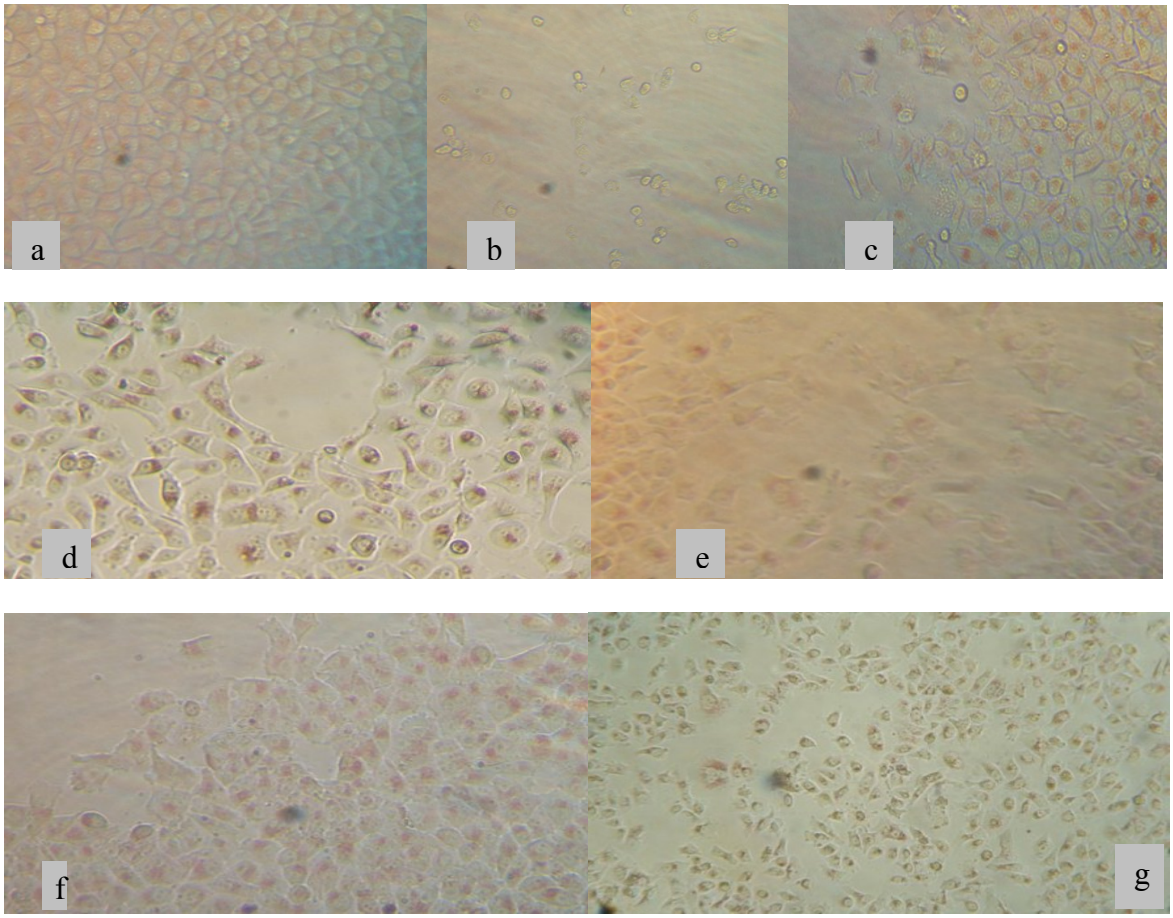

Figure 2. Hep-2 tumor cell line after 24 hrs (250X) treatment. (a) Control confluent monolayer (b) cells treated with 10000µg/ml Polyphenol mature fruit extracts (c) cells treated with 5000 µg/ml Polyphenol mature fruit extracts.(d) cells treated with 10000µg/ml aqueous mature fruit extracts (e) cells treated with 10000 µg/ml methanolic mature fruit extracts. (f) cells treated with 10000µg/ml rutin mature fruit extracts,(g) cells treated with 10000µg/ml alkaloids mature fruit extracts.

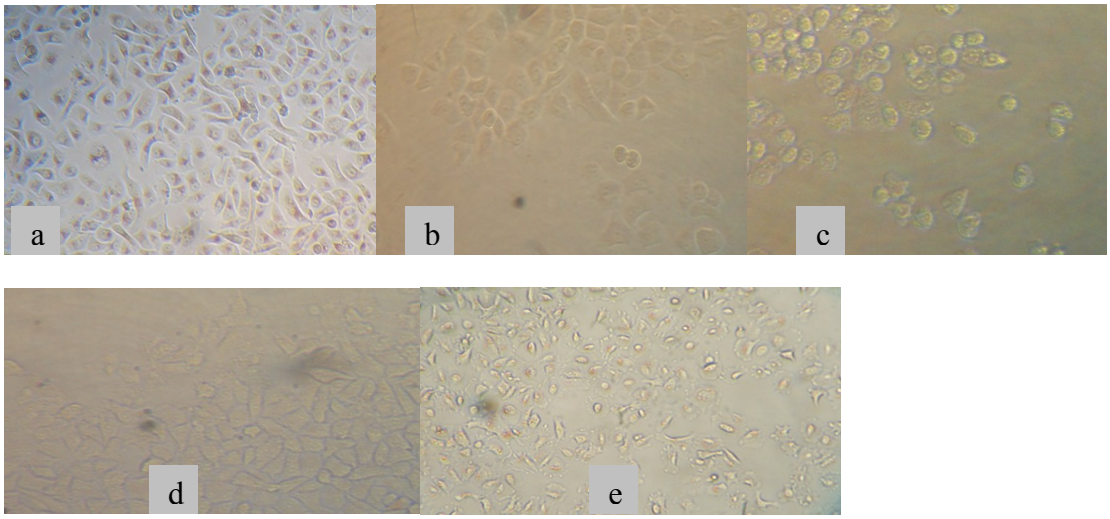

Figure 3. Hep-2 tumor cells (250X) after 48 hrs. treated with (a) 10000µg/ml aqueous mature fruit extracts (b) 10000 µg/ml methanolic mature fruit extracts (c) 10000µg/ml polyphenol mature fruit extracts (d) 5000µg/ml polyphenol mature fruit extracts (e) 10000 µg/ml rutin mature fruit extracts.

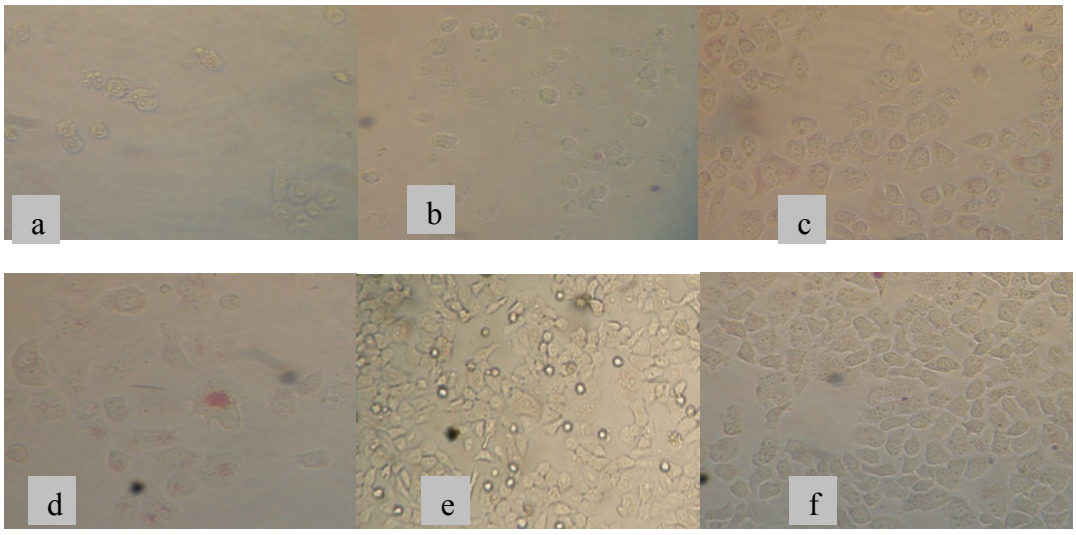

Figure 4. HeLa tumor cells(250X) after 48 hrs treated with (a) 10000µg/ml polyphenol mature fruit extracts (b) 5000µg/ml polyphenol mature fruit extracts (c)10000µg/ml methanol mature fruit extracts (d) 5000µg/ml methanol mature fruit extracts (e) 10000µg/ml alkaloids mature fruit extracts (f) 10000µg/ml rutin mature fruit extracts.

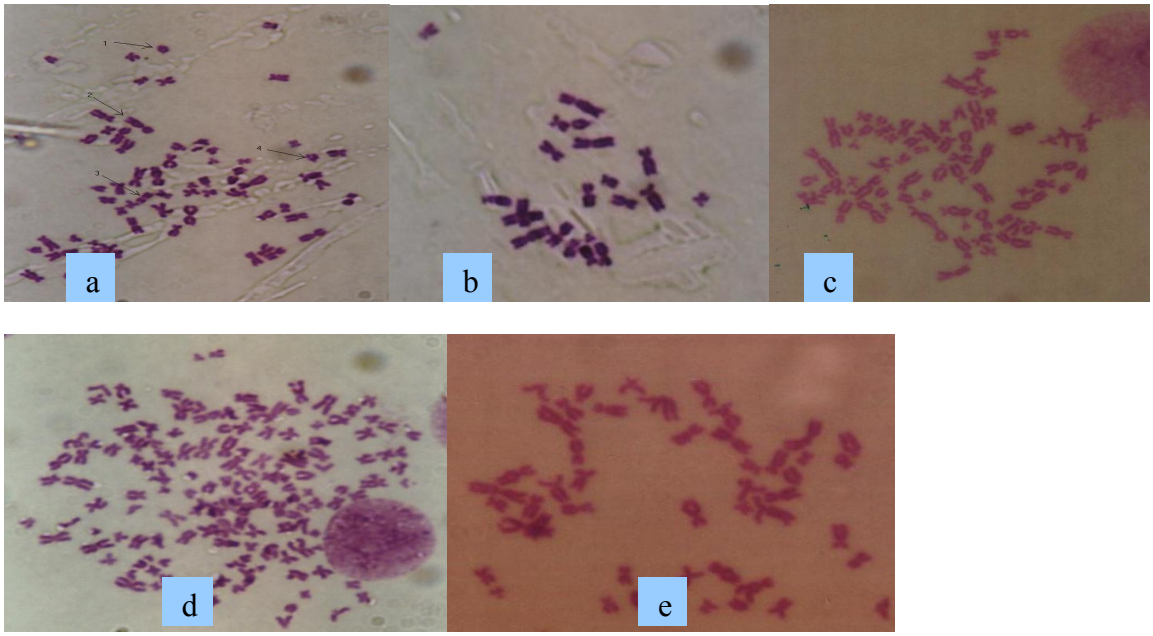

Figure 5. Chromosomes of HeLa tumor cell line untreated group(1000X, Giemsa stain) (a and b) structural chromosomal aberrations (a) 1-R. Ch.,2- D.C.Ch 3-Ch. B.W. F., and 4- gapCh. (b) symmetrical interchange of chromosome (c, d, and e) numerical chromosomal aberration (c) euploidy (triploid 3n) (d) octoploid, (e) aneuploidy(2n+2).

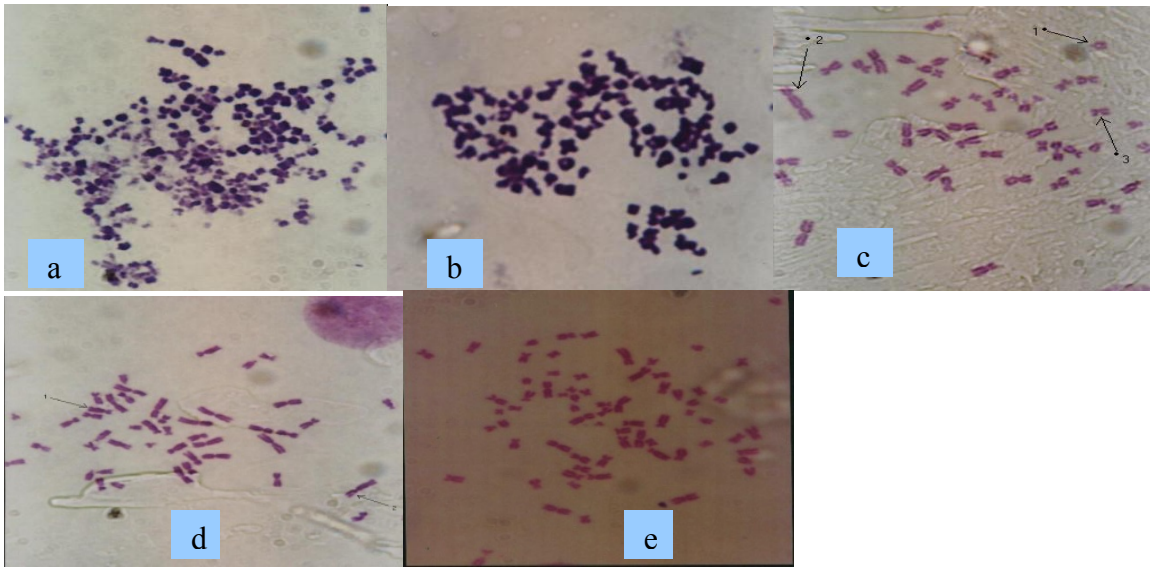

Figure 6. Chromosomes of HeLa tumor cell line(1000X, Giemsa stain) treated with polyphenol. mature fruit extracts (a and b) pulverization of chromosomes treated with 3550 and 1775µg/ml., respectively. (c, d and e) structural chromosomal aberration (c) 1-R. Ch., 2- D.C.Ch. 3-Chromtid gap in HeLa cells treated with 1775 µg/ml (d) 1-D. C.Ch 2- chromatide gap in HeLa cells treated with 887.5µg/ml (e)euploidy (3n) in HeLa cells treated with 887.5µg/ml

References

Adrienne C, Scheck K, Nicole C, Hank P and Clark W D. 2006. Anticancer activity of extracts derived from the mature roots of Scutellariabaicalensis on human malignant brain tumor cells. *BMC Complementary and Alternative Medicine*, **6**:27.

AICR. .2007. **Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective**. Washington.

Al-Assady AAB. 2007. Cytotoxic and cytogenetic effect of Capparis spinosa extract on tumor cell lines in vitro and in vivo. Ph.D. thesis. College of Education. University Of Duhok.Duhok,Iraq.

Al-Atby S M H. 2001. Effect of crude alcoholic extract of Withaniasomnifera dun on growth of cancer cell line in Vitro and on some physiological parameters in mice. Ph. D. Thesis, Faculty of Veterinary Medicine, University of Baghdad. Baghdad Iraq,In Arabic.

AL-Dabhawi AHA. 2005. Effect of crude extract of Artemisia herba alba on cancer cells growth inhibition in vitro and treatment of transplanted tumor in mice. Ph.D. Thesis College of Veterinary. University of Baghdad. Baghdad Iraq.

Al-Habbib OAM and Al-Asady AAB. 2009. Cytotoxic effect of aqueous extract of Echiumsericeum L. on tumor cell lines *in vitro*. *J. Dohok Univ*. **12 (1):**96-101.

Al-Shahaat N A Z. 1986. **Plants and Medicinal Herbs**. Dar AI-Behaar, Beirut.

Al-Soqeer A.2011.Antioxidant activity and biological evaluation of hot-water extract of Artemisia monosperma and Capparis spinosa against lead contamination. *Research J of Botany,*.**6**:11-20.

AngelaB, Julia HR, Lynn AGR, Steve S and Gric JF. 2007. Multiple cancer prevalence : A growing challenge in long-term survivorship.*Cancer Epidemiology, Biomarkers and Prevention*, **16**:588-589.

Aparicio-Fernández X, García-Gasca T, Yousef G G, LilaMA, De Mejia EG and Loarca-Pina G. 2006. Chemopreventive activity of polyphenolics from black jamapa bean (Phaseolus vulgaris L.) on HeLa and HaCaT Cells. *J. Agric. Food Chem*., **54 (6):** 2116-2122

Attila A and Ozcan M. 1999. Some compositional characteristics of Capparis (Capparisspp.) seed oil, *Grass Y.A. Ceite,* **50**: 42-52.

Berger W,Hauptmann E, Elbling L, Vetterlein M, Kokoschka EM and Micksche M.1997. Possible role of the multi-drug resistance-associated protein (MRP) in chemoresistance of human melanoma cells. *Int. J. Cancer*,**71**:108-115.

Betancur-Galvis LA, Saez J, Granados H, Salazar A and Ossa JE. 1999. Antitumor and antiviral activity of Colombian Medicinal Plant Extracts. *Mem. Inst.* , **94(4):** 101-106.

Bhargara and Soni. 1980. Chemical analysis of *C. spinosa*. *Rajasthan J. Med*. **19**: 23.

Çalis I, Kuruüzüm A and Rüedi P. 1999. 1H-Indole-3 acetonitrile glycosides from *C. spinosa* fruits. *Phytochem*., **50**:1205-1208.

Calis I, Kuruuzum A, Lorenzetto PA and Ruedi P. 2002. (6S)-Hydroxy-3-oxo-ionol glucosides from *C. spinosa* fruits. *Phytochem.,* **59(4):** 451-457.

Decker EA. 1995. The role of phenolics, conjugated linoleic acid, carnosine, and pyrroloquinolinequinone as nonessential dietary antioxidants. *Nutr. Rev*., **53**: 49-58.

Duesberg P and Rasnick D. 2000. Aneuploidy, the somatic mutation that makes cancer a species of its own. *Cell Motil. Cytoskeleton,* **47**:81-107.

Duthie G and Crozier A. 2000. Plant-derived phenolic antioxidants. *Curr. Opin. Clin. Nut*. **3**: 447-451.

Elisa VA, Rafaela TA, Manuel JE, Ma.LVO and Ruben RR. 2004. Cytotoxic Activity of Cupheaaequipetala. *Proc. West. Pharmacol. Soc.,* **47**:129-133.

Fayed L. 2008. Cervical cancer risk factors. Available from: (http://cervicalcancer.about.com/od/riskfactorsandprevention/a/cervica lrisk.htm).

Fresheny RIA. 1994. **Culture of Animal Cells. Amanual of Basic Technique,** 3rd ed. A John Wiley & sons. Ins .New York.

Fu XP, Wu T, Abdurahim M, Su Z, HouXL Aisa HA and Wu H. 2008. New spermidine alkaloids from *Capparis spinosa* roots. *Phytochem Let.*,**1(1):** 59-62.

Gayon PR. 1972. **Plant Phenolics**. 1st ed. Oliverand Boye. Edinburge.

Harborne J B. 1984. **Phytochemical Methods**. 2nd ed. Chapman and Hall, London.

HarborneJ B. 1993. **The Flavonoids: Advances in Research Since 1986.** Chapman and Hall, London, 87.

Hawk PB, Oser BL and Sumerson HW. 1954. **Practical Physiological Chemistry.** 13th ed. , McGraw-Hill, Book Comp. INC., New York.

Hayslett HT and Patrick M. 1981. **Statistics. Heinemann**. London, 246.

Henning SM, Fajardo C, Lee HW, Youssefian AA, Go VLW and Herber, D. 2003. Catechine content of 18 teas and a green tea extract supplement correlates with the oxidant capacity. *Nutr. Cancer,***45**:226-235.

Howard LR, Talcott ST , BrenesCH and Villalon B. 2000. Changes in phytochemical and antioxidant activity of selected pepper cultivars (Capsicum Species) as influenced by maturity.*J. Agric. Food Chem.,* **48 (5):** 17120-17122.

IARC. 2008. International Atomic Energy Agency IAEA. 2003. **Cancer Treatment in Developing Countries**

ICRC. 2000. Result of Iraq Cancer Registry. Ministry of Health, Central Public Health Laboratory, Baghdad-Iraq.

Khan SG, Katiyar SK, Agarwal R and Mukhtar H. 1992. Enhancement of antioxidant and phase II enzymes by oral feeding of green tea polyphenols in drinking water to SKH-1 hairless mice: possible role in cancer chemoprevention. *Cancer Res*., **52 (14):**4050-4052

KhanaviM, Hajimahmoodi M, Cheraghi-Niroomand M, Kargar Z, Ajani Y, Hadjiakhoondi A and Oveisi MR. 2009. Comparison of the antioxidant activity and total phenolic contents in some Stachys species. *J. Biotech*., **8 (6):** 1143-1147.

Kim KH, Lee KW, Kim DY, Park HH, Kwon JB and Lee HJ 2005. Optimal recovery of high-purity rutin crystals from the whole plant of Fagopyrum esculentum Moenoh (buckwheat) by extraction, fractionation and recrystallization. *Bio. Res.Technol.* , **96**: 1709-1712.

Kleinsmith LJ. 2006. **Principles of Cancer Biology**. San Francisco: Benjamin Cummings. Pp 312.

Lam SK, Han QFand Ng TB. 2009. Isolation and characterization of a lectin with potentially exploitable activities from caper (Capparis spinosa) seeds. *J. Art.*, **29(5):**293-299.

Lee JC, Kim J, Park JK, Chung GHand Jang YS. 2003. The antioxidant, rather than prooxidant, activities of quercetin on normal cells: quercetin protects mouse thymocytes from glucose oxidase-mediated apoptosis. *Exp. Cell Res*., **291**: 386-397.

Mahony DE, Gilliat E, Dawson S, Stockdale E and Lee SH. 1989. Vero cell assay for rapid detection of Clostridium perfringens enterotoxin. *Appl Environ Microbiol*., **55(9):** 2141- 2143.

Marja T. 2004. The formation and antimicrobial activity of nisin and plant derived bioactive components in lactic acid bacteria fermentations. PhD Thesis, Faculty of Agriculture and Forestry. University of Helsinki.

Mathur 1986. Chemical analysis of *C. spinosa*. *Curr Sci.* **, 55**: 899-901.

MatthausBand Ozcan M. 2005. Glucosinolates and Fatty acids, Sterol and tocopherol composition of seed oils from Capparisspinosavar. *Spinosa and C. ovate* Desf. Var. *Canescens* (Coss.) Heywood. *J. Agric. Food Chem.,* **53(18):** 7136-7141.

Mitchell JC. 1974. Contact dermatitis from plants of the caper Family, Capparidaceae. Effect on the skin of some plants which yield isothiocyanates, *Br. J. Dermatol.* „**91**: 13-20.

Modi,W.S., Nash,W.G., Ferrari, A.C. and O'Brien, S.J. 1987. Cytogenetic methodilogies for gene mapping and comparative analysis in mammalian cell culture systems. *Gene Anal.Tech*., **4**:75-85.

Montbriand MJ. 1997. Empowerment of seniors through improved communication about medication. In**: Proceedings of the Sixth Science in Health-Social Services for the Elderly and the Disabled**, Heumann, L.F. (ed.) University of Illinois of Urbana-Champaign, Chicago. IL, 258-264.

Montbriand MJ. 2000. Senior and health-professionals' mismatched perceptions and communication about prescription and non-prescription medication. *Cancer J. Aging*. **19**: 35-56.

Mukherjee AK, Basu S, Sarkar N and Ghosh AC. 2001. Advances in cancer therapy with plant based natural products. *Curr. Med. Chem .,* **8**: 1467-1486.

Newman DJ, Cragg GM, and Snader KM. 2000.The influence of natural products upon drug discovery. *Nat. Prod. Rep*.**,17(3):** 215-234.

Padilla-Nash HM, Heselmeyer-Haddad, K, Wangsa D, Zhang H, Ghadimi M, Macville M, AugustusM, Schrock E, Hilgenfeld E and Ried T. 2001. Jumping translocations are common in solid tumor cell lines and result in recurrent fusions of whole chromosome arms. *Genes, Chromosomes & Cance*., **30**: 349-363.

Pandey G and Madhuri S. 2006. Medicinal plants: better remedy for neoplasm. *Indian Drugs*, **43**: 869-874.

Parkin D M, Pisani P. and Forlay J. 1990. Estimates of the worldwide incidence of 25 major cancers in 1990. *Int J Cancer*, **80**: 827-841.

Pei-Wen Z, Lien-Chai C and Chun-ChingL. 2005.Apigenin induced apoptosis through p53-dependent pathway in human cervical carcinoma cells. *Life Sci*. **76:** 1367-1379.

RamezaniZ, Aghel N and Keyghobadi H. 2008. Rutin from different parts of Capparisspinosa growing wild in Khuzestan/Iran. *J. Bio. Sci..*, **11(5)**: 768-772.

Rastogi RP and Mehrotra BN. 1995**. Composition of Indian Medicinal Plants,** (Publication and information directorate, New Delhi). 147-148.

Richard AL, Linda AM, Leanna JC and Diane BH. 2001. **Pharmacology for Nursing Care**. 4th ed., W.B. Saunders company, New York.

Rocha-Guzman N, Gallegos-Infante J, González-Laredo R, Reynoso-Camacho R, Medina-Torres L and Cervantes-Cardozo V. 2009. Effect of air flow rate on the polyphenols content and antioxidant capacity of convective dried cactus pear cladodes (Opuntiaficusindica). *Int. J. Food Sci. Nut*., **60**(**2**): 80-87.

RomeoV, Ziino M, Giuffrida D., Condurso C and Verzera A. 2007. Flavour profile of capers (Capparisspinosa L.) from the Eolian Archipelago by HS-SPME/GC-MS. *Food Chem*., **101(3):** 1272-1278.

SAS 2000. **Statistical Analysis System**. SAS Institute Inc., Cary, NC. USA.

Sa'eed O. F.2004. The effect of green and black tea extracts on different cell line in vivo. M.Sc. Thesis, college of pharmacy, University of Mosul, Mosul/Iraq.

Satyanarayana T, Anjana A, Mathews A and Vijetha P. 2008. Phytochemical and pharmacological review of some Indian Capparis species. *Phcog. Rev*., **2(4)** : 36-45.

Schoene NW, Kelly MA, Polansky MM and Anderson RA.2005. Water-soluble polymeric polyphenols from cinnamon inhibit proliferation and alter cell cycle distribution patterns of hematologic tumor cell lines. *Cancer Lett*., **230**:134-140.

Sivalokanathan S,Ilayaraja M and Balasubra-Manium MP. 2005. Efficacy of Terminaliaarjuna (Roxb.) on N-nitrosodiethylamine induced hepato-cellular carcinoma in rats. Indian *J. Exp. Biol***., 43**: 264-267.

Winter KM. 2008.Identification of bioactive compounds in wheat. PhD Thesis. School of Environmental Science and Management. Southern Cross University. Lismore.NSW.

YangT,C.H.Wang,G.X.Chou,T.Wu,X.M.CHeng and Wang Z.T..2010. New alkaloids from Capparis spinosa:Structure and X-ray crystallographic analysis. *Food Chem*., **123**:705-710.

Yu Z and Dahlgren RA. 2000. Evaluation of methods for measuring polyphenols in copper foliage. *J. Chem. Ecol*., **26**: 2119-2140.

YuJI, Mo K, Wang and Zou Xiang .2008. Study on inhibitory effect by total alkaloids in Capparis spinosa on SGC-7901 *in vitro*. *J. of Shenyang Pharm. Univ*., **25(21):** 74-75.

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Analgesic and Anti-inflammatory Activities of Ethanolic Root Extract of *Swertia chirata* (Gentianaceae)

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Abstract

Swertia chirata, a Gentian species, can be traced through the medicinal history as a nontoxic and safe ethnomedicinal herb and has been mentioned in medical papyri to expel fever, relieve headache, to draw out inflammation and to stimulate CNS. The ethanolic root extract of *Swertia chirata* was chosen for pharmacological screening and analgesic and anti-inflammatory activities in animal models. The anti-inflammatory activity was assessed using the carrageenan-induced rat paw edema model. The analgesic effect was measured in mice using the acetic acid-induced writhing test and the radiant heat tail-flick method. In rat paw edema model induced by carrageenan, the extract was found to reduce significantly (p<0.001) the formation of edema at the 400 mg/kg dose level and showed 57.81% (p<0.001) inhibition of edema volume at the end of 3 h. In the acetic acid-induced writhing test in mice, the extract at 200 and 400 mg/kg doses level showed 41.76% (p<0.001) and 58.29% (p<0.001) inhibition of writhing, respectively. In radiant heat tail-flick method, the root extract produced 43.88% (p<0.001) and 64.81% (p<0.001) increase in reaction time 30 min after oral administration at the 200 and 400 mg/kg doses level, respectively. *Swertia chirata* possesses evident analgesic and anti-inflammatory activities. The results signify the traditional uses of *Swertia chirata*, for inflammation and pain.

Keywords: *Swertia chirata,* analgesic, anti-inflammatory, carrageenan, Gentianaceae.

1. Introduction

Swertia chirata belongs to the family Gentianaceae and it has an erect, about 2–3 ft long stem, the middle portion is round, while the upper is four-angled, with a prominent decurrent line at each angle. The stems are orange brown or purplish in color, and contain large continuous yellowish pith (Chaudhuri *et al*., 2007; Balaraju *et al*., 2011). The root is simple, tapering and stout, short, almost 7 cm long and usually half an inch thick (Clarke, 1985). Some authors have described *Swertia chirata* as an annual and others as biennial or pluri-annual (Keil *et al*., 2000; Edwards, 1993). It is widely used in India to treat fever, malaria and liver diseases (Banerjee *et al*., 2000). Concoction of *Swertia chirata* with cardamom, turmeric and kutki is given for gastrointestinal infections, and along with ginger it is considered good for fever (Keil *et al*., 2000). When given along with neem, manjishta and gotu kola, it serves as a cure for various skin problems. It is used in combination with other drugs in case of scorpion bite (Joshi and Dhawan, 2005).

Swertia chirata, a Gentian species, can be traced through the medicinal history as a nontoxic and safe ethnomedicinal herb utilized for its bitter bioactive compounds (Jensen and Schripsema, 2002). The chemical constituents of *Swertia chirata* include secoiridoid bitters, alkaloids, xanthones and triterpenoids (Wang *et al.*, 2003; Balasundari *et al.*, 2005; Brahmachari *et al.*, 2004). Amarogentin, amaroswerin, gentiopicroside and swertiamarin are the reported bitter secoiridoid glycosides of the plant (Friedhelm and Hans, 1956; Takino *et al.*, 1980; Friedhelm, 1955; Bhattacharya *et al.*, 1976). A xanthone rich extract of this plant has shown significant anti-inflammatory activity in acute, subacute, chronic and immunological models and swerchirin, a xanthone from *Swertia chirata* is a potent hypoglycaemic agent (Mandal *et al.,* 1992; Bajpai *et al.*, 1991; Saxena *et al.*, 1991). Methanol extracts of this plant having antidiabetic activity contain mangiferin, amarogentin, amaroswerin, sweroside and swertiamarin as active constituents (Suryawanshi *et al*., 2009). Xanthone derivatives like mangostin, isomangostin and mangostin triacetate are known to possess significant anti-inflammatory activities. Reports also suggest that several varieties of xanthones show potent anti-platelet, anti-cancer, CNS stimulant, anti-fungal and antimalarial effects (Banerjee *et al*., 2000).

Extract of *Swertia chirata* is used as anthelmintic and hepatoprotective agents whereas antimalarial and hypoglycemic activities of this medicinal plant are also

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known (Brahmachari *et al.,* 2004). It is also reputed for its antidiarrhoeal properties (Dahanukar *et al.,* 2000).

The present study is undertaken to investigate the analgesic and anti-inflammatory potentials of ethanolic root extract of *Swertia chirata* scientifically.

2. Materials and Methods

2.1. Plant collection

The root part of fresh unadulterated *Swertia chirata* was collected from Chawk bazaar, Dhaka and taxonomically identified by the National Herbarium of Bangladesh, Mirpur, Dhaka, Bangladesh. A fresh sample was dried at room temperature (25–30°C) for 10 days. The dried root sample was then ground in coarse powder using high capacity grinding machine in the Phytochemical Research Laboratory, Faculty of Pharmacy, University of Dhaka and preserved in air tight container.

2.2. Extraction of the plant materials and sample preparation

The dried and ground root (1.5 kg) part of the plant was macerated with ethanol (95%) for 15 days. Then the extract was filtered and concentrated with a rotary evaporator and was subsequently defatted to get the dried extract yielding 13% root (195 g) (Ahmed *et al*., 1991). For the pharmacological tests, the extract was dissolved in 0.1% Tween-80 in normal saline solution to prepare 200 mg/kg and 400 mg/kg concentrations.

2.3. Drugs and Chemicals

Aminopyrine, carrageenan and diclofenac were purchased from Sigma-Aldrich, Germany. Morphine was obtained from Square Hospital, Dhaka, Bangladesh following required formalities. Acetic acid was purchased from Merck, Germany.

2.4. Experimental animals

Swiss albino mice weighing 20-30 g and Long-Evans rats weighing 160-200 g were used in this study. They were obtained from the Animal Research Branch of the International Centre for Diarrhoeal Diseases and Research, Bangladesh (ICDDR, B). The animals were housed in polyvinyl cages with not more than six animals per cage and maintained under standard laboratory conditions (temperature 25 ± 2^0C) and a 12/12 h dark/light cycle and received feed, formulated by ICDDR, B and water *ad libitum*. To keep the hydration rate constant, food and water were stopped 12 h before the experiments. Experiments on animals were performed strictly in accordance with the guidelines provided by the Institutional Animal Ethics Committee.

2.5. Anti-inflammatory activity

2.5.1. Carrageenan-induced rat hind paw edema

The anti-inflammatory potential of the ethanolic root extract of *Swertia chirata* was assessed by the carrageenan-induced right hind paw edema method (Winter *et al*., 1962; Saha *et al*., 2007). Briefly, acute inflammation was produced by subplantar injection of 0.1 ml of 1% suspension of carrageenan in normal saline, in the right hind paw of the rats 1 h after the oral administration of test materials. The paw volume was measured by plethysmometer (Ugo Basile, Italy) at 0 h, and 3 h after the carrageenan injection (Winter *et al*., 1962). The extract was administered at 200 and 400 mg/kg body weight by gavage. Diclofenac at a dose of 25 mg/kg body weight was used as standard anti-inflammatory agent. The negative control group received 0.1% Tween-80 in saline solution.

The anti-inflammatory effect of the extract was calculated by the following equation (Asif and Kumar, 2009):

Anti-inflammatory activity (%) = (1-D/C) X100

Where, C= Mean paw volume of control,

D= Mean paw volume of test.

2.6. Analgesic activity

2.6.1. Acetic acid induced writhing method

The peripheral analgesic activity of root extract of *Swertia chirata* was measured by the acetic acid induced writhing test in mice (Saha and Ahmed, 2009; Koster *et al*., 1959). The abdominal writhing was induced by intraperitoneal injection of acetic acid solution (0.7%) at a dose of 0.1 ml/10 g of body weight to each mouse, a model of visceral pain. Aminopyrine at oral dose of 50 mg/kg was used as standard analgesic agent. The extract was administered at 200 and 400 mg/kg body weight. The extract, standard drug and control (normal saline solution, 1 ml/kg) were orally administered 1 h prior to the injection of acetic acid. The number of writhing was calculated for 10 min after the application of acetic acid.

2.6.2. Radiant heat tail-flick method

The central analgesic activity of the root extract was studied by measuring drug-induced changes in the sensitivity of the pre-screened (reaction time: 2-4 sec) mice to heat stress applied to their tails by using a Medicraft Analgesiometer Mask-N (D'Amour and Smith, 1941). The current intensity passing through the naked nichrome wire was maintained at 5 ampere. The distance between the heat source and the tail skin was 1.5 cm and cut-off reaction time was fixed at 10 sec to avoid any tissue damage. Morphine was used to compare the analgesic effect of the plant extract. The extract was orally administered at 200 and 400 mg/kg body weight. Morphine was administered sub-cutaneously at a dose of 2 mg/kg body weight.

2.7. Statistical analysis

Data were analyzed by one-way ANOVA followed by Dunnett's test and p value *of* 0.05 was considered statistically significant.

3. Results

3.1. Anti-inflammatory activity

The anti-inflammatory activity of the extract was measured at a dose of 200 and 400 mg/kg b.w. against acute paw edema induced by carrageenan. A strong inhibition of the paw edema was observed with the different doses of the extract and with diclofenac. The two doses tested (200 and 400 mg/kg) produced significant (p<0.001) anti-inflammatory activity and reduced the paw

volume by 37.76% and 57.81% respectively, whereas diclofenac caused 65.89% reduction when used as a reference drug (Table 1).

3.2. Analgesic activity

3.2.1. Acetic acid induced writhing method

The root extract of the plant *Swertia chirata* at the doses of 200 and 400 mg/kg b.w. and aminopyrine 50 mg/kg b.w induced a significant (p<0.001) decrease in the number of writhes when compared to control untreated groups. The two doses tested (200 and 400 mg/kg) produced significant (p<0.001) analgesic activity and reduced the paw volume by 41.76% and 58.29% respectively, whereas aminopyrine caused 63.77% reduction when used as a reference drug (Table 2).

3.2.2. Radiant heat tail-flick method

In the radiant heat tail-flick test, the root extract prolonged the heat stress tolerance capacity of the mice, indicating the possible involvement of a higher center (Whittle, 1964). In radiant heat tail-flick test, the root extract produced 43.88% (p<0.001) and 64.81% (p<0.001) elongation of the reaction time to tail flicking 30 min after oral doses of 200 and 400 mg/kg body weight respectively. After 60 min the extract caused 30.81% (p<0.001) and 46.44% (p<0.001) increase in reaction time to tail flicking of 200 and 400 mg/kg body weight respectively and after 120 min the extract caused 13.34% and 19.69% (p<0.01) increase in reaction time to tail flicking of 200 and 400 mg/kg body weight respectively. Morphine caused 78.88% (p<0.001), 54.00% (p<0.001) and 25.13% (p<0.001) increase in reaction time to tail flicking after 30, 60 and 120 min respectively when used as a reference drug at 2 mg/kg body weight (Table 3).

Table 1. Effects of *Swertia chirata* extract (SCE) on carrageenan induced rat paw edema.

Group	Dose (mg/kg)	Paw volume increase after 3 h (ml)[a]	Percentage (%) of inhibition
Control	-	0.64 ± 0.032	-
SCE	200	0.40 ± 0.025***	37.76
SCE	400	0.27 ± 0.014***	57.81
Diclofenac	25	0.22 ± 0.017***	65.89

[a]Each datum represents the mean paw volume increase after 3 h (ml) ± SEM (n = 6)

***p<0.001 compared with the control group (Dunnett's test)

Table 2. Effects of *Swertia chirata* extract (SCE) on acetic acid induced writhing response in mice.

Group	Dose (mg/kg, p.o.)	Writhing[a]	Percentage (%) of inhibition
Control	-	21.17 ± 0.477	-
SCE	200	12.33 ± 0.333***	41.76
SCE	400	8.83 ± 0.703***	58.29
Aminopyrine	50	7.67 ± 0.494***	63.77

[a]Each datum represents the mean writhing number ± SEM (n = 6)

***p<0.001 compared with the control group (Dunnett's test)

Table 3. Effects of *Swertia chirata* extract (SCE) on radiant heat tail-flick response in mice.

Group	Dose (mg/kg)	Reaction time (sec)[a]		
		30 min (% elongation)	60 min (% elongation)	120 min (% elongation)
Control	-	4.50 ± 0.24	4.62 ± 0.18	4.97 ± 0.23
SCE	200	6.32 ± 0.46*** (43.88)	6.05 ± 0.28**(30.81)	5.63 ± 0.18 (13.34)
SCE	400	7.22 ± 0.39*** (64.81)	6.55 ± 0.25*** (46.44)	5.93 ± 0.21** (19.69)
Morphine	2	8.15 ± 0.86*** (78.88)	7.15 ± 0.31*** (54.00)	6.20 ± 0.12*** (25.13)

[a] Each datum represents the mean reaction time (sec) ± SEM (n = 6)

***p<0.001, **p<0.01 compared with the control group (Dunnett's test)

4. Discussion

Pain and inflammation are associated with the pathophysiology of various clinical conditions such as arthritis, cancer and vascular diseases. Inflammatory reactions are not only the response of living tissues to injury and infection, but also are relevant to disease developments, such as asthma, multiple sclerosis, colitis, inflammatory bowel disease and atherosclerosis. Many natural products are used in traditional medical systems to relieve the symptoms from pain and inflammation (Kaplan *et al*., 2007; Marrassini *et al*., 2010).

Results from the present study shows that the ethanolic root extract of *Swertia chirata* has a potent antinociceptive effect against chemical pains provoked by acetic acid and a good activity against mechanic pain induced by heat. The extract also presents important anti-inflammatory effects on acute edema induced by carrageenan.

The paw edema induced by carrageenan involves several chemical mediators such as histamine, serotonin, bradykinin, and prostaglandins (Vinegar *et al*., 1987; Chang *et al*., 2011). In the carrageenan-induced rat paw edema model, root extract of *Swertia chirata* showed significant inhibitory effect on the edema formation. This effect started from the first hour and was maintained in all the inflammatory phases, suggesting that the main mechanism of action of the tested extract may involve prostaglandin biosynthesis pathway and may influence other mediators of inflammation. The extract is found to be less active than diclofenac even when used in higher doses.

As the carrageenan-induced inflammation model is a significant predictive test for anti-inflammatory agents acting by inhibiting the mediators of acute inflammation, these results are an indication that *Swertia chirata* can be an effective for acute inflammatory disorders (Mossa *et al.*, 1995).

In the acetic acid-induced writhing test, local peritoneal receptors are postulated to be partly involved in the abdominal writhing response and the mechanism of the reaction to this nociceptive stimulus seems to be related to the prostanoid system (Nguemfo *et al*., 2007). The constriction response of abdomen produced by acetic acid is a sensitive procedure for peripheral analgesic agents, and has also been associated with prostanoids in general, for example, increased levels of PGE2 and PGF2α in peritoneal fluids (Ronaldo *et al*., 2000; Deraedt *et al*., 1980) as well as lipoxygenase products (Levini *et al*., 1984; Dhara *et al*., 2000). The extract of *Swertia chirata* and aminopyrine exhibit marked inhibitory effect on the writhing response induced by acetic acid. These results strongly suggest that the extract possesses peripheral analgesic activity and its mechanism of action may be mediated through inhibition of local peritoneal receptors or arachidonic acid pathways, involving cyclo-oxygenases and/or lipoxygenases.

The phytochemical analysis of this extract revealed that it contains xanthones flavonoids, terpenoids, iridoids, secoiridoid glycosides and saponin (Wang *et al.*, 2003; Balasundari *et al.,* 2005; Brahmachari *et al.,* 2004; Ghosal *et al.*, 1973; Phoboo *et al*., 2010; Bhargava *et al*., 2009). Of these, flavonoids and saponins are well known for their ability to inhibit pain perception. Flavonoids also have anti-inflammatory properties due to their inhibitory effects on enzymes involved in the production of the chemical mediator of inflammation (Owoyele *et al*., 2005). Flavone and its methoxy derivatives exhibited significant dose-dependent analgesic activity (Thirugnanasambantham *et al*., 1990). Previous studies showed that ethanolic root extract of *Swertia chirata,* is rich in xanthone and xanthone derivatives has anti-inflammatory activity (Banerjee *et al*., 2000; Wang *et al.*, 2003; Balasundari *et al.*, 2005). It was also reported that mangiferin was a potent anti-inflammatory compound. Therefore, the activity of *Swertia chirata* can be attributed to magniferin (Kumar *et al*., 2003).

In conclusion, this study has shown that the ethanolic root extract of *Swertia chirata* possesses significant analgesic and anti-inflammatory effects that may be mediated through inhibition of cell mediators such as bradykinin, and prostaglandins. These results support the traditional use of this plant in some painful and inflammatory conditions.

References

Ahmed M, Datta BK, Rouf ASS and Jakupovic J. 1991. A flavone and α-santalene derivatives from *Polygonum flaccidum*. *Phytochemistry,* **30**: 3155-3156.

Asif M and Kumar A. 2009. Anti-inflammatory activity of ethanolic extract of *Dalbergia sissoo (roxb.)* Bark. *Malaysian J of Pharmaceutical Sciences,* **7(1):** 39–50

Bajpai MB, Asthana RK, Sharma NK, Chatterjee SK and Mukherjee SK. 1991. Hypoglycemic effect of swerchirin from the hexane fraction of *Swertia chirayita*. *Planta Med.,* **57**: 102-104.

Balaraju K, Saravanan S, Agastian P and Ignacimuthu S. 2011. A rapid system for micropropagation of *Swertia chirata* Buch-Ham. ex Wall.: an endangered medicinal herb via direct somatic embryogenesis. *Acta Physiol Plant*, **33 (4):**1123–1133.

Balasundari P, Singh S and Kavimani S. 2005. Free radical scavenging of xanthones from *Swertia chirata* Buch-ham and tumor cell growth inhibition. *Main Group Chemistry,***4(3):** 177-185.

Banerjee S, Sur TK, Mandal S, Das PC and Sikdar S. 2000. Assessment of the anti-inflammatory effects of *Swertia chirata* in acute and chronic experimental models in male albino rats. *Indian J of Pharmacol.,* **32**: 21-24.

Bhargava S, Rao PS, Bhargava P and Shukla S. 2009. Antipyretic potential of *Swertia chirata* buch ham. root extract. *Sci Pharm*., **77:** 617–623.

Bhattacharya SK, Reddy PKSP, Ghosal S, Singh AK and Sharma PV. 1976. Chemical constituents of gentianaceae XIX: CNS-depressant effects of swertiamarin. *J of Pharmaceutical Sciences,* **65**: 1547–1549.

Brahmachari G, Mandal S, Gangopadhyay A, Gorai D, Mukhopadhyay B, Saha S and Brahmachari AK. 2004. *Swertia* (Gentianaceae): Chemical and pharmacological aspects. *Chemistry and Biodiversity,* **1(11):** 1627–1651.

Chang HY, Sheu MJ and Yang CH. 2011. Analgesic effects and the mechanisms of anti-inflammation of hispolon in mice. *Evidence-Based Complementary and Alternative Medicine*, **478246**: 1-8.

Chaudhuri RK, Pal A and Jha TB. 2007. Production of genetically uniform plants from nodal explants of *Swertia chirata* Buch.-Ham. ex Wall.—an endangered medicinal herb. *In Vitro Cell Dev Biol-Plant*, **43**:467–472.

Clarke CB. 1985. Gentianaceae. In: Hooker JD,(Ed). **The Flora of British India**, vol. IV. London; Reeve L & Co. p.93-132.

Dahanukar SA, Kulkarni RA and Rege NN. 2000. Pharmacology of medicinal plants and natural products. *Indian J of Pharmacol.,***32**: S81-S118

D'Amour FE and Smith DL. 1941. A method for determining loss of pain sensation. *J. Pharmacol. Exp. Ther*., **72**: 74-79.

Deraedt R, Jouquey S, Delevallee F, Flahaut M. 1980. Release of prostaglandins E and F in an algogenic reaction and its inhibition. *European J of Pharmacol.,* **61(1):** 17–24.

Dhara AK, Suba V, Sen T, Pal S and Chaudhuri AKN. 2000. Preliminary studies on the anti-inflammatory and analgesic activity of the methanolic fraction of the root extract of *Tragia involucrata* Linn. *J of Ethnopharmacol.,* **72**: 265–268.

Edwards DM. 1993. The marketing of non-timber forest product from the Himalayas: The trade between East Nepal and India. *Rural development Forestry Network*. pp. 1–21.

Friedhelm K. 1955. Characteristic plant constituents. IX. Amarogentin, a new bitter principle from Gentianaceae. *Chem. Ber.,* **88**:704-707.

Friedhelm K and Hans GS. 1956. Chemical classification of plants XII. Amarogentin. *Chem. Ber.,* **89**: 2404-2407.

Ghosal S, Sharma PV, Chaudhuri RK, and Bhattacharya SK. 1973. Chemical constituents of the gentianaceae V: Tetraoxygenated xanthones of *Swertia chirata* buch.-ham. *J of Pharmaceutical Sciences,* **62**: 926–930.

Jensen SR and Schripsema J. 2002. Chemotaxonomy and pharmacology of Gentianaceae. In: Struwe Land Albert VA(Eds.), **Gentianaceae— Systematics and Natural History**, vol V Cambridge University Press, Cambridge 574–631.

Joshi P and Dhawan V. 2005. *Swertia chirayita* – an overview. *Current Science.* **89 (4):** 635-640.

Kaplan M, Mutlu E, Benson M, Fields J, Banan Aand Keshavarzian A. 2007. Use of herbal preparations in the treatment of oxidant-mediated infl ammatory disorders. *Complement Ther Med.,* **15:** 207–216.

Keil M, Härtle B, Anna G and Psiorz M. 2000. Production of amarogentin in root cultures of *Swertia chirata*. *Planta Med.,* **66**: 452-457.

Koster R, Anderson M and DeBeer EJ. 1959. Acetic acid analgesic screening. *Fed Proc.,* **18**: 418–420.

Kumar IVMLRS, Paul BN, Asthana R, Saxena A, Mehrotra S and Rajan G. 2003. *Swertia chirayita* mediated modulation of interleukin-1beta, interleukin-6, interleukin-10, interferon-gamma, and tumor necrosis factor-alpha in arthritic mice. *Immunopharmacol Immunotoxicol.,* **25**: 573-83.

Levini JD, Lau W, Kwait G and Goetzl EJ. 1984. Leukotriene B4 produces hyperalgesia that is dependent on the polymorphonuclear leucocytes. *Science,***225**: 743–745.

Mandal S, Das PC and Joshi PC. 1992. Antiinflammatory action of *Swertia chirata*. *Fitoterapia,* **63**: 122-128.

Marrassini C, Acevedo C, Miño J, Ferraro G and Gorzalczany S. 2010. Evaluation of antinociceptive, antiinfl-amatory activities and phytochemical analysis of aerial parts of *Urtica urens* L. *Phytother Res*., **24**: 1807–1812.

Mossa JS, Rafatullah S, Galal AM and Al-Yahya MA. 1995. Pharmacological studies of *Rhus retinorrhaea. Int. J. Pharmacog*., **33**: 242-246.

Nguemfo E, Dimo T and Azebaze A. 2007. Anti-inflammatory and anti-nociceptive activities of the stem bark extracts from *Allanblackia monticola* STANER L.C. (Guttiferae*). J Ethnopharmacol.,* **114**: 417–424.

Owoyele VB, Oloriegbe YY, Balogun EA and Soladoye AO. 2005. Analgesic and anti-inflammatory properties of *Nelsonia canescens* leaf extract. *J Ethnopharmacol.,* **99**:153-156.

Phoboo S, Silva Pinto MD, Bhowmik PC, Jha PK and Shetty K. 2010. Quantification of major phytochemicals of *Swertia chirayita*, a medicinal plant from Nepal. *Ecoprint*., **17**: 59-68.

Ronaldo AR, Mariana LV, Sara MT, Adriana BPP, Steve P, Ferreira SH and Fernando QC. 2000. Involvement of resident macrophages and mast cells in the writhing nociceptive response induced by zymosan and acetic acid in mice. *Eur J Pharmacol*., **387:** 111-118.

Saha A, Masud MA, Bachar SC, Kundu JK, Datta BK, Nahar L and Sarker SD. 2007. The analgesic and anti-Inflammatory activities of the extracts of *Phyllanthus reticulatus* in mice model. *Pharm Biol*., **45**: 355-359.

Saha A and Ahmed M. 2009. The analgesic and anti-inflammatory activities of the extract of *albizia lebbeck* in animal model. *Pak. J. Pharm. Sci*., **22(1):** 74-77.

Saxena AM, Bajpai MB and Mukherjee SK. 1991.Swerchirin induced blood sugar lowering of streptozotocin treated hyperglycemic rats. *Indian J. Exp. Biol*., **29**: 674-675.

Suryawanshi S, Asthana RK and Gupta RC. 2009. Assessment of systemic interaction between *Swertia chirata* extract and its bioactive constituents in rabbits. *Phytotherapy Research,* **23**: 1036–1038.

Takino Y, Koshioka M, Kawaguchi M. 1980. Quantitative determination of bitter components in *Swertiae* herba. *Planta Med*., **38**: 351-355.

Thirugnanasambantham P, Viswanathan S, Mythirayee C, Krishnamurty V, Ramachandran S and Kameswaran L. 1990.

Analgesic activity of certain flavone derivatives: a structure-activity study. *J Ethnopharmacol.,* **28**: 207-214.

Vinegar R, Truax JF, Selph JL, Johnston PR, Venable AL and McKenzie KK. 1987. Pathway to carrageenaninduced inflammation in the hind limb of the rat. *Federation Proceedings,* **46(1):** 118–126.

Wang, CZ, Maier UH, Keil M, Zenk MH, Bacher A, Rohdich F and Eisenreich W. 2003. Phenylalanine-independent biosynthesis of 1,3,5,8-tetrahydroxyxanthone. *European J of Biochem.,* **270**: 2950–2958.

Whittle BA. 1964. The use of changes in capillary permeability in mice to distinguish between narcotic and non-narcotic analgesics. *Br J Pharmacol Chemotherp*., **22**: 246-253.

Winter CA, Risley EA and Nuss GW. 1962. Carrageenan induced edema in hind paw of the rat as an assay for anti-inflammatory drugs. *Proc Soc Exp Biol Med*., **111**: 544-547.

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Protective Role of Omega-3 Fish Oil against the Toxicity of Ifosfamide in Male Rats

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Abstract

Ifosfamide IFO) is a cytotoxic alkylating drug used for the treatment of a variety of cancers but has reported to cause certain hematological, hepatotoxic and nephrotoxic side effects. For protection against these side effects, different antioxidants were used. This study is performed to evaluate the ability of omega-3 fatty acids (Omega-3 FAs) to attenuate ifosfamide (IFO) toxicity. Thirty male albino rats were randomly divided into six groups. Group 1: control, Group 2: omega-3 (4gm/kg diet), Group 3: IFO (50mg/kg b.wt.), Group 4: IFO(80mg/kg b.wt.), Group 5: IFO (50mg/kg b.wt.) plus omega-3, Group 6: IFO (80mg/kg b.wt.) plus omega-3. Ifosfamide was administrated intraperitoneally (i.p.), while omega-3 was given with the diet. The duration was five consecutive days for IFO, and six consecutive days for omega-3 oil. A significant increase in the body weight gain of the rats has been recorded after applying omega-3 to both doses of IFO when compared to each IFO group. In the IFO group, the levels of serum creatinine, phosphorous, glutamate-oxaloacetate transaminase (GOT), glutamate-pyruvate transaminase (GPT) and malondialdehyde (MDA) were increased, while serum glucose decreased significantly. In the Omega-3 and IFO plus omega-3 groups, these variations didn't show significant changes. Hematologically, Omega-3 has recovered the decrease in WBC, RBC and blood platelets caused by IFO after giving omega-3 to the IFO treated rats. IFO has induced many histological alterations in the liver such as degeneration of the cells, inflammatory cells infiltration and dilation in the sinusoidal lumen, while it caused severe inflammation, degeneration in the kidney tubule lining cells and hypertrophy of these tubules. The histological structure of liver and kidney was found to be protected from this effect of IFO when a combination of Omega-3 FAs and IFO was administrated. Interestingly, Omega-3 didn't interfere with the antimitotic property of IFO, suggesting a very important role of this oil in the future of cancer chemotherapy.

Keywords: IFO, omega-3 fish oil, hepatotoxicity, Nephrotoxicity.

1. Introduction

Although Ifosfamide (IFO) is a highly effective chemotherapeutic agent for treating a variety of pediatric and adult solid tumors (Straka *et al.*, 2003), it has been shown to induce many side effects such as hepatotoxicity (Paschke *et al.*, 1988) and nephrotoxicity (Chen *et al.*, 2008; Hanly *et al.*, 2009). Nephrotoxicity may present in more severe cases as Fanconi syndrome (Skinner *et al.*, 1993; Loebstein and Koren, 1998). This renal disorder is characterized by urinary loss of amino acids, glucose, phosphate and bicarbonate (Loebstein *et al.*, 1999; Rossi *et al.*, 1999; Skinner, 2003). The clinical consequences may finally necessitate a kidney transplant (Skinner, 2003).The IFO-induced animal model of Fanconi syndrome was described by Nissim and Weinberg (1996).

IFO has been found to cause oxidative damage in renal and bladder tissues (Sener *et al.*, 2004; Knouzy *et al.*, 2010), for this reason, researchers for improving the therapeutic efficacy of IFO, used different antioxidants such as, taurine (Badary, 1998), L-Histidinol (Badary, 1999a), thymoquinone (Badary, 1999b), resveratrol (Şehirli *et al.*, 2007), N-Acetylcysteine (Chen *et al.*, 2008) and melatonin (Casado-Zapico*et al.*, 2010). Against neurotoxicity caused by IFO, methylene blue (Hamadani and Awan, 2006) and dexmedetomidine (Bernard *et al.*, 2010) have been used.

Omega-3 fatty acids (Omega-3 FAs) is considered as a strong antioxidant (Calviello and Serini, 2010) and its role as an anticancer agent has been extensively confirmed in

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most of the human malignancies (Shaikh *et al.*, 2010).Its role in enhancement cytotoxicity of anticancer drugs to tumor cells and protection of normal cells was previously reported (Pardini, 2006). Furthermore, the anti-inflammatory potential of long chain Omega-3 FAs in many chronic diseases has been suggested (Calder, 2009; Wall *et al.*, 2010). The role of Omega-3 FAs in inhibiting proliferation, inducing apoptosis and promoting differentiation in many cancers have been recently studied (Edwards and O'Flaherty, 2008; Sun *et al.*, 2009).In addition, recent findings indicate that omega3-FA acts synergistically with certain chemotherapeutic agents (Wendel and Heller, 2009).The present investigation was undertaken to study the protective role of omega-3 fish oil against the IFO toxicity in animal model which can be considered, as far we searched, as the first trial for this oil in this field.

2. Materials and Methods

2.1. Experimental animals

Thirty adult Wister albino male rats (8 weeks old weighing 200-250 gm) were used in this study. They fed a standard laboratory chow and allowed to drink water. They were divided randomly into six groups (each contained five rats). Group 1, control: injected daily with 0.9% NaCl (1 ml) (i.p.) for 5 days. Group 2: Omega-3: administered omega-3 in diet (4gm/kg diet) daily for 6 days. Group 3 and 4: two doses of IFO (50and 80mg/kg b.wt., respectively) administrated intraperitoneally (i.p.).Group 4: IFO (80mg/kg b.wt).Group 5: Omega-3 + IFO (50mg/kg b.wt.). Group 6: Omega-3 + IFO (80mg/kg b.wt).

2.2. Anesthesia, dissection and removal of organs

All animals were anesthetized with ketamine hydrochloride (100mg/Kg b.wt.) and sacrificed, blood was collected then liver and kidneys were surgically removed, cut into small pieces (approximately 0.5cm in thickness) and put in fixative. The body weight of the rats in all groups was recorded twice; at the beginning and at the end of the experiment for calculating the body weight gain.

2.3. Histological preparations

2.3.1. Light Microscopy (Paraffin Method)

Samples of the organs were directly fixed in Bouin's fluid for 24hours and then processed for paraffin method by dehydrating through ascending concentrations of ethanol (50%, 70%, 95% and 100%), Cleared in xylene, and embedded in paraffin . The 4µmthick sections were stained by haematoxylin and eosin (H&E) (Murice-Lambert *et al.*, 1989).

2.3.2. Light Microscopy (Resin Method)

Tissue samples (<1mm3) were fixed in 2.5% glutaraldehyde in 0.1Mcacodylate buffer pH 7.2 - 7.4 for 24 hours, postfixed in 1% osmium tetroxide for 1 hr., dehydrated through a graded series of ethanol (50%, 70%, 95%, and 100%), cleared in propylene oxide and embedded in Araldite mixture. Plastic sections (0.5-1µm) were stained by 1% toluidine blue in 1% Borax for light microscopy.

2.4. Bone marrow smear preparation

Another separate six groups of male rats (5 rats in each group) were used for this preparation. Rat femur was subcutaneously injected with colchicine (1.0mg/kg) 2hours before sacrifice (El-Habit *et al.*, 2000). Bone marrow cells from control and experimental animals were processed for analysis of chromosomal aberrations by the method of Sharma and Sharma (1994).The bone marrow from the femurs was flushed into a centrifuge tube containing 0.9% saline and centrifuged at 500g for 5min.The supernatant was removed and hypotonic KCl was added to the sediment. After incubation for 20 min at 37C°, the contents were centrifuged for 5 min and the sediment was fixed in methanol–acetic acid (3:1v/v).Three changes of fixative were given prior to slide preparation. The slides were air-dried, stained in 5%Giemsa solution and scored blindly. Bone marrow cells were examined for mitotic figures by scoring the number of cells in mitosis per 1000 bone marrow cells, then the percentage of mitotic cells were obtained.

2.5. Blood collection

Blood samples were taken from the rats through cardiac puncture some into chilled tubes with ethylene diaminetetraacetic acid (EDTA) for hematological parameters and another into chilled tubes without EDTA for serum collection (biochemical test); later centrifuged at 3000 rpm for 15 minutes at 4°C, then the sera were stored at -55°C.

2.6. Biochemical analysis

2.6.1. Determination of serum malondialdehyde (MDA):

The level of serum MDA was determined spectrophotometrically by thiobarbituric acid (TBA) solution. In brief: 150µl serum sample was added to the followings: 1ml trichloroacetic acid (TCA) 17.5%, 1ml of 0.66 % TBA, then mixed well by vortex, incubated in boiling water for 15 minutes, and then allowed to cool. One ml of 70% TCA was added and left to stand at room temperature for 20 minutes, centrifuged at 2000 rpm for 15 minutes, and the supernatant was taken out for scanning spectrophotometrically (Weinstein *et al.*, 2000).

2.6.2. Determination of serum creatinine

Colorimetric reaction of creatinine with alkaline picurate was measured kinetically at 490 nm (490-510) nm. The kit was obtained from BIOLABO SA, Maizy, France.

2.6.3. Determination of serum phosphorous

In an acid medium, phosphate ions form a phosphomolydic complex with the ammonium molybdate. The absorbance measured at 340 nm is proportional to the concentration of phosphate ions in the specimen. The kit was obtained from BIOLABO SA, Maizy, France.

2.6.4. Determination of serum glucose

The absorbance was measured at 505 nm. The kit was obtained from Plasmatic laboratory products LTD.

2.6.5. Determination of Serum glutamate-pyruvate transaminase (GPT):

The GPT, also called alanine aminotransferase (ALT), was determined in serum and it relies on the following principle: the α-Oxoglutarate reacts with L-Alanine in the presence of ALT to form L-Glutamate plus Pyruvate. The kit was obtained from BIOLABO SA, Maizy, France. The absorbance was measured at 505 nm.

2.6.6. Determination of Serum glutamate-oxaloacetate transaminase (GOT)

The GOT is also known as aspartate aminotransferase (AST).The determination of serum GOT is based on the principle that the α-Oxoglutarate reacts with L-Aspartate in the presence of AST to form L-Glutamate plus Oxaloacetate. The kit was obtained from BIOLABO SA, Maizy, France. The absorbance measured at 505 nm.

2.7. Statistical analysis

All data were expressed as means ± standard error of mean (M ± SE) and statistical analysis was carried out using statistically available software (SPSS version 11.5). One-way analysis of variance (ANOVA) was performed to test for significance followed by Duncan's multiple range comparison tests for comparisons between the groups. P values ≤ 0.05 and 0.01 were considered significant.

3. Results

As shown in Figure 1, both IFO doses caused a significant decrease in the body weight gain of rats when compared to the control group. On contrast, both IFO plus omega-3 treated groups showed a significant increase in the body weight gain of rats in comparison to the IFO treated rats. Hair loss has been seen in all the treated groups except the control.

Table 1 shows some serum biochemical results (MDA, creatinine, phosphorous, glucose, GOT, and GPT) after administration of both doses of IFO in combination with omega-3.Compared to the control group, both IFO treated groups caused a significant increase in the level of serum MDA, while these high levels of serum MDA decreased significantly in both IFO plus omega-3 treated groups when compared to the IFO treated groups. Both IFO doses caused a significant increase in the level of serum creatinine when compared to the control group, while when omega-3 was added to the diet of IFO treated rats, these levels of creatinine were significantly decreased when compared to the IFO treated groups. With respect to phosphate level, both doses of IFO caused significant increase when compared to the control group, while these levels of serum phosphorous significantly decreased after giving omega-3 to the IFO treated groups. A significant decrease in serum glucose level of both IFO treated groups were reported when compared to the control group, while both IFO plus omega-3 treated groups showed a significant increase in the level of serum glucose when compared to the IFO treated groups. The level of serum GOT was significantly increased in both IFO treated groups when compared to the control group, while both IFO plus omega-3 treated groups showed significant decrease in serum GOT level when compared to the IFO treated groups. Only the higher dose of IFO treated group showed statistical increase in serum GPT level when compared to the control group, while the lower dose of IFO treated group showed non-significant increase in serum GPT when compared to the control group. Similarly, both IFO plus omega-3 treated groups showed non-significant decrease of serum GPT in comparison to the IFO treated groups.

As shown in Table 2, both IFO treated groups caused significant decrease in blood WBC, RBC, HGB, and PLT count when compared to the control group. On the other hand, both IFO plus omega-3 treated groups showed a significant increase in the level of these blood parameters (except RBC) when compared to the IFO treated groups.

As shown in Figure 2, all groups that have received IFO alone or in combination with omega-3 showed approximately the absence of mitotic division, while omega-3 alone didn't cause significant decrease of mitotic division in the bone marrow when compared with the control group.

The histological figures have shown normal structure of rat liver in control group (Figure 3 A and B).On the other hand, histological figures of both doses of IFO treated rats showed degeneration of hepatocytes, in which the higher dose of IFO(i.e. 80mg/kg) has showed higher degenerative effect (Figure 3 C-F). In very rare occasions, few mitotic figures have been seen in IFO treated groups. Other histological alterations due to IFO administration included infiltration with inflammatory leukocytes, congested blood vessels (Figure 3 C).

Both paraffin and plastic sections showed well-designated glomeruli and tubuli (PCT and DCT) in the kidney of control group (Figure 4 A and B). Medullary region of the control kidney also showed normal appearance of tubules. The IFO treated groups showed various histological alterations such as dilatation of the cortical tubule lumens (Figure 4 C).Other histological changes included the appearance of inflammatory areas in the cortex and medulla (Figure 4 D and E). Dilatation in the lumen of kidney tubules has been appeared in higher level in the IFO 80mg/kg treated rats (Figure 4 F and G) as well as necrosis, congestion of blood vessels with blood cells (Figure 4 G) and anisonucleosis of the nuclei of kidney tubular cells(i.e. the nuclei sizes were variable) (Figure 4 H).

In comparison to the IFO treated groups, the histological figures of the IFO plus omega-3 treated rats showed that the structure of the glomeruli and cortical tubuli (PCT and DCT) has been approximately normalized (Figure 5 A-E). Although the kidney structure in the IFO 80mg/kg plus omega-3 showed approximately normal structure in which no inflammatory cells were detected(Figure 5 E), some alterations were still exist such as degeneration of some kidney tubular cells and shrinkage of glomerular tuft(Figure 5 F).

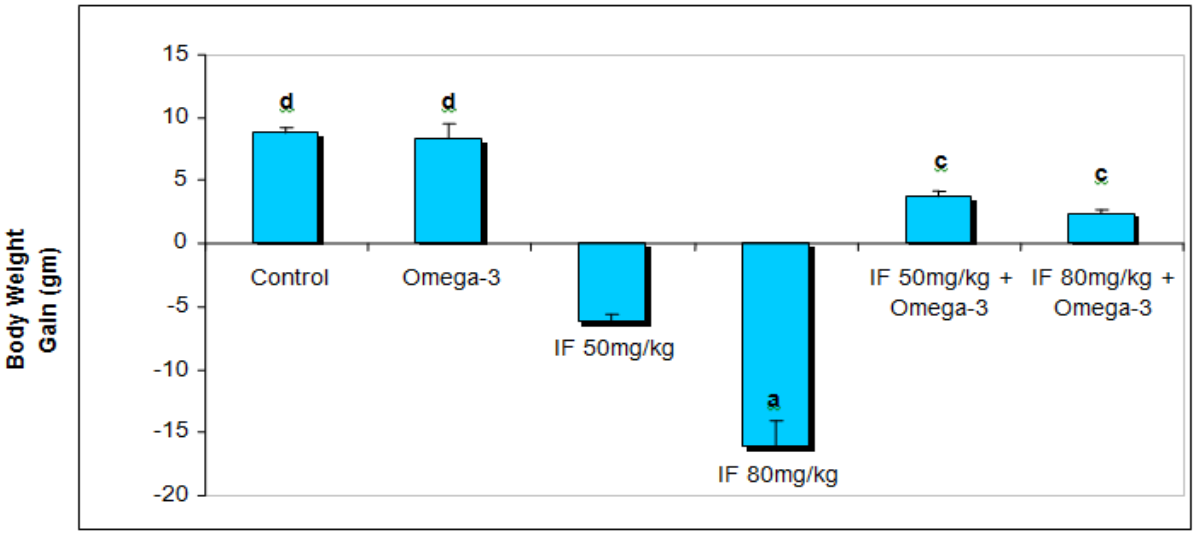

Figure 1. Effect of IFO and/or Omega-3 on body weight gain

Table 1. Effect of IFO and/or Omega-3 on Some Biochemical Parameters.

Groups	MDA µmol/L.*	Creatinine Mg/dl**	Phosphor Mg/dl**	Glucose Mg/dl**	GOT IU/L*	GPTIU/L**
Control	1.63±0.14^{a}	0.52± 0.07^{a}	5.98±0.21^{a}	158.16±1.9^{e}	64.21± 4.47^{a}	7.71±2.37^{a}
Omega-3	1.74±0.11^{a}	0.58±0.04^{ab}	7.8± 0.16^{b}	152.83±0.89^{e}	77.01 ±7.93^{ab}	7.52±1.14^{a}
IF(50mg/kg)	2.23±0.22^{bc}	1.26± 0.04^{d}	12.1±0.41^{d}	114.16±1.84^{b}	134.03± 6.08^{c}	11.41±0.66^{a}
IF(80mg/kg)	2.54±0.1^{c}	1.52±0.06^{e}	12.88±0.16^{d}	98.36±1.48^{a}	185.08± 14.78^{d}	20.39±1.02^{b}
IF(50mg/kg)+Omega-3	1.84±0.13^{ab}	0.76±0.02^{bc}	9.88±0.52^{c}	141.96±0.59^{d}	67.36±1.39^{a}	7.22±1.05^{a}
IF(80mg/kg)+Omega-3	1.93±0.05^{ab}	0.84±0.07^{c}	10.32±0.08^{c}	130.7± 0.51^{c}	112.63±11.86^{bc}	17.26±1.14^{b}

*The values represented by mean ± SE, N=5, Duncan's test used to compare between groups. Different letters in the same column refer to significant changes, while similar letters refer to non-significant changes. ** ($p<0.01$) and * ($p<0.05$).*

Table 2. Effect of IFO and/or Omega-3 on Some Hematological Parameters.

Groups	WBC**	RBC*	HGB*	PLT**
Control	6.52 ± 0.49^{c}	7.52 ±0.17^{b}	14.2± 0.28^{c}	508.66 ±21.36^{b}
Omega-3	5.86 ± 0.6^{c}	7.38 ±0.22^{b}	13.76 ± 0.31^{c}	489.33± 31.13^{b}
IF(50mg/kg)	0.63 ± 0.08^{a}	5.87± 0.17^{a}	10.2 ± 0.35^{a}	361 ± 27.75^{a}
IF(80mg/kg)	0.52±0.02^{a}	5.65±0.13^{a}	9.95±0.12^{a}	345±21.33^{a}
IF(50mg/kg) + Omega-3	4.43 ±1.53^{b}	6.45 ±0.29^{a}	11.76 ± 0.76^{b}	573 ± 19.73^{b}
IF(80mg/kg) + Omega-3	3.8± 0.9^{b}	6.13±0.17^{a}	10.9±0.46^{b}	432±11.07^{b}

The values represented by mean ± SE, N=5, Duncan's test used to compare between groups. Different letters in the same column refer to significant changes, while similar letters refer to non-significant changes. ** ($p<0.01$) and * ($p<0.05$).

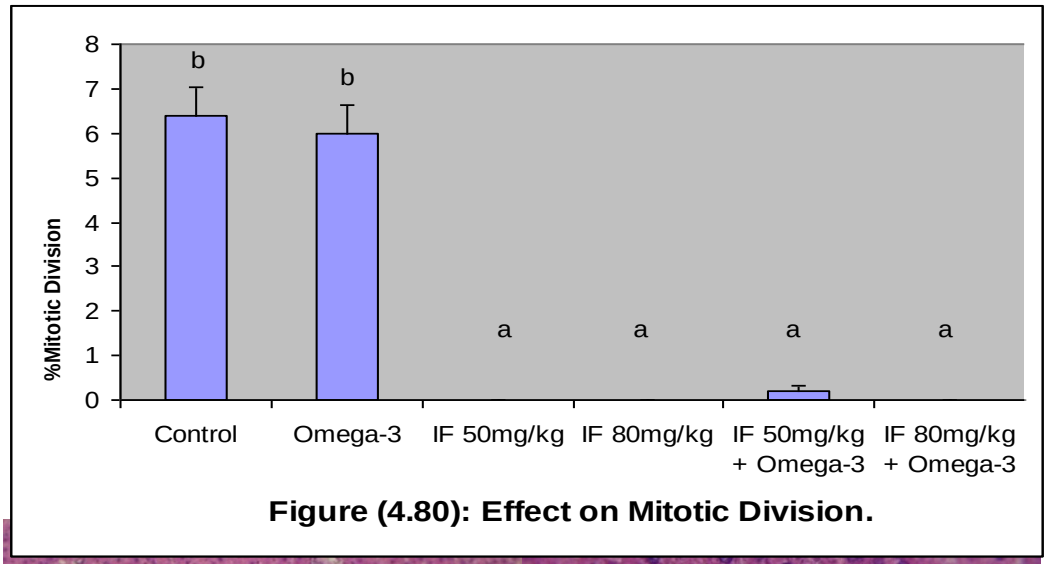

Figure (4.80): Effect on Mitotic Division.

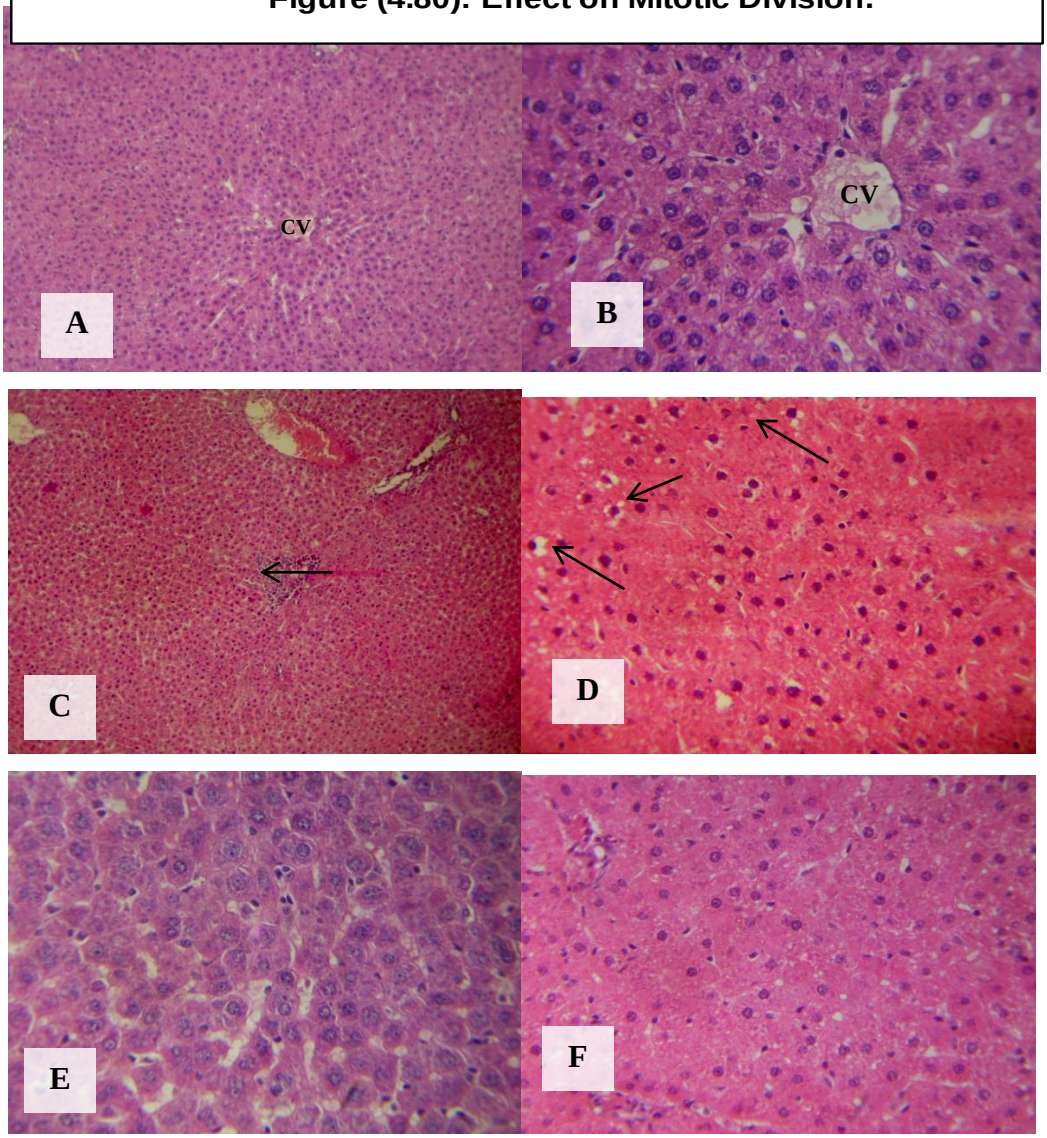

Figure 3. Paraffin sections through the liver of rats: A) control, 100X, B) control,400X, (CV):central vein, C):group3,IFO(50mg/kg.b.wt) showing inflammatory leucocyte infiltration (arrow),100X, D) Group 4, IFO(80mg/kg.b.wt) showing degenerated hepatocytes with vacuolated cytoplasm and condensed nuclei(arrows),400X,E)Group 5, IFO(50mg/kg.b.wt) plus omega-3 showing well protected liver structure,400X, F) Group6: (80mg/kg.b.wt) plus omega-3 showing approximately protected liver structure, although some unhealthy hepatocytes still exist,400X.

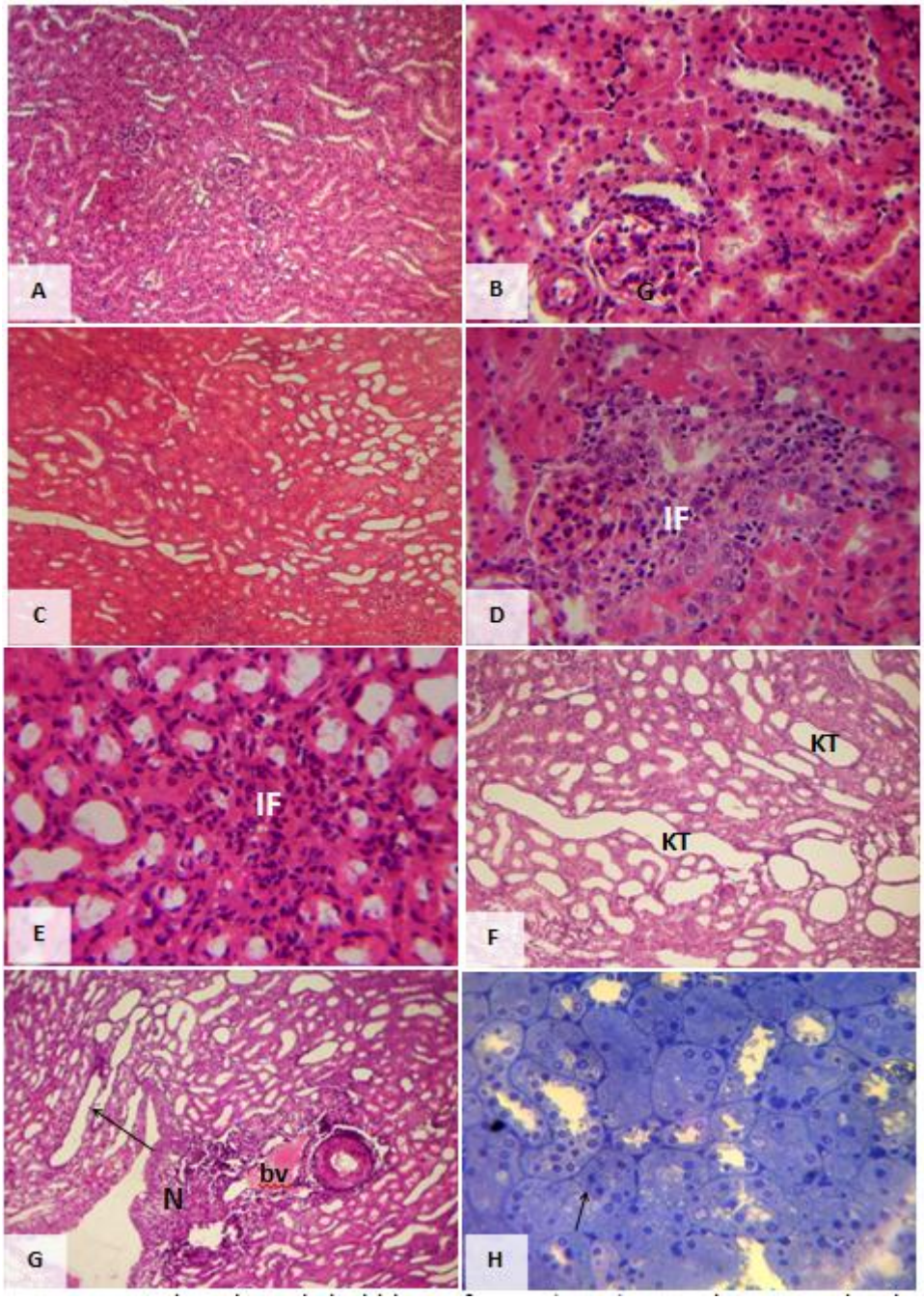

Figure 4. Sections through the kidney of rats: A) & B) Control group sowing the normal structure of kidney in the cortical region, 100X & 400X respectively ,C)Group3: IFO(50mg/kg.b.wt) showing dilatation of kidney tubules,100X,D) & E) Group3: IFO(50mg/kg.b.wt) large area of inflammatory cells(IF) in the cortex and medullary regions respectively,400X, F)Group 4: IFO(80mg/kg.b.wt) showing highly dilated kidney tubules(KT),100X, G)same later group with dilatation of kidney tubules(arrow),necrosis of the kidney cells(N) and congestion of blood vessels(bv),100X, H) Plastic section of the kidney tubular epithelial cells in the later group showing high number of dead cells(arrow),notice the nuclei of kidney tubular cells showing anisonucleosis,400X.

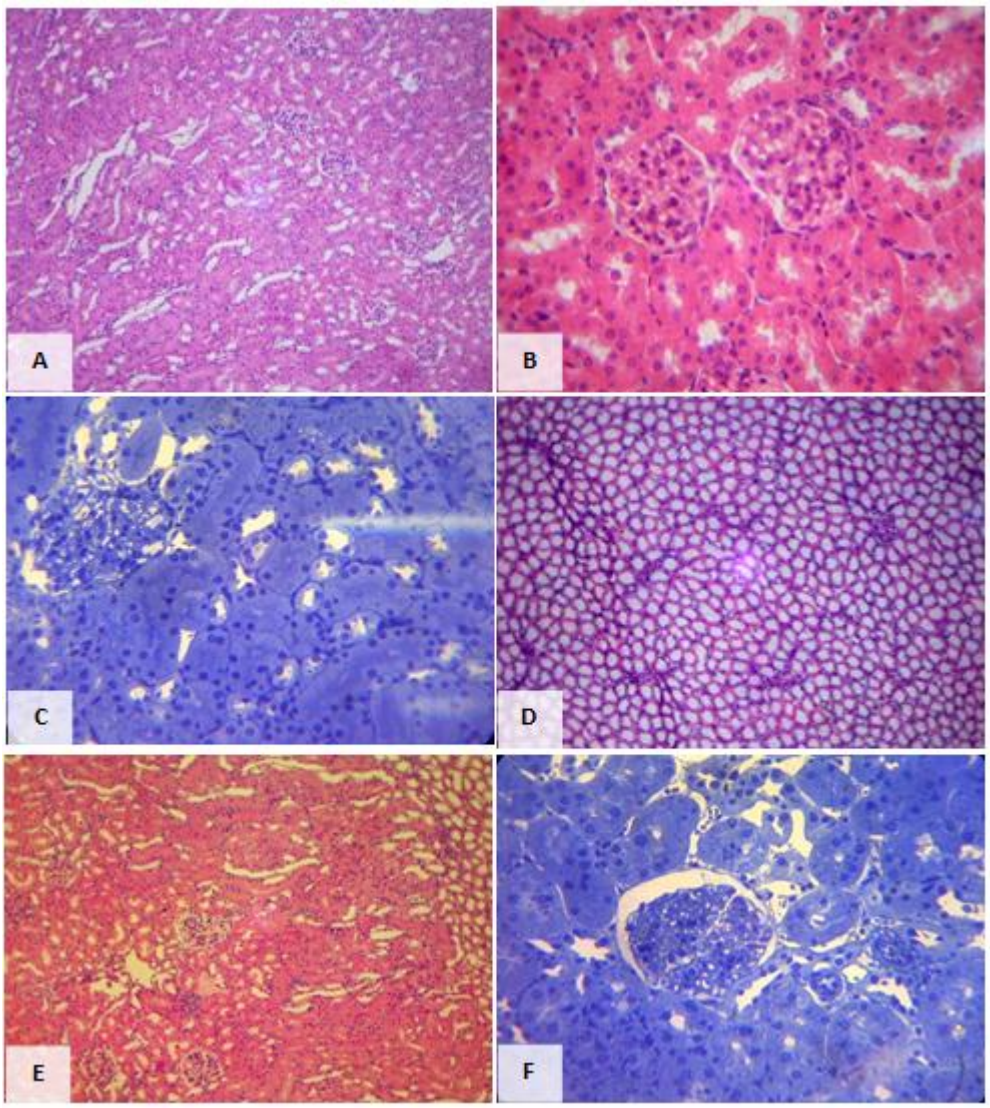

Figure 5: Sections through the kidney of rats treated with IFO plus omega-3 oil: A) & B) IFO (50mg/kg.b.wt) plus omega-3 showing approximately normal structure of the cortical region especially the glomeruli,100X and 400X respectively, C) plastic section of the kidney in the later group showing approximately healthy kidney tubular epithelial cells,400X, D) Normal cortical structure in the kidney of the later group,100X,E) Group 6,(80mg/kg.b.wt) plus omega-3 showing approximately normal structure with no inflammation or dilation of kidney tubule lumen,100X,F) same later group showing slight alteration in the structure of the kidney compared to control, notice the degeneration of some kidney tubule cells and slightly shrunken glomerular tuft,400X.

4. Discussion

The significant dose dependent decrease in body weight due to ifosfamide (IF) treatment, which is found in the present work and also recorded by other investigators (Springate and Van Liew, 1995) may be related to the effect of exposure to this drug on the appetite of the rats and also wastage in muscle mass as a result of the induced physiological changes (Chen *et al.*, 2008). As it will be declared later, degeneration of the liver and kidney which caused by IFO treatment may another reason for this decrease in the body weight gain.

As revealed by the current investigation, both doses of IFO (50 and 80 mg/kg.b.wt.) have caused Fanconi syndrome which included the higher levels of serum creatinine and phosphorous and the lower level of glucose in the IFO treated groups compared with the control (Skinner *et al.*, 1993; Loebstein and Koren, 1998; Rossi *et al.*, 1999; Skinner, 2003; Şehirli *et al.*, 2007; Chen *et al.*, 2008). The degeneration of kidney tubule cells due to IFO action may be the reason for this renal dysfunction (Chen *et al.*, 2008).

The levels of serum MDA in the IFO treated rats were significantly higher than those of the control group. This elevation in MDA (thereby elevation in lipid peroxide level) indicates the presence of oxidative tissue damage as a result of impaired antioxidant defense mechanism (Şehirli *et al.*, 2007) and this oxidative stress may cause the death of cells in general (Ramaekers *et al.*, 1997).

Elevation of the two liver enzymes GOT and GPT in the serum of IFO treated rats refers to the hepatic cellular injury (Green and Flamm, 2002; Clark *et al.*, 2003) because damaged liver cells develop leaky membranes, allowing for escape of intracellular enzymes (including GOT, GPT and other enzymes) into the bloodstream and

this will raise the levels of these enzymes in the serum (Amacher, 1998).

The degeneration of hepatocytes which has been detected in the IFO treated rats may be related to the toxic metabolites of IFO, especially chloroacetaldehyde (CAA), which may induce cell death through the depletion of hepatocellular GSH, ATP and enhanced lipid peroxidation rate (Sood and O'Brien, 1994), although the pathophysiology of this toxicity is not fully understood (Knouzy *et al.*, 2010). It has been found that CAA collapsed the mitochondrial membrane, induced the release of cytochrome C from mitochondria to the cytosol and significantly reduced cellular ATP level that triggers cell death. The mechanism of such cell death follows the apoptotic cell death (Takahashi *et al*., 2007), although necrotic mechanism of cell death has also been mentioned by other workers (Daniel *et al.*, 1992).

Similarly, the death of PCT cells in the IFO treated rats are mainly due to the toxic effects of IFO metabolites, acroline and CAA, and not by IFO itself (Schwerdt *et al.*, 2005)through a possible mechanism which is the depletion of reduced GSH and ATP (Nissim *et al.*, 2006; Brüggemann *et al.*, 2006; Dubourg *et al.*, 2001), because the depletion of tissue GSH is one of the primary factors that permit lipid peroxidation to occur in cell membranes, devastating the functional integrity of the cellular structure, and if the damage is sever, cell death is inevitable (Şehirli *et al.*, 2007). Elevation of MDA level reported by the present work confirms the above mechanism.

In accordance with the present results, some further histological changes in IFO treated rats which include inflammation in the renal tissue and degeneration of proximal tubules with desquamated epithelium was also observed by other investigators (Şehirli *et al.*, 2007; Chen *et al.*, 2008). This inflammation which has been seen in the liver and kidney is due to the necrotic mechanism caused by IFO (Schwerdt *et al.*, 2007; Chen *et al.*, 2008).

On the other hand, Omega-3 FAs through their antioxidant properties reduced the toxicity of IFO metabolites especially CAA. This was also detected in previous works that the toxicity of IFO lowered by using certain antioxidants (Nissim and Weinberg, 1996; Badary, 1998, 1999a, 1999b; Şehirli *et al.*, 2007; Chen *et al.*, 2008; Casado-Zapico *et al.*, 2010). The antioxidant and/ or anti-inflammatory effects of omega-3 FA through scavenging of free radicals and inhibiting lipid peroxidation have been reported previously (Ernest and Magdalena, 2008).This oxidant/antioxidant theory may explain the protection role of Omega-3 FAs against the hepatotoxicity and nephrotoxicity of IFO. The anti-inflammatory property of omega-3 fatty acids (Omega-3 FAs) is due to the action of eicosapentaenoic acid, which is one of the components of omega-3. The anti-inflammatory action is achieved by reducing the pro-inflammatory cytokines like intrleukin-1(IL-1) and tumor necrosis factor alpha (TNFα) (Wardle, 2000) and it is effects on transcription factors that regulate inflammatory gene expression (e.g. Nuclear Factor Kappa B) (Calder, 2009).

The most peculiar result in the present work was the normal existence of mitotic division in the bone marrow of omega-3 group and the absence of mitotic division in the IFO plus omega-3 group. The antimitotic effect of Omega-3 oilhas been mentioned previously (Hardman 2002). If we consider this and the anticancer activity of this oil (Simopoulos, 2003) and its role in increasing the differentiation of the myeloid progenitor cells in the bone marrow without having an adverse effect on peripheral white blood cells counts(Varney *et al.*,2009) beside the findings of the current work which included the protective role of this oil against the side effects of IFO (i.e. hepatotoxicity, nephrotoxicity, changes in hematological parameters and the oxidative stress), a unique protective agent against IFO toxicity can be suggested in comparison to the previously studied antioxidants.

References

Amacher DE.1998. Serum transaminase elevations as indicators of hepatic injury following the administration of drugs. *Regul Toxicol Pharmacol.,* **27(2):**119-130.

Badary OA.1998.Taurine attenuates Fanconi syndrome induced by IFO without compromising its antitumor activity. *Oncol Res.,* **10**:355-360.

Badary OA. 1999a. L-Histidinol attenuates Fanconi syndrome induced by IFO in rats. *Exp Nephrol.,* **7**:323-327.

Badary OA. 1999b. Thymoquinone attenuates IFO-induced Fanconi syndrome in rats and enhances its antitumor activity in mice. *J Ethnopharmacol.,* **67(2):**135-142.

Bernard PA, McCabeT, Bayliff S and Hayes D. 2010.Successful treatment of IFO neurotoxicity with dexmedetomidine.*J Oncol Pharm Pract.,* **16(3):**151-159.

Brüggemann SK, Radike K, Braasch K, Hinrichs J, Kisro J, Hagenah W, Peters SO and Wagner T.2006. Chloroacetaldehyde: mode of antitumor action of the IFO metabolite. *Cancer Chemother Pharmacol.,* **57(3)**:349-356.

Calder PC. 2009. Polyunsaturated fatty acids and inflammatory processes: New twists in an old tale. *Biochim*, **91(6):**791-795.

Calviello G and Serini S. 2010.**Dietary Omega-3 Polyunsaturated Fatty Acids and Cancer.**Springer Dordrecht Heidelberg London.

Casado-Zapico S, Rodriguez-Blanco J, Garcia-Santos G, Martin V, Sánchez-Sánchez A M, Antolín I, Rodriguez C. 2010.Synergistic antitumor effect of melatonin with several chemotherapeutic drugs on human Ewing sarcoma cancer cells: potentiation of the extrinsic apoptotic pathway. *J Pineal Res*,**48(1**):72-80.

Chen N, Aleksa K, Woodland C, Rieder M and Koren G. 2008.N-Acetylcysteine prevents IFO-induced nephrotoxicity in rats. *Br J Pharmacol*,**15**:1-9.

Clark JM, Brancati FL and Diehl AM.2003.The prevalence and etiology of elevated aminotransferase levels in the United States. *Am J Gastroenterol*,**98**:960-967.

Daniel FB,De Angelo AB, Stober JA, Olson GR and Page NP.1992. Hepatocarcinogenicity of chloral hydrate, 2-chloroacetaldehyde, and dichloroacetic acid in the male B6C3F1 mouse. *Fund Appl Toxicol*,**19(2):**159-168.

Dubourg L, Michoudet C, Cochat P and Baverel G.2001. Human kidney tubules detoxify chloroacetaldehyde, a presumed

nephrotoxic metabolite of IFO. *J Am Soc Nephrol*,**12(8):**1615-1623.

Edwards IJ and O'Flaherty JT. 2008. Omega-3 Fatty Acids and PPAR gamma in Cancer. *PPAR Res*.**358052**:1-14.

El-Habit OH, Sayed-Ahmed M, Gabry MS, Khalil M and Osman A.M. 2000. Modulation of induced cardiocytotoxicity and genotoxicity of Doxorubicin in rat by L-Carnitine. *J Egypt Nat Cancer Inst.* **12(4)** :267-274

Ernest KJP and Magdalena K. 2008. Fatty Acid Facts, Part III: Cardiovascular Disease, or, a Fish Diet is not Fishy. *Drug News Perspect*,**21(10):**552.

Green RM and Flamm S.2002. AGA Technical review on the evaluation of liver chemistry tests.*Gastroenterol*,**123**:1367-1384.

Hamadani M and Awan F. 2006. Role of thiamine in managing IFO-induced encephalopathy. *J Oncol Pharm Pract*,**12(4):**237-239.

Hanly L, Chen N, Rieder Mand Koren G. 2009.IFO nephrotoxicity in children: a mechanistic base for pharmacological prevention. *Exp Opinon Drug Safty*,**8(2):**155-168.

Hardman WE. 2002. Omega-3 Fatty Acids to Augment Cancer Therapy. *J Nutr.*,**132**:3508S-3512S.

Knouzy B, Dubourg L, Baverel G and Michoudet C.2010.Targets of chloroacetaldehyde-induced nephrotoxicity.*Toxicol In Vit*, **24(1):** 99-107.

Loebstein R and Koren G.1998.IFO-induced nephrotoxicity in children: critical review of predictive risk factors. *Pediatr.*, **101**:E8-E12.

Loebstein R, Atanackovic G, Bishai R, Wolpin J, Khattak S, Hashemi G,Gobrial M, Baruchel S, Ito S and Koren G..1999. Risk factors for long-term outcome of IFO-induced nephrotoxicity in children. *J Clin Pharmacol.*,**39**:454-461.

Murice-Lambert E, Banford A Band Folger R L.1989. Histological preparation of implanted biomaterials for light microscopic evaluation of the implant-tissue interaction.*Biotech Histoch.*, **64(1):**19-24.

Nissim I and Weinberg J M.1996. Glycine attenuates Fanconi syndrome induced by maleate or IFO in rats. *Kid Int*, **49(3):**684-695.

Nissim I, Horyn O, Daikhin Y, Nissim I, Luhovyy B, Phillips PC and Yudkoff M.2006. IFO-induced nephrotoxicity: mechanism and prevention. *Cance Res.* , **66(15):** 7824-7831.

Pardini RS. 2006. Nutritional intervention with omega-3 fatty acids enhances tumor response to anti-neoplastic agents. *Chemico-Biol. Interact.* **162**:89–105

Paschke R, Worst P, Brust J and Queisser W.1988.[Hepatotoxicity with etoposide-IFO combination therapy]. *Onkol*, **11(6):**273-275.

Ramaekers VT, Bosman B, Jansen GA, Wanders JR..1997. Increased plasma malondialdehyde associated with cerebellar structural defects. *Arch. Dis. Child.*,**77:**231-234.

Rossi R, Pleyer J, Schäfers P, Kuhn N, Kleta R, Deufel T and Jürgens H.1999. Development of IFO-induced nephrotoxicity: prospective follow-up in 75 patients. *Med Pediatr Oncol.*, **32(3):** 177-182.

Schwerdt G, Gordjani N, Benesic A, Freudinger R, Wollny B, Kirchhoff A and Gekle M.2005.Chloroacetaldehyde and acrolein-induced death of human proximal tubule cells.*Pediatr Nephrol.*,**21**:60-67.

Schwerdt G, Kirchhoff A, Freudinger R, Wollny B, Benesic A and Gekle M.2007. Mensa or cysteine prevents chloroacetaldehyde-induced cell death of human proximal tubule cells. *Pediatr Nephrol.*,**22(6):**798-803.

Şehirli Ö,Şakarcan A, Velioğlu-Öğünç A, Çetinel Ş, Gedik N, Yeğen BÇ and Şener G. 2007. Resveratrol improves IFO-induced Fanconi syndrome in rats. *Toxicol Appl Pharmacol.*,**222**:33-41.

Sener G, Sehirli O, Yegen BC, Cetinel S,Gedik N and Sakarcan A.2004. Melatonin attenuates IFO-induced Fanconi syndrome in rats. *J Pineal Res.*,**37 (1)**:17-25.

Shaikh IA, Brown I, Wahle KWand Heys SD.2010. Enhancing cytotoxic therapies for breast and prostate cancers with polyunsaturated fatty acids. *Nutr Canc*, **62(3):** 284-296.

Sharma AK and Sharma A. 1994.**ChromosomeTechniques.A manual**. Switzerland Harwood Academic Publishers.

Simopoulos AP. 2002. Omega-3 Fatty Acids in Inflammation and Autoimmune Diseases. *J Am Coll Nutr.*,**21(6):**495-505.

Simopoulos AP. 2003. Omega-3 fatty acids and cancer. *Indoand Built Environ*, **12(6):**405-412.

Skinner R.2003. Chronic IFO nephrotoxicity in children. *Med Pediatr Oncol.* ,**41(3):**190-197.
Skinner R, Sharkey IM, Pearson AD and Craft AW.1993.IFO, mesna, and nephrotoxicity in children. *J Clin Oncol.*, **11**:173-190.

Sood C. and O'Brien PJ.1994. Chloroacetaldehyde-induced hepatocyte cytotoxicity: Mechanisms for cytoprotection. *Biochem Phrmacol.*,**48(5):**1025-1032.

Springate J E and Van Liew J B.1995. Nephrotoxicity of ifosfamide in rats.*J Appl Toxicol.*, **15(5):**399-402.

Straka C, Hebart H, Adler-Reichel S, Werding N, Emmerich B and Einsele H. 2003.Blood stem cell collections after mobilization with combination chemotherapy containing IFO followed by G-CSF in multiple myeloma.*Oncol*, **65**(Suppl. 2):94-98.

Sun W, Chen G, Ou X, Yang Y, Luo C, Zhang Y, *et al.* 2009. Inhibition of COX-2 and activation of peroxisome proliferator-activated receptor γ synergistically inhibits proliferation and induces apoptosis of human pancreatic carcinoma cells. *Canc Lett.*, **275(2):**247-255.

Takahashi K, Sakurai K, Takahashi K, Tanaka H and Fujimoto Y. 2007.Necrotic pathway in human osteosarcoma Saos-2 cell death induced by chloroacetaldehyde. *Anti-Canc Drug*,**18(5):**543-553.

Varney ME, Hardman WE and Sollars VE.2009. Omega-3 fatty acids reduce myeloid progenitor cell frequency in the bone marrow of mice and promote progenitor cell differentiation. *Lipid Heal Dis.*, **8(9):**1-6.

Wall R, Ross RP, Fitzgerald GF and Stanton C.2010. Fatty acids from fish: the anti-inflammatory potential of long chain omega-3 fatty acids. *Nutr Rev.*, **68(5):**280-289.

Wardle EN. 2000. Ways of Treating IgA Nephropathies. *Saudi J Kid Dis Transpl,* **11(3):**325-333.

Weinstein T, Chagnac A, Korzets A, Boaz M, Ori Y, Herman M, Malachi T, Gafter U.2000.Hemolysis in haemodialysis patients: evidence for impaired defense mechanisms against oxidative stress. *Nephro Dial Transplant,* **15**:883-887.

Wendel M and Heller AR. 2009. Anticancer action of omega-3 fatty acids-current state and future perspectives. *Anticancer Agent Med Chem*., **9(4):**457.

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Physico-Chemical and Some Trace Metal Analysis of Ogba River, Benin City, Nigeria

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Abstract

The physico-chemical parameters and some trace metal contents of Ogba River in Benin City, Nigeria were investigated from January to August 2008. Four stations were studied from upstream to downstream using standard methods. A total of twenty-six physico-chemical characteristics and trace metal contents were examined; Air and water temperatures, depth, transparency, turbidity, flow velocity, pH, total alkalinity, conductivity and dissolved solids. Other includes dissolved oxygen, oxygen saturation, biochemical oxygen demand, chloride, phosphate, nitrate, sulphate, potassium, calcium, magnesium, iron, lead, copper, zinc and manganese Thirteen (13) parameters exhibited clear seasonal variations. However, there were significant differences ($p < 0.05$) in the values of depth, flow velocity, BOD5, sulphate, phosphate, nitrate and sodium among the stations. The Benin City wastewater had negatively impacted at station 2 of the river, although the recorded values were still within acceptable limits.

Keywords: Physico-chemical parameters, trace metal, anthropogenic activities, water quality, acceptable limits, Nigeria.

1. Introduction

The quality of any water body is governed by its physicochemical and heavy metal factors. The monitoring of physicochemical characteristics of a water body is vital for both long term and short evaluation of its quality (Wood, 1995). Lakes, rivers and streams have important multi – usage components, such as sources of drinking water, irrigation, fishery and energy production (Iscen *et al*., 2008). Water is a scarce and fading resource, and its management can have an impact on the flow and the biological quality of rivers and streams (Prat and Munné, 2000). Expanding human population, industrialization, intensive agricultural practices and discharges of massive amount of wastewater into the rivers and streams have resulted in deterioration of water quality (Herschy, 1999). The impact of these anthropogenic activities has been so extensive that the water bodies have lost their self-purification capacity to a large extent (Sood *et al*., 2008). Freshwater ecosystems have been used for the investigation of factors controlling the distribution and abundance of aquatic organisms. The physical and chemical characteristics of water bodies affect the species composition, abundance, productivity and physiological conditions of aquatic organisms (Bagenal, 1978).

In Nigeria, studies on the physicochemical quality of water bodies have been reported extensively (Mustapha and Omotosho, 2005; Omoigberale and Ogbeibu, 2007; Yusuf and Osibanjo, 2007; Asonye *et al*., 2007; Davies and Otene, 2009). However, less attention has been given to smaller rivers like Ogba River, which are scattered all over the country and contain a significant proportion of the nation's aquatic biodiversity. Ogba River plays important roles in the lives of the surrounding inhabitants. Fishing, farming, bathing, washing/laundry, car/motorbike washing, refuse disposal, municipal wastewater and human waste disposal and religious activities are constantly going on within and around this river. The objective of the present study was therefore to evaluate the physico-chemical parameters and some trace metal contents of Ogba River, Benin City, Nigeria.

2. Materials and Methods

2.1. Study area and sampling stations

Ogba river is a forth order (4^o) river, located at the Southwest region of the outskirt of Benin City in Edo State, Nigeria between Latitude 6.20^oN and Longitude 5.34^oE (Fig. 1). The river is about 42 km long and takes its source at Ekewan and flows in a South East direction through Ogba village and empties to Osiomo River, into Benin River, which in turn empties into the Atlantic Ocean. This study was carried out in the upper part of the river stretching from Ogbe Ibuya area to Ogba community. Four sampling stations were chosen for the study; Station 1 located at Ogbe Ibuya area was open to a number of

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aquatic macrophytes within the water and around the edges blocking the northern part completely. No human activity was observed during the study. Station 2, also located at Ogbe Ibuya area, 0.66km downstream of station 1.

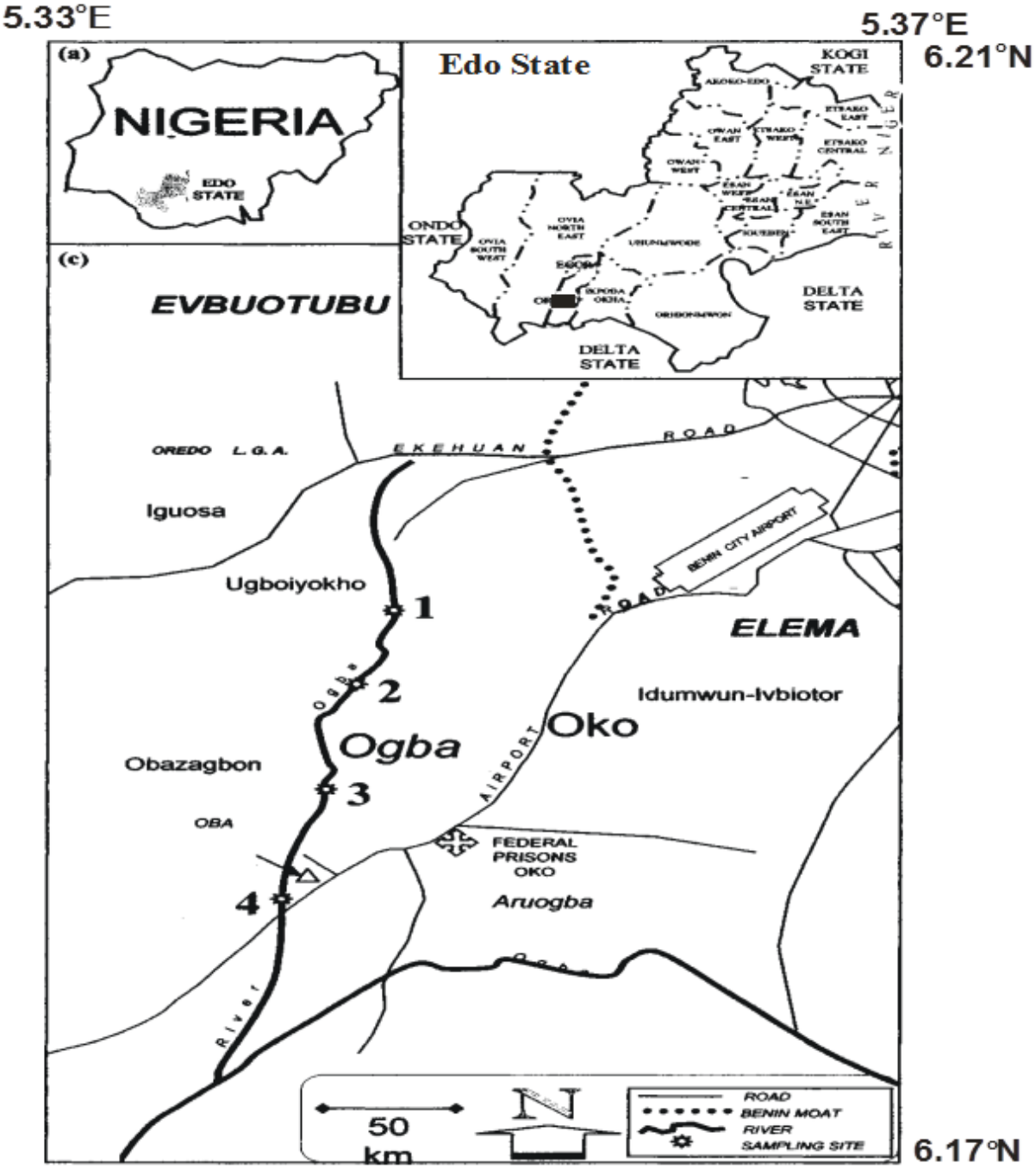

Figure 1: Map of Benin City showing location of sampling stations 1 – 4 in the Ogba River.

2.2. Samples collection and analyses

Benin City wastewater from GRA area discharges into the river at this point. Station 3, located behind Oko Open Prison, 0.6km downstream of station 2; the human activities include bathing, washing and farming by the inmates of the prison. Station 4 located within Ogba community by the bridge, 2km downstream of station 3 where human activities include: bathing, washing of clothes, cars and motor bikes as well as idol worshiping and water baptism.

Samples were collected once a month between January and August 2008. Water samples were collected with a 2 liter plastic hydrobios water sampler and transferred to clean 2 litre polyethylene containers. All samples were transported in ice chests and analyzed for pH and conductivity within 12 hours of collection. Other physicochemical parameters were analyzed later using refrigerated samples. They were analyzed according to standard methods (APHA, 1998) for physicochemical

parameters. The determination of heavy metal was carried out in two stages. The samples were digested in concentrated Nitric Acid and analyzed by Atomic Absorption Spectrometer (Varian Techtron Spectra B). The data obtained were subjected to analysis for means, standard error and significance between the means at 95% probability level. These were carried out using Microsoft Excel (2003) package.

3. Results

The physicochemical parameters and trace metal contents of the studied river are presented in Tables 1 and 2, respectively. The air temperature ranged between 15^{o}C and 31.5^{o}C while the surface water temperature ranged between 20.0oC and 27.5oC. The pattern of fluctuation for both air and water temperatures was similar in all the stations. Water depth ranged from 21.0cm to 113cm and station 2 was significantly different ($p<0.001$) while transparency and turbidity ranged from 12.5cm to 100.0cm and 0.11NTU to 53.08 NTU respectively. The mean flow velocity was lowest in station 1 (1.41 m/s) while stations 2 (4.90 m/s), 3 (4.73 m/s) and 4 (4.63 m/s) had high mean values which decreased spatially. Temporally, there was a distinct seasonal pattern in the variation of both transparency and turbidity. While transparency values were highest in the dry season months, the reverse is the case for turbidity.

The pH values revealed that the water was moderately acidic to moderately alkaline with a range of 5.4 to 8.1 while the total alkalinity values ranged between 6.1mgl^{-1} and 183mgl^{-1}. The conductivity values ranged from 23.3µScm^{-1} to 116.5µScm^{-1} while total dissolved solids which followed the same spatial variation trend, ranged between 14.48mgl^{-1} and 66.4mgl^{-1}. There was a clear seasonal variation in both conductivity and total dissolved solids; values increased from dry season to rainy season. The dissolved oxygen content ranged from 4.8mgl^{-1} to 6.9mgl^{-1}; concentrations generally increased with the rains while oxygen saturation values confirmed that water was well saturated. The oxygen saturation in all the stations throughout the study period was above 50%. The values of BOD5 ranged between 1.7mgl^{-1} and 4.8mgl^{-1} with station 2 being significantly different ($p<0.05$). The temporal variation of DO and oxygen saturation was similar while that of BOD5 was irregular. The chloride concentration ranged between 19.5mgl^{-1} and 106mgl^{-1} in an irregular temporal variation. Nitrate values ranging from 0.001mgl^{-1} to 0.3mgl^{-1} were observed throughout the period of study. Peak values were recorded in the rainy season months and the lower values in the dry season months while sulphate and phosphate values observed ranged from 0.60mgl^{-1} to 6.39mgl^{-1} and from 0.10mgl^{-1} to 1.44mgl^{-1}, respectively. There was no clear seasonal pattern observed in sulphate and phosphate fluctuations. There was a significant difference in the values recorded in all the stations ($p<0.05$) for nitrate, sulphate and phosphate. The cations such as sodium and potassium values ranged from 1.15mgl^{-1} to 2.80mgl^{-1} and 0.18mgl^{-1} to 0.91mgl^{-1} respectively. There was a significant difference in the values of sodium in all the stations ($p<0.05$). On the other hand, the concentrations of calcium in all the stations were relatively close (between 9.60 and 12.83 mgl^{-1}) while magnesium values ranged between 1.57mgl^{-1} and 5.83mgl^{-1}. In both cases, no significant differences were observed in the stations.

The values of the heavy metals were generally low. Iron values ranged from 1.00mgl^{-1} to 4.23mgl^{-1} with higher value recorded in the rainy season months. Lead ranged from 0.012mgl^{-1} to 0.20mgl^{-1}, most of the high lead values were recorded during the dry season months. The highest copper value was 0.505mgl^{-1} while the lowest value was 0.256mgl^{-1}. The trend showed that in all the stations, the values of copper increased with increase in rains and dropped with slight decrease in rains in August 2008. The highest zinc concentration was 0.15mgl^{-1} while the lowest was 0.09mgl^{-1}. The value of manganese ranged from 0.22mgl^{-1} to 0.53mgl^{-1}. Zinc and manganese did not show any clear trend in their temporal variations. There were no significant differences observed among the heavy metals.

4. Discussion

Atmospheric temperatures recorded in the study were lower in January and February, 2008 with a relatively higher value in March before decreasing in April in all the stations; increasing and stabilizing with minimal fluctuations from May to August, 2008. Low atmospheric temperature recorded in the months of January and February 2008 were probably due to the North East trade wind that blows across the Sahara, often referred to as the Harmattan while that of April could be attributable to early rains. This condition is typical of tropical weathers (Awachie, 1980). The surface water temperature followed the same trend that was observed in the air temperature.

Station 3 recorded the highest depth in March while station 2 recorded the lowest in June 2008. The variations in depth are usually associated with the rainfall pattern of the drainage basin. Lower depths recorded during the rainy season especially in stations 2 to 4 could be attributed to heavy siltation associated with increase in rainfall exacerbated by the increased floodwater entering the river system from Benin City wastewater drain in station 2 and moving down to station 4 (Mligo, 2007).

Transparency values recorded indicated that stations 1, 3 and 4 were clear zones with high transparency values. In this study, transparency showed a seasonal pattern, lower transparency values were recorded in the rainy season months while higher transparency values were recorded in the dry season months. This is primarily due to the fact that in the rainy season, rivers receive run-offs from nearby terrestrial environment thereby increasing the suspended solids load. This trend is consistent with reports from most Nigerian inland waters (Imoobe and Oboh, 2003).

In this study, turbidity showed seasonal and spatial patterns; relatively higher turbidity values were observed during the rainy season period due to increased suspended solids loads laden run-offs while the mean value increased from station 1 to 4; this could be attributable to the level of human activity in the river system, which is highest in station 4.

Table 1: Mean and standard error of physicochemical parameters of Ogba River, Benin City, Nigeria during the eight (8) months study period

Parameter	LEVEL RECORDED/DETECTED					Maximum Permissible Levels	
	Station 1 $\overline{X}$ ± S.E.	Station 2 $\overline{X}$ ± S.E.	Station 3 $\overline{X}$ ± S.E.	Station 4 $\overline{X}$ ± S.E.	*P* - Value	FEPA**	SON***
Air Temperature (°C)	22.85±1.35 15.0–25.5)	23.89±1.38 (18.0–30.5)	24.79±1.38 (18.9–31.5)	26.09±1.06 (20.5–31.5)	$p > 0.05$	-	-
Water Temperature (°C)	24.72±0.97 (20.0–27.5)	24.44±094 (20.2–25.6)	24.83±0.92 (20.5–26.9)	24.79±0.82 (20.6–26.7)	$p > 0.05$	< 40	Ambient
Depth (cm)	85.83±4.13 (71.0–104.0)	41.5±5.58 (21.0–62.0)	70.1±10.23 (40.0–113.0)	76.91±5.26 (52.0–98.0)	$p < 0.001$*	-	-
Transparency (cm)	63.38±8.31 (22.0–94.0)	33.63±5.20 (16.0–57.0)	50.24±12.05 (12.5–100.0)	51.43±7.09 (22.0–82.3)	$p > 0.05$	-	-
Velocity (m/s)	1.41±1.79 (10.8–25.6)	4.90±3.89 (28.2–65.6)	4.73±1.95 (37.7–53.0)	4.63±1.61 (37.7–51.4)	$p < 0.001$*	-	-
pH	6.78 ±0.10 (5.4–7.5)	6.23±0.05 (5.7–6.9)	6.83 ± 0.15) (5.9–8.1)	6.45± 0.09 (5.7–7.2)	$p > 0.05$	6.0 – 9.0	6.5 – 8.5
Conductivity (µS/cm)	50.6±11.22 (23.3–116.5)	45.73±4.06 (33.4–69.7)	47.6±3.67 (31.0–74.7)	40.8±6.18 (29.3–78.2)	$p > 0.05$	-	1000
Turbidity (NTU)	7.30±2.58 (0.11–17.18)	8.51±3.81 (0.63–28.2)	9.19±4.11 (0.22–32.8)	11.07±6.11 (1.7–53.08)	$p > 0.05$	10	15
Total Dis. Solids (Mgl^{-1})	30.11±6.33 (14.5–66.4)	27.66±2.43 (20.1–41.8)	28.95±3.42 (19.2–44.9)	26.96±3.74 (18.1–47.0)	$p > 0.05$	2000	500
Dissolved Oxygen (Mgl^{-1})	5.59±0.25 (4.9–6.9)	5.53±0.15 (4.8–6.0)	5.95±0.13 (5.4–6.5)	5.78±0.16 (4.9–6.3)	$p > 0.05$	5	-
Oxygen Saturation (%)	73.3±3.86 (58.1–86.5)	67.3±1.96 (60.6–74.8)	73.0±1.75 (68.5–81.7)	71.3±2.91 (56.9–80.8)	$p > 0.05$	-	-
BOD_5 (Mgl^{-1})	2.16±0.15 (1.7–3.0)	4.13±0.15 (3.5–4.8)	4.04±0.23 (2.9–4.8)	2.56±0.24 (1.7–3.9)	$p < 0.001$*	10	-
Alkalinity (Mgl^{-1})	35.08±9.83 (12.2–100.7)	50.33±19.43 (18.3–183.0)	38.13±7.38 (15.3–82.4)	33.17±10.79 (6.1–97.6)	$p > 0.05$	-	-
Chloride (Mgl^{-1})	34.80±3.19 (19.5–49.7)	35.72±3.68 (21.5–53.3)	41.78±9.60 (21.8–106.5)	34.98±5.39 (21.3–71.0)	$p > 0.05$	2000	250
Sulphate (Mgl^{-1})	2.83±0.30 (1.98–4.11)	6.06±0.10 (5.49–6.39)	0.90±0.03 (0.76–1.00)	0.66±0.02 (0.60–0.72)	$p < 0.001$*	200 – 400	100
Phosphate (Mgl^{-1})	1.08±0.01 (1.00–1.11)	1.24±0.03 (1.12–1.44)	0.23±0.12 (0.10–1.10)	0.86±0.17 (0.10–1.18)	$p < 0.001$*	5	-
Nitrate (Mgl^{-1})	0.02±0.01 (0.001–0.05)	0.13±0.05 (0.004–0.3)	0.04±0.01 (0.02–0.08)	0.03±0.01 (0.011–0.06)	$p < 0.01$*	20	50
Calcium (Mgl^{-1})	12.28±0.72 (9.62–16.03)	12.39±0.94 (9.60–16.05)	11.79±0.48 (9.60–12.83)	12.92±1.16 (9.26–19.24)	$p > 0.05$	-	-
Magnesium (Mgl^{-1})	2.23±0.25 (1.76–3.89)	2.58±0.26 (1.94–3.60)	2.05±0.23 (1.57–3.75)	3.08±0.51 (1.85–5.83)	$p > 0.05$	30 – 150	0.20
Potassium (Mgl^{-1})	0.44±0.06 (0.27–0.72)	0.46±0.08 (0.18–0.91)	0.43±0.69 (0.20–0.72)	0.43±0.05 (0.27–0.68)	$p > 0.05$	75 – 200	-
Sodium (Mgl^{-1})	1.27±0.02 (1.15–1.35)	1.88±0.15 (1.38–2.80)	1.64±0.06 (1.29–1.82)	1.40±0.04 (1.23–1.56)	$p < 0.001$*	-	200

* Significantly different means ($p < 0.05$). Range in parenthesis

** Nigerian Water Quality Standard for Inland Surface Water. Federal Environmental Protection Agency (FEPA), 2003.

***Nigerian Standard for Drinking Water Quality. Standards Organisation of Nigeria (SON), 2007

Table 2: Mean and standard error of some trace metal contents of Ogba River, Benin City, Nigeria during the eight (8) months study period

Parameter	LEVEL RECORDED/DETECTED					Maximum Permissible Levels	
	Station 1 $\overline{X}$ ± S.E.	Station 2 $\overline{X}$ ± S.E.	Station 3 $\overline{X}$ ± S.E.	Station 4 $\overline{X}$ ± S.E.	*P* - Value	FEPA**	SON***
Copper (Mgl^{-1})	0.41±0.03 (0.27–0.50)	0.39±0.03 (0.26–0.50)	0.40±0.02 (0.30–0.46)	0.40±0.03 (0.26–0.49)	$p > 0.05$	0.05 – 1.5	1
Zinc (Mgl^{-1})	0.12±0.004 (0.11–0.14)	0.11±0.003 (0.09–0.12)	0.12±0.008 (0.09–0.15)	0.11±0.003 (0.10–0.12)	$p > 0.05$	5 – 15	3
Iron (Mgl^{-1})	2.09±0.36 (1.00–3.95)	2.61±0.39 (1.23–4.23)	2.00±0.36 (1.03–3.82)	2.02±0.19 (1.06–2.87)	$p > 0.05$	20	0.3
Lead (Mgl^{-1})	0.09±0.02 (0.012–0.20)	0.06±0.08 (0.02–0.09)	0.10±0.02 (0.07–0.18)	0.07±0.01 (0.03–0.13)	$p > 0.05$	0.01 – 1.0	0.01
Manganese (Mgl^{-1})	0.36±0.02 (0.27–0.43)	0.36±0.02 (0.30–0.45)	0.39±0.02 (0.33–0.53)	0.34±0.02 (0.22–0.45)	$p > 0.05$	0.05 – 0.5	0.2

* Significantly different means ($p < 0.05$). Range in parenthesis

** Nigerian Water Quality Standard for Inland Surface Water. Federal Environmental Protection Agency (FEPA), 2003.

***Nigerian Standard for Drinking Water Quality. Standards Organisation of Nigeria (SON), 2007

The flow velocity of water in station 1 was generally low, though the flow velocity increased generally during the rainy season in all the stations. The highest flow velocity was recorded in station 2; this could be as a result of Benin City wastewater entering the river at that point. The pH recorded in this study indicates that the water was moderately acidic to moderately alkaline. The range observed in this study is close to those recorded elsewhere in most Nigerian inland water bodies (Onwudinjo, 1990; Ogbeibu, 1991; Odum, 1992). There was no clear predictable seasonal pattern in the pH values recorded in this study, though in some stations a number of high pH values (>7) were recorded during the rainy season. The highest total alkalinity value was recorded in January 2008 at station 2, the entry point of the Benin City wastewater. Akin-Oriola (2003) reported values as high as 346.8 and 175.8 mgl^{-1} respectively in Ogunpa and Ona Rivers in Ibadan while Radojevics and Bashkin (1999) reported that untreated domestic wastewaters are normally alkaline with alkalinities between 50 and 2000mgl^{-1}. This can also explain at least partly the high pH values recoded in some stations during the rainy seasons.

Conductivity showed that the water is fresh in all the station but an indication of negligible impact of human activities in the area. The study revealed that the monthly values were generally lower in months of January to March than in the months of April to August 2008 in all the stations except in station 1 in January 2008, which had the highest value that could not be explained.

Total dissolved solids in this study revealed a similar trend with conductivity. Egborge (1994) observed higher values in rainy season months than in the dry season - a phenomenon common in most Nigerian inland waters. However, low dissolved oxygen was recorded during March at station 2, could be attributed to the extent of flora composition, organic pollution and population density of fauna. Concentrations below 5mgl^{-1} may adversely affect the functioning and survival of biological communities and below 2mgl^{-1} may lead to the death of most fishes (Chapman, 1996). Dissolved oxygen can also be expressed in terms of percentage saturation. The highest value of oxygen saturation (86.5%) was record in August 2008 while the lowest value (58.1%) was recorded in January 2008, both in station 1. The lowest values of BOD_5 recorded throughout the sampling period was 1.7mgl^{-1} at stations 1 and 4, both in January 2008 while the highest value recorded was 4.8mgl^{-1} in January 2008 at station 2 and in February 2008 at station 3. This could be attributed to entrance of high organic materials from the Benin City wastewater and prison farms, respectively.

The chloride values showed that the river was completely freshwater type in all the study stations. The values were relatively similar to the values of Osse River, Owo River and Ologe lagoon (Omoigberale and Ogbeibu, 2007; Yusuf and Osibanjo, 2007). The nitrate – nitrogen concentration obtained was relatively low (0.001 to 0.3mgl^{-1}), and similar to those reported for some Nigerian inland waters (Omoigberale and Ogbeibu 2007). The irregular higher values obtained during the study especially in station 2 can be attributed to the increased nutrients coming in through the Benin City wastewater drain. These high values of nitrate observed in station 2 corresponded with the findings of Obhahie *et al.* (2007) whose station 4 is station 2 in this study. The other potential factor is the disposal of human wastes in the immediate watershed, which is washed into the river in small volumes via run-offs.

The reason for the decreased phosphate value, compared to other aquatic systems, may involve heterotrophic uptake by micro-organisms, sediment adsorption and removal by the currents. Omoigberale and Ogbeibu (2007) reported higher values of 0.28mgl^{-1} to 3.52mgl^{-1} for Osse River. In contrast to what is obtainable in most Nigerian inland waters, peak value was observed

in April 2008 as against rainy season months when allochthonous phosphorus containing materials are introduced by surface run-offs. An important source of phosphate in Ogba River is likely to be from soaps and detergents used in washing of cars and motorcycles, bathing and other laundry activities, which commonly take place in the river as well from the Benin City wastewater. High sulphate values were observed in station 2 throughout the period of study. Obhahie *et al.* (2007) recorded lower monthly mean values of between 0.06 and 0.13mgl^{-1} which was similar to that recorded in station 2.

Sodium values were low compared to those of some freshwater bodies in Nigeria (Ogbeibu and Edutie, 2002; Omoigberale and Ogbeibu, 2007 and Ebadin, 2006). Many surface waters, including those receiving waste waters, have concentrations well below 50mgl^{-1}. There was no marked seasonal pattern of lower values in dry season and higher values in rainy season as observed by Egborge (1978), but high values were generally recorded for station 2. The level of potassium recorded in this study ranged from 0.18mgl^{-1} to 0.91mgl^{-1} with no clear pattern of seasonality. The highest value was recorded in August 2008 in station 2. Some recent studies reported higher values (Ogbeibu and Edutie, 2002; Ebadin, 2006; Omoigberale and Ogbeibu, 2007). Calcium values are relatively high compared to 1.11mgl^{-1} to 9.62mgl^{-1} for Osse River (Omoigberale and Ogbeibu, 2007) but higher values have also been recorded in some Nigerian waters. Ogbeibu and Edutie (2002) recorded a range of 4.80mgl^{-1} to 25.0mgl^{-1} in Ikpoba River and Ebadin (2006) reported values of 0.40mgl^{-1} to 19.24mgl^{-1} for Utor River. The level of magnesium recorded in this study are comparable to some Nigerian water bodies (Okogwu and Ugwumba, 2006; Omoigberale and Ogbeibu, 2007). No seasonality was observed. The results of the present study show that the major cations in Ogba River are in the order of Ca > Mg > Na > K. This order is consistent with the common trend observed in Nigerian inland freshwaters in which calcium and magnesium are the most important cations (Imevbore, 1970; Egborge, 1971).

The results showed that water had low concentrations of heavy metals. The concentrations of the heavy metals in the Ogba River were below the Nigerian standard for drinking water quality.

In conclusion, the physico-chemical parameters and some heavy metal content of Ogba River as observed from this study are within the acceptable limits of Federal Environmental Protection Agency (FEPA) guidelines and Nigerian standard for drinking water quality. The impact of the Benin City wastewater was observed at the point of entry (station 2) in relation to depth, velocity, Biochemical Oxygen Demand (BOD), sulphate, phosphate, nitrate and sodium which were statistically different from the other stations but are still within the limits.

References

Ajayi O and Osibanjo O. 1981. Pollution on Nigeria Rivers, II: Water quality of some Nigerian Rivers. *Environ Pollution Series,* **2***:* 87-95.

American Public Health Association (APHA). 1998. **Standard Methods for the Examination of Water and Wastewater**. American Public Health Association. New York, USA. 1268 pp.

Asonye CC, Okolie NP, Okenwa EE and Iwuanyanwu UG. 2007. Some physico-chemical characteristics and heavy metal profiles of Nigerian rivers, streams and waterways. *African J of Biotechnol.*, **6(5)**: 617-624.

Awachie JBE. 1980. Running Water Ecology in Africa. In: Lock MA and William DD (Eds), **Perspective in Running Water Ecology**. Plenum Press. New York & London. 339 – 366.

Bagenal TB. 1978. Fecundity in Eggs and Early Life History (Part 1) In: Bagenal TB (Ed), **Methods for Assessment of Fish Production in Freshwaters**.. 3rd edition, 166 - 178.

Chapman D. 1996. **Water Quality Assessment**: A guide to the use of biota, sediments and water in environmental monitoring. 2nd Edition. Taylor and Francis, London and New York, 626 pp.

Davies OA and Otene BB. 2009. Zooplankton Community of Minichinda Stream, Port Harcourt, Rivers State, Nigeria. *European J of Scientific Res.*, **26(4)**: 490 – 498.

Ebadin FH. 2006. Physico-chemical Characteristics and Crustacean Zooplankton of Utor River, Edo State, Southern Nigeria (M.Sc. Thesis). University of Benin, Benin City, Nigeria. 143pp.

Egborge ABM. 1971. The chemical hydrology of the River Oshun, Western State, Nigeria. *Freshwater Biol.*, **1**: 257 – 271.

Egborge ABM. 1978. The hydrology and plankton of Lake Asejire, Nigeria (Ph.D. Thesis). University of Ibadan, Ibadan, Nigeria. 230pp.

Egborge ABM. 1994. Salinity and the distribution of rotifers in the Lagos Harbour Badagry Creek system, Nigeria. *Hydrobiologia*, **272**: 95-104.

Federal Environmental Protection Agency (FEPA) 2003. **Guidelines and Standards for Environmental Pollution Control in Nigeria**. 238 pp.

Herschy RW. 1999**. Hydrometry: Principles and Practices**. 2nd Edition. John Wiley and Sons, Chichester. 200p.

Imevbore AMA. 1970. The chemistry of the River Niger in the Kainji reservoir area. *Archive Fur Hydrobiologie*, **67(3):**412 - 431.

Imoobe TOT and Oboh IP. 2003. Physical and chemical hydrology of Jamieson River, Niger Delta, Nigeria. *Benin Sci. Dig*., **1**: 105 – 119.

Iscen CF, Emiroglu O., Ilhan S, Arslan N, Yilmaz V, Ahiska, S. 2008. Application of multivariate statistical techniques in the assessment of surface water quality in Uluabat Lake, Turkey. *Environ Monitoring and Assessment*, **144 (1-3):** 269-276.

Mligo C. 2007. Environmental Flow Assessment (EFA), Wami River Sub-Basin, Tanzania: Vegetation Survey of the Ecosystem along the Wami River. 2007. Wami-Ruvu Basin Water Office. 29p.

Mustapha MK and Omotosho JS. 2005. An assessment of the physico – chemical properties of Moro Lake. *AJAZEB* , **7**: 73 – 77.

Obhahie AI, Ugwaka KA, Ugwu LL and Adesiyan FA. 2007. Effects of Industrial Effluents and Municipal Wastes on Water Conductivity and Total Dissolved Solids, Sulphate and Phosphate Ions Concentration of Ogba River, Benin City, Nigeria. *J of Fisheries International*, **2 (4):** 277 – 283.

Odum O. 1992. The Ecology and biology of the fishes of Ethiope River, Nigeria (Ph.D Thesis). University of Benin, Benin City, Nigeria. 222pp.

Ogbeibu AE. 1991. Hydrological characteristics of water bodies in the Okomu Forest Reserve (Sanctuary), Benin City, Nigeria (Ph.D. Thesis). University of Benin, Benin City, Nigeria. 257pp.

Ogbeibu EA and Edutie LO. 2002. Effects of brewery effluents on water quality and rotifers of the Ikpoba River, Southern Nigeria. *African J of Enviro, Pollution and Health*, **1(1):** 1 – 12.

Okogwu OI and Ugwumba OA. 2006. The zooplankton and environmental characteristics of Ologe Lagoon, South West Nigeria. *The Zoologist*, **1 (4):**86 – 92.

Omoigberale MO and Ogbeibu AE. 2007. Assessing the Environmental Impacts of Oil Exploration and Production on the Water Quality of Osse River, Southern Nigeria. *Global J of Environ Sciences*, **6(1):**1 – 13.

Onwudinjo CC. 1990. The hydrology and plankton of Benin River, Nigeria (Ph.D. Thesis). University of Benin, Benin City, Nigeria. 355pp.

Prat N and Munne A. 2000. Water use and quality and stream flow in a Mediterranean stream. *Water Res.*, **34 (15):** 3876-3881.

Radojevic M and Bashkin VN. 1999. **Practical Environmental Analysis**. Royal Society of Chemistry, Cambridge, UK. 466pp.

Sood A, Singh KD, Pandey P, Sharma S. 2008. Assessment of bacterial indicators and physicochemical parameters to investigate pollution status of Gangetic river system of Uttarakhand (India). *Ecological Indicators*, **8**: 709-717.

Standards Organisation of Nigeria 2007. Nigerian standard for drinking water quality. Nigerian Industrial Standard (NIS 554). Standards Organisation of Nigeria (SON), Abuja, Nigeria. pp. 14 – 17.

Yusuf KA and Osibanjo O. 2007. Nutrients in a Freshwater lagoon, Lagos, Nigeria. *Pakistan J of Scientific and Industrial Res.*, **50 (4):** 232 – 239.

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Lysozyme Turnover during the Development of Chicken Oocyte

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Abstract

Lysozyme, the major oocyte enzyme was studied using the lysoplate assay, the turbidimetric assay, SDS-PAGE assay and native gel electrophoresis coupled with activity gel overlay system. Using the lysoplate assay, lysozyme activity was shown to exist in all developmental stages of oocytes although to various degrees. Both eggwhite and yolk, had lysozyme activity. Using the less sensitive quantitative turbidimetric lytic assay, lysozyme activity started to appear in the yolk fractions collected from the caudal region of the infundibulum. For the egg white, lysozyme activity started to appear in samples collected from the anterior portion of the magnum. It increased sharply when the ovum was found in the posterior portion of the magnum. Using the SDS-PAGE, a lysozyme band was not detectable in all the follicular stages of oogenesis. A very faint band of lysozyme was detected in all the yolk fractions collected from the different segments of the oviduct. For the egg white lysozyme started to appear in the fractions collected from the anterior portion of the magnum; this continued in all fractions until egg maturation. In native gel electrophoresis, coupled with the activity gel overlay, a distinct lysozyme activity band appeared in all fractions including egg yolk and egg white collected during the process of oogenesis .To our knowledge, this is the first report of the existence of lysozyme in both egg white and yolk, in all the developmental stages of the oocyte.

keywords: Lysozyme, egg white, egg yolk, oogenesis, developmental regulation.

1. Introduction

Hen oogenesis provides an ideal model system for studying protein turnover for obvious reasons: chicken ova are visible and easy to obtain and manipulate during essentially all stages of their development. Their size is rather convenient to fractionate and determine the various molecular components at every stage *in vitro*.

Lysozyme is one of the most extensively studied enzymes (Jolles and Jolles, 1984). Its ubiquitous distribution, ease of isolation, high stability and relatively small size made it an ideal model system in a diverse number of investigations (Prager and Jolles, 1996).

In pharmaceutical investigations, hen egg white lysozyme was found to protect the body against bacterial, viral and inflammatory diseases (Sugahara *et al*., 2000). It has been used in aerosols for the treatment of bronchopulmonary disease, as a prophylactic treatment for dental caries, for nasal tissue protection and was incorporated in various therapeutic creams for the protection and topical reparation of certain dystrophic and inflammatory lesions of the skin and soft tissue (Lacono *et al*., 1980). Oral administration of lysozyme has been shown to have immunostimulatory effect and an antihistaminic effect (Namba *et al*., 1981). Most recently, lysozyme has been found to have an anti HIV effect (Lee-Huang *et al*., 1999); this effect was attributed to a nonapeptide isolated from a digest of lysozyme (Lee-Huang *et al*., 2005).

Furthermore, lysozyme has been used as a marker in the course of several diseases. Serum lysozyme levels have been determined and used to assist in the diagnosis of several diseases including different types of leukemias (Osserman, 1973) as well as sarcoidosis (Canfield *et al*., 1974). Urinary lysozyme measurement is useful in evaluating patients with renal diseases, especially tubular dysfunctions (Hayslett *et al*., 1968). Lysozyme level determination has also been performed in several secretions including tears and cerebrospinal fluid (Sen and Sarin, 1982). The degeneration of tear glands in keratoconjunctivits sicca reduces the tear fluid production as well as the tear lysozyme concentration (Sen and Sarin, 1982). In the cereribrospinal fluid, the concentration of lysozyme is used as a sensitive index of inflammatory and neoplastic disease of the central nervous system (Harrison *et al*., 1973). Point mutations in human lysozyme gene were found to cause hereditary systemic amyliodosis, a disease in which amyloid deposition of the lysozyme variant in the viscera occurs (Pepys *et al*., 1993).

Previously, we have investigated lysozyme turnover during the process of embryo development (Eshbailat *et al*., 2004). However, to our knowledge,

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lysozyme activity has not been described in the early developmental stages of chicken oocytes. Therefore the present study was conducted to investigate the turnover of lysozyme during chicken ova development.

The significance of this study stems from the fact that lysozyme in the developing Oocyte could act as a prototype innate immune system for the protection of the Oocyte from infection.

2. Materials and methods

2.1. Materials

All chemicals were purchased from BDH Chemicals Ltd. or Sigma Chemical Company unless otherwise indicated. Chicken egg white lysozyme (three times recrystalized) and *Micrococcus Luteus* (freeze-dried bacteria) were from Sigma Chemical Company.

Molecular weight standards were obtained from Pharmacia Fine Chemicals. Poly-L-lysine Hydrochloride was also from Sigma Chemical Company. Single-comb white leghorn hens (*Gallus Domesticus*) 38 to 50 weeks of age, laying regular sequences of four or more eggs were brought from the university research farm, college of agriculture, University of Jordan.

2.2. Methods

2.2.1Rearing of experimental animals

A total of 25 hens were used in this study. Hens were individually housed in laying batteries with free access to feed and water under a controlled photoperiod of 17 hours light, 7 hours dark (lights on at midnight). Approximate time of oviposition was monitored daily and visually at 30 minute intervals so that it became possible to determine the prospective ovulation time. Each hen was sacrificed by cervical dislocation to collect eggs of both preovulatory and postovulatory stages of the hen ovulatory cycle.

2.2.2Preparation of oocyte extracts

Extracts from ovarian follicles at different stages of the follicular hierarchy of hen ovulatory cycle were prepared. One hen was dissected for that purpose at 20 minutes after oviposition. After dissection, the ovary was removed and immediately immersed in a beaker containing 0.9% physiological saline solution cooled on ice. Preovulatory follicles (9-32mm diameter) and prehierarchal follicles (2-8.9mm diameter) were removed from the ovary and their diameter was measured under the dissecting microscope by using a caliper. Follicles from 2- 10 mm diameter were sorted into 2 - 2.9 mm (10 follicles), 3 - 3.9mm (9 follicles), 4 - 4.9mm (6 follicles), 5 - 5.9mm (4 follicles), 6 - 6.9mm (2 follicles), 8 - 8.9mm (1 follicle) and 9 - 9.9mm (1 follicle) diameter groups. The follicles in each group were immersed according to their sizes in 0.006M PBS, pooled as one follicle pool and homogenized at low speed using the sorvall omni-mixer. The homogenate was then centrifuged twice at 6000 x g for 20 minutes at 4 °C to get a clear supernatant which was aliquoted and stored at -20°C to be used in electrophoresis and enzyme assays. Follicles above that diameter range; including the largest preovulatory follicle (32mm diameter) were cut down their stigma with a scalpel blade and the yolk was extruded from the membrane and transferred into falcon tube. Each yolk sample was then diluted with two volumes of 0.006M PBS and homogenized at low speed on ice. The homogenate was centrifuged twice at 6000 x g for 20 minutes at 4°C. The clear supernatant was aliquoted and finally stored at -20°C.

The oviducts and the oviductal eggs which represent the sequential stages of the postovulatory cycle were removed from adult hens several hours after prospective ovulation time. The character of the egg varies according to the time of collection from hens after prospective ovulation. At these stages of development, it was possible to separate egg yolk from egg white except for the egg collected from the posterior (caudal) portion of the infundibulum.

Each time, the eggs were collected on ice and each egg yolk was manually separated from the egg white and carefully rolled with a paper towel to remove all albumins from the vitelline membrane.

The membrane was then cut with a scalpel blade and the content of the yolk collected, diluted with two volumes of 0.066M PBS, 0.3M NaCl pH 6.2 and gently homogenized at low speed on ice. The homogenate was then centrifuged at 6000 x g for 30 minutes at 4°C. The supernatant was stored at -20 °C and recentrifuged next day to get a clear supernatant. The clear supernatant was collected, aliquoted and kept at -20°C. Egg white samples were collected after their separation from egg yolk, diluted with two volumes of PBS and gently homogenized at low speed on ice. The homogenate of the samples that contain mucin fibers was then centrifuged at 6000x g for 20 minutes at 4°C. The supernatant was aliquoted and stored frozen at -20 °C to be used in electrophoresis and enzyme assays.

2.2.3 Lysozyme activity assays

Lysozyme activity was detected in non-denaturing gels before staining by use of the substrate *Micrococcus Leutus* incorporated into an overlaying polyacrylamide gel as previously described (Reisfeld *et al*., 1962). To optimize sensitivity and suitability for photography a 4 step procedure was used as previously described (Saleh and Ibrahimi 1995). The overlaying gel was fixed in a solution containing 1% SDS, 0.1M NaCl and 0.05M ethylenediamine tetraacetic acid (EDTA) for 20 minutes.

Lysozyme activity in solution was determined by the lysis of bacterial cells of *Micrococcus Leutus* (Dobson *et al*., 1984). The mixture contained 0.75 mg of freeze-dried bacteria in 1.5ml of 0.066M phosphate buffer pH 6.2 plus 0.5ml of 0.3M NaCl. The decrease in turbidity at 540 nm every 0.5minute for a total of 12 minutes in 1ml assay at room temperature was recorded. One unit of lysozyme activity was defined as 1% increase in transmittance per minute, and was estimated from the initial slope of the line which relates turbidity to time. The bacterial suspension and lysozyme solution were always prepared fresh with Sigma chicken lysozyme used as a standard to correct for the variations in the assay.

The lysoplate method was also adapted to measure the lysozyme activity in agar plate, which was proven to be more sensitive to low lysozyme concentrations (Eshbailat *et al*., 2004). The solution mixture contained 0.05M sodium phosphate buffer pH 6.2, 0.02M NaCl, 0.02% (w/v) *Micrococcus Leutus*, 0.02% (w/v) sodium azide and

1.5% (w/v) agar. It was heated at 100°C in a water bath for 1 hour. Then, 14ml of the suspension were poured in disposable petri dishes. After solidification of the mixture, the plates were transferred to a drying cabinet for 15min at 60°C to remove the condensed steam from the surface of the plates. Wells of 0.5 cm in diameter were made in the plates and 17 µl of lysozyme solution were applied in each well. Finally the plates were incubated for 6 hours at 37°C to allow diffusion of lysozyme and formation of clearance zones around the well. Lysozyme activity was measured with chicken egg white lysozyme as a standard.

2.2.4 Electrophoresis

Denaturing polyacrylamide gel electrophoresis with SDS was carried out based on the method of Laemmli (Laemmli, 1970) and was generally done through 12% gels at pH 8.3 as outlined before (Guilmineau *et al*., 2005). The samples were prepared for electrophoresis after precipitation with trichloroacetic acid followed by solubilization and reduction with dithiothreitol (DTT) at 70°C for 5 minutes and alkylation with iodoacetamide at 37°C for one hour. Electrophoresis was carried out at a constant current of 20 mA for 6-7 hours. The gels were then stained with Coomassie Brilliant Blue G-250 and destained with a solution of 35% methanol, 19% acetic acid in distilled water.

For non-denaturing gel electrophoresis the procedure was compiled and kindly provided by Dr. Ellen Prager from the University of California, CA. Electrophoresis was performed at 4°C in vertical polyacrylamide gel slabs 0.1cm thick, 15cm long and 13cm wide, using the pH 4.3 β-alanine acetate buffer system (Reisfeld *et al*., 1962). No stacking gel was used. The sample buffer, which made up one third of the total volume, contained 0.1 (w/v) methylgreen in 50% (v/v) glycerol and water. For electrophoresis in the cathodal direction the buffer contained in addition 5mg/ml of poly-L-lysine to minimize smearing of low lysozyme concentration samples (Jolles *et al*., 1990).

3. Results

The determination of the volumes of the yolk and diameter of follicles during different stages of follicular hierarchy as well as the volumes of egg yolk and egg white during postovulatory (oviductal) stages of chicken oogenesis have been previously reported (Salman, 2009).

3.1. Lysozyme activity and protein assays

3.1.1Lysoplate assay

The activity of lysozyme was determined during different sequential stages of chicken oogenesis by the lysoplate assay which was proven to be more sensitive than the turbidimetric assay. A wide variation in the levels of lysozyme activity was detected in the different stages. This ranged from very low, barely detectable, levels of activity in the follicular stages to a very high level of activity in the postovulatory stages. For the yolk samples which were collected from the ovarian follicles in the early stages of oogenesis, low detectable levels of lysozyme activity were noticed in the yolk samples collected from the prehierarchal follicles (2-8.9mm in diameter). The activity was maintained all over the samples collected from preovulatory follicles (9-29.1mm in diameter) until the largest preovulatory follicle (32mm in diameter) prepared for ovulation (Fig.1).

In samples of egg white collected from the oviductal stages, high levels of activity were detected in samples collected from the anterior portion of the magnum, approximately the same activity of the chicken standard lysozyme. A significant increase in the activity was noticed in the egg white samples from the sequential stages of the oviduct compared to the activity obtained from the chicken standard lysozyme, 1.0mg/ml. (Fig. 2). The yolk samples from these stages had a consistently higher activity compared to that of the yolk from previous follicular stages, and lower activity compared to the egg white from oviductal stages (Fig. 2).

3.1.2 Turbidemetric assay:

The activity of lysozyme in egg yolk and egg white of various sequential stages of chicken oogenesis was also determined by the turbidimetric lytic assay. Lysozyme activity per ml and lysozyme activity per fraction as well as the total activity per oocyte at different stages was calculated (Table 1). During the hierarchal and preovulatory stages of oogenesis, it was not possible to detect the activity of lysozyme in the yolk samples collected from all ovarian follicles regardless of their diameter.

During the postovulatory stages, lysozyme activity was detected in both egg yolk and egg white. For the egg yolk,the activity started to appear in the yolk fraction collected from the caudal region of the infundibulum; were the highest level of activity was recorded. As the egg moved down the anterior portion of the magnum, the activity started to decrease gradually were the first concentric coats and layers of albumin are added. In the posterior portion of the magnum, 2.5 hours postovulation, when almost all the layers of albumin are deposited, there was a decline in the activity of lysozyme which continued until the ovum has entered the shell gland were the activity has reached its minimum values. For the egg white, the activity appeared in the sample from the anterior portion of the magnum. It increased sharply when the ovum was found in the posterior portion of the magnum, and then the activity started to decrease as the ovum entered the magnum isthmus junction, and no change in the activity was detected in the egg white from the isthmus. Throughout the presence of the ovum in the shell gland, the activity of lysozyme decreased with time.

3.1.3SDS-PAGE

Using SDS- PAGE, a lysozyme band (14.4 kDa) was not detectable in all the follicular stages of chicken oogenesis (results not shown). It should be noticed however that lysozyme activity was detected in those stages of follicular development in the very sensitive lysozyme activity assays. This suggests the presence of lysozyme in those stages but in a very low concentration. A very faint lysozyme band was detected in all the yolk fractions collected from the different segments of the oviduct. This could be due to low lysozyme concentrations in these fractions and the low sensitivity of the stain used. For the egg white a band having a molecular weight of 14.4 kDa started to appear in the fractions which were collected from the anterior portion of the magnum (Fig. 3).

The band remained present in the subsequent fractions collected from other portions of the oviduct. This band comigrated with the standard chicken egg white lysozyme; it was deduced that it is a c-type lysozyme.

3.1.4 Non-denaturing gel electrophoresis

Egg yolk and egg white were assayed for lysozyme activity during different stages of oogenesis by the gel overlay method at pH 4.3. All yolk samples obtained from the different ovarian follicles including the small white prehierarchal follicles (2-5mm in diameter), the small yellow prehieracchal follicles (6-8mm in diameter), and the preovulatory follicles (9-32mm in diameter) showed clear cut lytic lysozyme activity bands on the overlay activity profiles (Fig.4). The activity bands were highly reduced in the ovarian preovulatory follicles (24.8 to 32mm in diameter) (Fig. 4). This could be due to great yolk deposition in these stages of ovarian follicle development. All of the bands in these stages did not correspond to protein bands in the leftover native gel (results not shown). This suggests the presence of lysozyme in the earliest stages of chicken oogenesis but at very low concentrations.

The egg yolk samples obtained from the different segments of the oviduct showed a clear lytic lysozyme activity band (Fig. 5). This indicates the presence of lysozyme in the mature yolk throughout its transport in the chicken oviduct. All of these bands corresponded to very faint protein bands in the leftover protein gel suggesting an increase in lysozyme concentration compared to very low concentrations of lysozyme in the earliest follicular stages of oogenesis. Lysozyme activity bands also appeared in the egg white samples collected from the different sequential segments of the chicken oviduct (Fig.5).

4. Discussion

4.1. The activity levels of lysozyme in different stages of the follicular hierarchy of chicken oogenesis :

The activity of lysozyme was determined for egg yolk fractions collected from the various ovarian follicles during sequential stages of the follicular hierarchy by the lysoplate assay, turbidimetric lytic assay and non-denaturing gel electrophoresis using *Micrococcus Leteus* as a substrate.

Using the lysoplate assay, the activity of lysozyme was detected in all the yolk fractions obtained from chicken ovarian follicles regardless of their diameter as indicated by the different lysis zones. These follicular stages included the small white prehierarchal follicles (2-5.9mm in diameter), small yellow prehierarchal follicles (6-8.9mm in diameter) and the preovulatory follicles (9-32mm in diameter). Based on the diameter of the lysis zones, yolk fractions obtained from these ovarian follicles exhibited a low lysozyme activity compared to the activity observed for standard chicken lysozyme (1.0mg/ml) used in the assay, reflecting a very low concentration of lysozyme in these samples.

In order to further assess the levels of lysozyme activity in these follicular stages, the turbidimetric lytic assay and overlay assay on non-denaturing gels were applied.

Based on the results of turbidimetric assay, these stages of development did not show any levels of activity. On the other hand, the same stages showed very clear activity bands in the overlay assay. The appearance of activity by this method and the absence of activity by the turbidimetric assay are due to the sensitivity of the overlay assay to very low concentrations of lysozyme. No corresponding lysozyme bands were detected in SDS PAGE. This can be interpreted by the very low concentration of the enzyme in these fractions and the very low sensitivity of the stain used in these gels (results not shown).

The ovarian follicle of the laying hen is a highly vascularised part of the hen ovary; which serves a nutritive function during the process of oogenesis. Blood vessels enter the follicle through the pedicle or the stalk and extend into all portions if the follicle, except for a narrow band, the stigma, opposite the pedicle. Later, when the most rapid deposition of yolk is taking place, the follicle passes the materials necessary for yolk formation from the blood to the oocyte (Romanoff and Romanoff, 1949). Furthermore the follicle may possibly elaborate some of the materials and nutritive substances across the intercellular bridges to the cytoplasm of the oocyte (Sturkie, 1976). So it can be deduced that lysozyme may probably be one of the intracellular enzymes in the ovarian follicles, and is produced by the granulose cells with the materials needed for yolk formation. It also may pass via blood circulation inside the follicle. Lysozyme distribution in rabbit and human blood among different blood elements and serum has also been studied (Flanagan and Lionetti, 1955). Appreciable values of lysozyme activity were found in both leukocytes, especially the granulocytes, and blood plasma. This indicates that lysozyme is an integral part of the cell and that the liberation of the activity into the plasma is a good index of the white blood cell integrity. In a previous investigation (Margaret *et al*., 1950), lysozyme distribution in chicken blood serum and egg yolk has been studied by a quantitative immunological technique. In this method, rabbit antisera were produced specifically against lysozyme and no lysozyme could be detected in these systems. Moreover, lysozyme presence was detected in chicken blood serum using the turbidimetric assay (Bazlamit, 2009). These inconsistencies could be due to differences in the methods applied; immunological reactions need certain concentrations of the protein to give positive results compared to very specific technique used for lysozyme activity. Our results demonstrate, for the first time, that chicken lysozyme is synthesized in the ovarian follicles during early stages of oogenesis but at considerably lower levels compared to later stages of this process.

4.2. The activity levels of lysozyme in different postovulatory stages of chicken oogenesis:

For the egg white and egg yolk from the different postovulatory stages, the fractions collected from different portions of the chick oviduct were assayed for lysozyme activity by the lysoplate assay, the overlay assay and the turbidemetric assay as described in methods. For the egg yolk, the activity was detected in all the fractions examined. It was higher than the activity observed in the counterpart fractions collected from the ovarian follicles as indicated by the lysis zones. For the egg white high detectable levels of lysozyme were also observed in all fractions examined.

Using the overlay assay on native gels, a definite lysozyme activity band was detected in all egg white and egg yolk fractions examined. To further determine the activity of lysozyme in these fractions, the turbidimetric assay was also performed as described in methods.

For the egg white, the highest level of activity was noticed in the egg white sample collected from the posterior portion of the magnum reaching as high as 1485 units per ml as would be expected, since the magnum of the laying hen is the site were the lysozyme is secreted and concentrated (Gilbert, 1971). The egg white from the magnum isthmus junction and the isthmus showed a lower level of activity (806 units per ml). A notable decrease in the activity was observed in the shell gland were water is added in a considerably rapid rate during the process of plumbing (King and McLelland, 1975). This aids in the dilution of egg white and eventually the decrease in the total number of lysozyme molecules per volume of egg white. From the result obtained, the activity levels in the egg yolk were decreasing as the ovum moved down the oviduct. This gradual decrease in activity can be interpreted as being the result of transfer of some egg white proteins to the egg yolk during the passage of the ovum along the oviduct (Mann *et al*., 2008). On the other hand, lowest levels of the enzyme activity were noticed in the shell gland, this is also a result of yolk dilution and passage of liquid to the yolk during the plumbing process.

Using the SDS-PAGE, a clear band was observed in the egg white fractions collected from the anterior part of the magnum up to the shell gland with very faint bands in the yolk fractions. On the other hand remarkable activity bands for lysozyme were observed in all the yolk fractions using the very sensitive lysoplate and overlay assays. The presence of lysozyme in egg yolk was recently studied by proteomic analysis. This analysis has identified 119 different proteins in the egg yolk. Lysozyme c was one of the major proteins identified (Mann *et al*., 2008).

Table 1. Lysozyme activity during different postovulatory (oviductal) stages of chicken oogenesis by the turbidimetric lytic assay

Site of sample collection from the oviduct	Lysozyme activity (units/ml)		Lysozyme activity/fraction		Total lysozyme activity/egg
	Egg white (units/ml)	Egg yolk (units/ml)	Egg white	Egg yolk	
Infundibulum	ND*	105	ND*	1575	1575
Anterior portion of the magnum	696	70	1392	1085	2477
Posterior portion of the magnum	1485	60	21622	887	22509
Magnum-Isthmus junction	806	36	12902	582	13485
Isthmus	806	26	16934	441	17375
Shell gland(1)**	672	17	18816	336	19152
Shell gland(2)***	546	17	19110	336	19446

* Not determined; because the amount of egg white collected was too small to use in the biochemical assays.

** Egg white and egg yolk fractions collected from the shell gland 10 hours post-ovulation.

***Egg white and egg yolk fractions collected from the shell gland 18 hours post-ovulation.

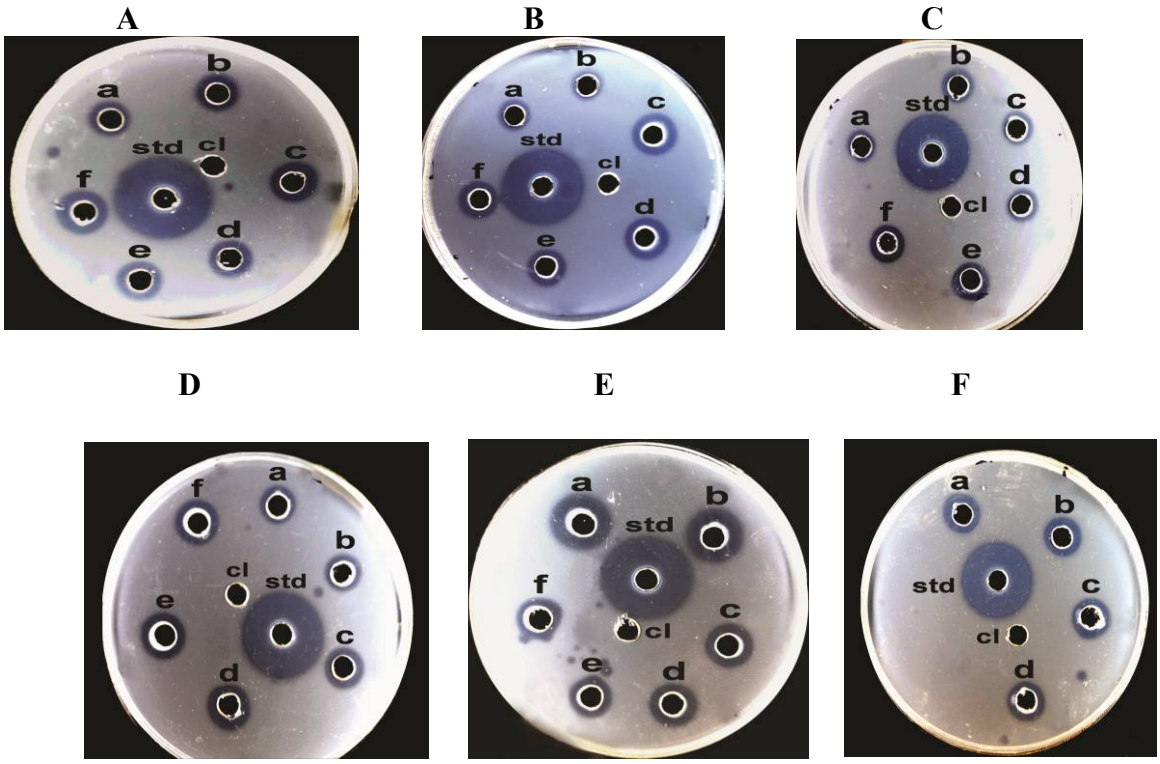

Figure 1. Lysoplate results of lysozyme activity in yolk samples during successive preovulatory (follicular) stages of Oogenesis

Lysoplate assay was done as described in methods. 17 follicles were assayed in duplicates ranging in size from 2-32mm in diameter as shown in A to F successively. a and b, c and d, e and f are duplicates in each case. Std is a positive control and cl is a negative control.

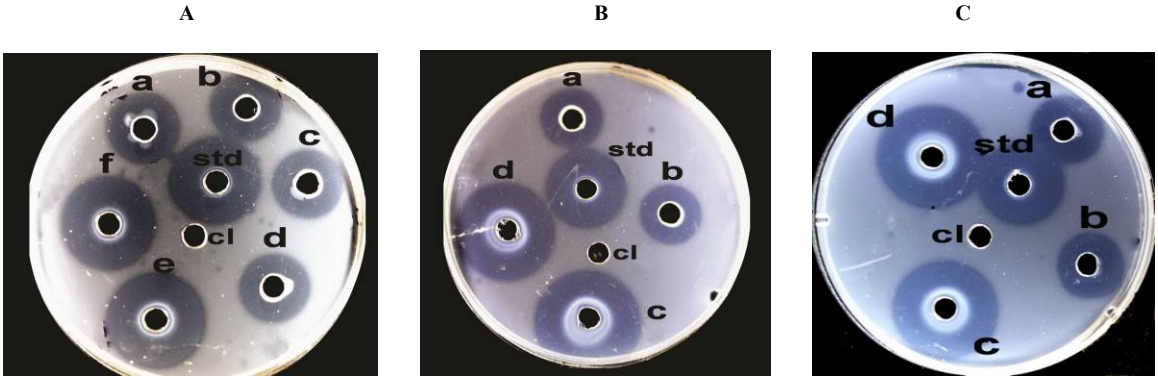

Figure 2. Lysoplate results of lysozyme activity during successive postovulatory (oviductal) stages of oogenesis

The results include both egg white and egg yolk. Each plate included a positive (std) and a negative (cl) control. The stages are:

A: a and b contained egg yolk in the ova from the posterior region of the infundibulum; c and d contained egg yolk in the ova from the anterior part of the magnum; e and f contained the egg white in the ova from the anterior part of the magnum.

B: a and b contained the egg yolk in the ova from the posterior part of the magnum; c and d contained the egg white in the ova from the posterior part of the magnum.

C: a and b contained the egg yolk in the ova from the magnum-isthmus junction; c and d contained the egg white in the ova from the magnum-isthmus junction.

***Figure 2 continues next page…….**

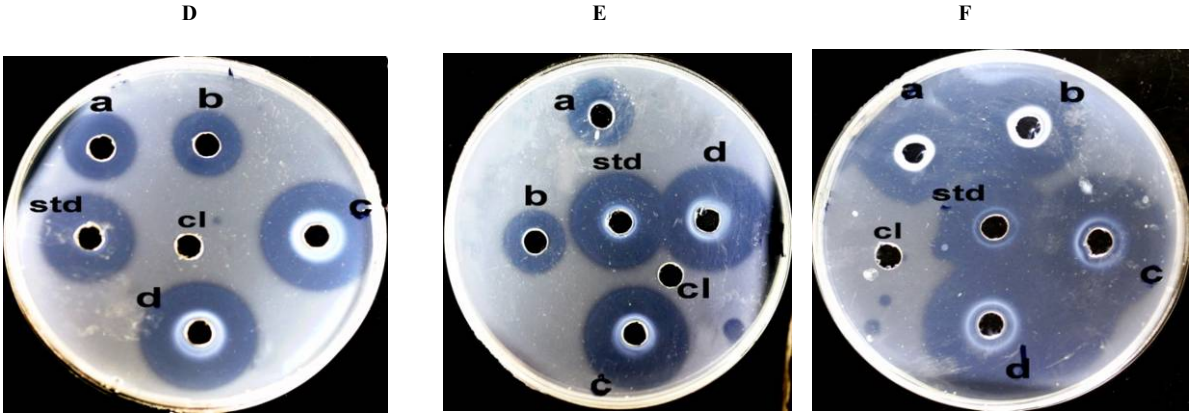

Figure 2. Lysoplate results of lysozyme activity during successive postovulatory (oviductal) stages of oogenesis

The results include both egg white and egg yolk. Each plate included a positive (std) and a negative (cl) control. The stages are:

D: a and b contained the egg yolk in the ova from the isthmus; c and d contained the egg white in the ova from the isthmus.

E: a and b contained the egg yolk in the ova from the shell gland 10 hrs post ovulation; c and d contained the egg white in the ova from the shell gland 10 hrs post ovulation.

F: a and b contained the egg yolk in the ova from the shell gland 18 hrs post ovulation; c and d contained the egg white in the ova from the shell gland 18 hrs post ovulation.

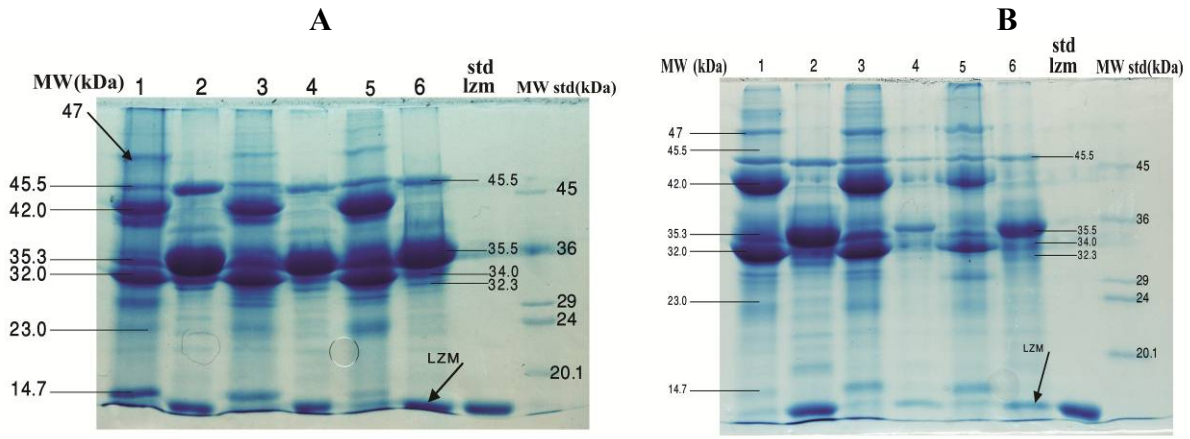

Figure 3. SDS-PAGE results of lysozyme in the egg yolk and egg white of successive postovulatory (oviductal) stages of oogenesis

A: lanes 1 and 2 contained egg yolk and egg white in the ova from the anterior part of the magnum, respectively.Lanes 3 and 4 contained egg yolk and egg white in the ova from the posterior part of the magnum, respectively. Lanes 5 and 6 contained egg yolk and egg white in the ova from the magnum-isthmus junction, respectively.

B: lanes 1 and 2 contained egg yolk and egg white in the ova from isthmus respectively.

Lanes 3 and 4 contained egg yolk and egg white in the ova from the shell gland 10 hrs post ovulation. Lanes 5 and 6 contained egg yolk and egg white in the ova from the shell gland 18 hrs post ovulation. STD is standard chicken lysozyme and MW is molecular weight standard

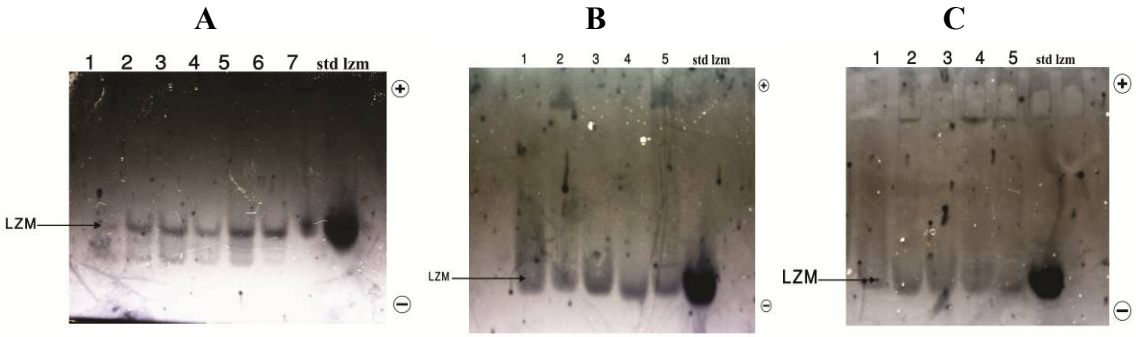

Figure 4. Non-denaturing gel electrophoresis coupled with the overlay lysozyme activity gel for successive preovulatory (follicular) stages of oogenesis. 17 successive follicular stages ranging in size from 2-32 mm in diameter were assayed as shown in A, B and C. A distinct lysozyme activity bands is shown in all stages.

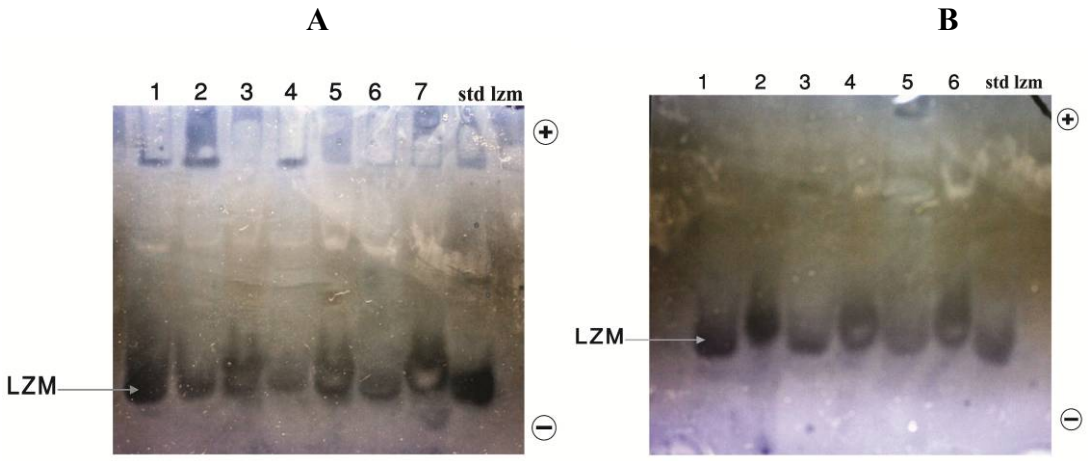

Figure 5. Non-denaturing gel electrophoresis coupled with the overlay lysozyme activity gel for the successive postovulatory (oviductal) stages of oogenesis ranging from posterior part of the infundibulum to the shell gland. Even numbers represent the egg yolk extract and odd numbers represent the egg white extract.

A: lane 1 contained egg yolk from posterior region of the infundibulum. Anterior portion of the magnum: Lane 2 egg yolk and lane 3 egg white. Posterior portion of the magnum: lane 4 egg yolk and lane 5 egg white, magnum-isthmus junction: lane 6 egg yolk and lane 7 egg white.

B: isthmus: lane 1 egg yolk and lane 2 egg white, shell gland 10 hrs post ovulation: lane 3 egg yolk and lane 4 egg white, shell gland 18 hours post ovulation: lane 5 egg yolk, lane 6 egg white.

A distinct lysozyme activity band is seen in all stages.

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References

Bazlamit Z. 2009. In: The distribution of lyszyme in certain fluids and tissues of the chick and goose with special reference to polyymorphism.M.Sc. Thesis, University of Jordan, Jordan.

Canfield R E, Collins J C and Sobel J H. 1974. Human leukemia lysozyme. In: Osserman E F, Canfield, R E and Beychok S. (Eds), **Lysozyme**. Academic Press, New York.

Dobson D E, Prager EM and Wilson A C. 1984. Stomach lysozyme of ruminants: Distribution and catalytic properties. *J. Biol. Chem*., **259**: 11607-11616.

Eshbailat S, Haj Hand Ibrahimi I. 2004. Turnover of lysozyme during the development of chicken embryo. *Dirasat, medical and biological sciences*, **31(2):** 103-115.

Flanagan P and Lionetti F. 1955. Lysozyme distribution in blood. *Blood*, **10**: 497-501.

Gilbert A B. 1971. The ovary. In: Bell, D.J. and Freeman, B.M. (Eds), **Physiology and Biochemistry of the Domestic fowl**. Academic press, New York. Pp. 1163-1208.

Guilmineau F, Krause I and Kulndozik U. 2005. Efficient analysis of egg yolk proteins and their thermal sensitivity using sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing and nonreducing conditions. *JAgric Food Chem.*, **53**: 9329−9336.

Harrison JF, Parker RQ and Desilva K L. 1973. Lysozymuria and acute disorders of renal function. *J Clin Pathol*, **26**: 279-284.

Hayslett J P, Perllie P E and Finch S C. 1968. Urinary muramidase and renal disease: correlation with renal histology and implication for the mechanism of enzymuria. *N Engl J Me*d., **279**: 506-512.

Jollés P and Jollés J. 1984. What's new in lysozyme research? Always a model system, Today as yesterday (Review). *Mol Cell Biochem.*, 63: 165-189.

JollésJ, PragerE M, Alnemri E S, Jollés P, Ibrahimi I M and Wilson A C.1990. Amino acid sequence of stomach and non-stomach lysozyme of ruminants. *J Mol Evol.*, **30**: 370-382.

King A S and McLelland J. 1975. Female Reproductive System. In: **Outline of Avian Anatomy.** The Macmillan Publishing Company Inc., New York, Pp. 65-73.

Lacono V J, Mackay B J, Dirienzo S and Pollock J J. 1980. Selective antibacterial properties of lysozyme for oral microorganisms. *Infection and Immunity*, **29(2):** 523-532.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680-685.

Lee-Huang S, Huang P, Sun Y, Kung H, Blithe D and Chen H. 1999. Lysozoyme and RNases as an anti HIV components in β-core preparations of human chorionic gonadotropin. *PNAS*, **96**: 2678-2681.

Lee-Huang S, Maiorov V, Huang P, Angela N, Lee H, Chang Y, Kallenbach N, Huang P and Chen H. 2005. Structural and functional modeling of human lysozyme reveals a unique nonapeptide H, with anti-HIV activity. *Biochem.*, **44:** 4648-4655.

Mann K, Olsen J V, Maček B, Gnad F and Mann M 2008. Identification of new chicken egg proteins by mass spectrometry-based proteomic analysis. *World's Poultry Science J*, **64**: 209-218.

Margaret E, Marshall E and Deutsch H F. 1950. Distribution of egg white proteins in chicken blood serum and egg yolk. *The J of Biol Chem.*, **24**: 1-9.

Namba Y, Hidaka Y, Taki K and Morimoto T. 1981. Effect of oral administration of lysozyme on digested bacterial cell walls on immunostimulation in guinea pigs. *Infection Immunity*, **31(2):** 580-583.

Osserman E F. 1973. Monocytic and monomyelocytic leukemia with increased serum and urine lysozyme as a late complication in plasma cell myeloma. *British Medical J.*, **8**: 215-222

Pepys M B, Hawkins P N, Booth D R, Vigushin D M, Tennent G A, Soutar A K, Nguyen O, Blake CCF, Terry C J, Zalin A M and Hsuan J J. 1993. Human lysozyme gene mutations cause hereditary systemic amyloidosis. *Nature*, **362:** 553-557.

Prager E M and Jollés P. 1996. Animal lysozyme C and G: An overview. In: Jolles P (Ed), **Lysozyme: Model Enzymes in Biochemistry and Biology**. Birkhauser Venag Baser, Switzerland. Pp. 9-31.

Reisfeld R A, Lewis U J and Williams D E. 1962. Disc electrophoresis of Basic proteins and peptides on polyacrylamide gels. *Nature*, **195**: 281-283.

Romanoff A L and Romanoff A J. 1949. Egg formation. In: **The Avian Egg**. (Edited by John Wiley & Sons, Inc.) New York, Pp. 199-251.

Saleh A and Ibrahimi I. 1995. Electrophoretic polymorphism in rabbit tear lysozyme. *Comp Biochem Physiol.,* **112 B** (1): 21-30.

Salman H M. 2009. In: Protein turnover during chick oogenesis with special reference to lysozyme. M. Sc. Thesis, University of Jordan, Jordan.

Sen D R and Sarin G S. 1982. Immunoassay of tear lysozyme in conjunctivital diseases. *BrJ Ophthalmol.,* **66**: 732-735.

Sturkie P D. 1976. Reproduction in the female and egg formation. In: Sturkie P D. (Ed) **Avian Physiology**. Springer-Verlag, New York Heidelberg Berlin. Pp. 303-326.

Sugahara T, Murakami F, Yamada Y and Sasaki T. 2000. The mode of action of lysozyme as an immunoglobulin production stimulation factor. *Biochimica et Biophysica Acta*, **1475**: 27-34.

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Prevalence of Anemia among Jordanian Pregnant Women and the Effect of Early Pregnancy on Alkaline Phosphatase Activity

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Abstract

A cross-sectional study was conducted on 1030 pregnant women in the age of 16-40 years for the assessment of their hemoglobin status. One hundred pregnant women in their first trimester were selected from the whole sample to study the effect of their pregnancy on alkaline phosphatase activity. The overall prevalence of anemia and the mean hemoglobin (Hb) concentrations in the investigated sample were found to be 56.7% and 9.8±1.4g/dL, respectively. The highest prevalence of severe anemia was found among pregnant women of the Eastern region as well as among pregnant women of third trimester. The prevalence of anemia was found lower among pregnant women in the developed region (Amman; 41.4%) than in other less developed regions in particular Eastern, Southern and Western being 54.0%, 63.4% and 67.9%, respectively. The obtained results of the study also showed that anemia was greater among pregnant women of age 16-19 years. Anemia was of higher prevalence among multipara than among primigravida. Prevalence of anemia was greater the more advanced the gestation. The activity of alkaline phosphatase was normal in 80.0% of the investigated sample, where as 20.0% of the sample showed extremely high concentrations of alkaline phosphatase. The significant increase in alkaline phosphatase activity ($p<0.01$) accompanied the early pregnancy complications such as diabetes mellitus, preeclampsia, proteinuria and diabetes with hypertension. This suggests that alkaline phosphatase activity could be used as a monitor for status of pregnancy in its first trimester.

k**eywords:** Anemia, pregnancy, alkaline phosphatase activity, Jordan.

1. Introduction

Iron deficiency anemia is a problem of serious public health affecting more than 700 million in the world (Dawood *et al*., 1990). It is considerably more prevalent in the developing than in the industrialized world, being 59% in the developing region and 14% in the developed region with global prevalence 51% (DeMaeyer, 1989). Anemia is the second highest cause of maternal mortality in Asia (Sanghvi *et al.*, 2010).

Pregnancy constitutes a major drain on the iron reserves of women. The loss of iron in normal pregnancy, delivery and lactation shifts the balance of reproductive women to the side of negative iron balance (Passmore and Eastwood, 1986; Halper, 1987). In pregnant women, iron deficiency increases the risk for a preterm delivery and delivering a low birth-weight baby (CDC, 1998; Banhidy *et al*., 2011). Studies on iron deficiency anemia among pregnant women in some countries revealed prevalence of 78% in Liberia (Jackson and Lantham, 1982), 73.9% in Guyana (Johnson *et al.*, 1982), 61% in Jamaica (Simmons *et al*., 1982), 50.0% in Bahrain (Aldallal, 1984), 39.7% in Kuwait (Dawood *et al.,* 1990), 44.9% versus 31.1% in the Gaza Strip and the West Bank, respectively (Khader *et al.*, 2009), 32.6% in China (Zhang *et al.*, 2009) and 89.8% in Monchegorsk, Russia (Chumac and Grjibovski, 2010).

Iron supplementation during pregnancy raises and maintains the serum ferritin above 10 µg/L, thus, resulting in a substantial reduction in proportion of women with hemoglobin level below 10 or 10.5 g/dL in late pregnancy (Mahomed, 1997; Sloan *et al*., 2002). The bone and placental alkaline phosphatase have been identified as the source of the increase in alkaline phosphatase activity in normal pregnancy in its second and third trimesters, respectively (Okesina *et al.*, 1995). Fenuku and Foli (1975), reported significant decrease in the concentration of alkaline phosphatase in the second trimester and an increase in the third trimester of pregnancy. Aleem (1972) suggested that the increase in alkaline phosphatase activity may accompany threatened abortion. Very low activities of alkaline phosphatase in first trimester indicated affected fetus (Muller *et al.*, 1991). Rosenau *et al*. (1994) reported greater elevation of alkaline phosphatase in cases of vomiting during first trimester.

The main aim of this study was to assess the hemoglobin status in Jordanian pregnant women and the impact of pregnancy duration and repeated pregnancies on the prevalence of anemia among pregnant women. This study was also concerned with the effect of pregnancy in its first trimester on the activity of alkaline phosphatase.

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2. Materials and Methods

2.1. The Sample

A representative sample of 1030 pregnant women in the age of 16-40 years was randomly selected from four health regions of the country.

2.2. Technique

2.2.1. Collected data and blood samples

A questionnaire was completed for each pregnant investigated woman and the following data was collected:

- Age at pregnancy
- Duration of present pregnancy
- Total number of pregnancies Every woman investigated was requested to offer a finger prick capillary blood sample.

2.2.2. Determination of hemoglobin (Hb) concentration

Assessment of hemoglobin concentration was carried out by cyanmethemoglobin technique (Makarem, 1974).

2.2.3. Cut-off level of anemia

The World Health Organization (1972) cut-off level for diagnosis of anemia among pregnant women was used in the present study.

Anemia was categorized into three classes of severity:

- Severe: Hb. concentration < 8.0 g/dL
- Moderate: Hb. Concentration 8.0-9.0 g/dL
- Mild: Hb. concentration 9.0-10.9 g/dL

2.2.4. Determination of alkaline phosphatase activity

Blood samples were collected from 100 pregnant women in their first trimester. The serum was separated from each blood sample upon centrifugation at 2500 rpm for 10 minutes. Alkaline phosphatase activity was determined according to the procedure of American Association for clinical chemistry (Tietz, 1983). The reference range of alkaline phosphatase activity was 26.0-99.0 UIL at 30°C.

2.2.5. Statistical Analysis

All the statistical analyses were performed using the *student t test*.

3. Results

Table 1 shows the prevalence of anemia and the mean hemoglobin concentrations among Jordanian pregnant women in different regions of the country. The lowest prevalence was observed in the capital Amman (41.4%; mean Hb concentrations 11.4 ± 1.2 g/dL) and become higher in the Eastern (54.0%; mean Hb concentrations 9.5 ± 1.4 g/dL), Southern (63.4%; mean Hb concentrations 9.1 ± 1.5 g/dL), and Western (67.9%; mean Hb concentrations 9.3 ± 1.3 g/dL). The differences were found to be statistically significant (P <0.01). Eastern region showed highest prevalence of severe anemia (4.4%).

Table 1. Prevalence of Anemia among Pregnant women in different regions of Jordan

Region	Anemic (%)				Non-Anemic (%)	Hb (Mean ± SD) (g/dL)	No. of Sample
	Severe	Moderate	Mild	Total			
Amman (Capital)	0.0	6.3	35.0	41.4	58.6	11.4 ± 1.2	400
Eastern	4.4	15.2	48.0	54.0	46.0	9.4 ± 1.4	120
Southern	3.0	12.6	43.0	63.4	36.6	9.1 ± 1.5	150
Western	0.0	5.2	52.0	67.9	32.1	9.3 ± 1.3	360
Mean	1.85	9.8	45.0	56.7	43.3	9.8 ± 1.4	
Total							1030

Table 2 represents the prevalence of anemia and the mean hemoglobin concentration by duration of pregnancy. The overall prevalence of anemia was found to be 47.0% during the first trimester (mean Hb concentration 11.0 ± 1.6 g/dL), 56.1 % during the second trimester (mean Hb concentration 10.1 ± 1.3 g/dL) and 66.9% during the last trimester (mean Hb concentration 8.7 ± 1.4 g/dL), which also showed a higher prevalence of severe anemia.

A statistically significant increase (P<0.01) was found between the prevalence of anemia during the second and third trimesters versus the first trimester.

Table 2. Prevalence of Anemia among Pregnant women by duration of pregnancy

Pregnancy Duration	Anemic (%)				Non-Anemic (%)	Hb (Mean ± SD) (g/dL)	No. of Sample
	Severe	Moderate	Mild	Total			
First Trimester	0.3	7.2	39.5	47.0	53.0	11.0 ± 1.6	305
Second Trimester	2.1	9.6	44.4	56.1	43.9	10.1 ± 1.3	515
Third Trimester	3.2	12.6	51.1	66.9	33.1	8.7 ± 1.4	210
Mean	1.86	9.8	45.0	56.7	43.3	9.9 ± 1.4	
Total							1030

The prevalence of anemia among pregnant women and mean hemoglobin concentrations by the number of pregnancies are shown in table 3. A statistically different increase (P<0.01) was found between the overall prevalence of anemia in Multipara women (64.0%) compared to primigravida women (49.3%). The mean hemoglobin concentration was found to be 11.0 ± 1.4 g/dL for primigravida, but, it decreased to 8.7 ± 1.3 g/dL for multipara women. The prevalence of anemia among pregnant women by pregnancy age was demonstrated in table (4). The prevalence was greater among pregnant women of ages 16-19 (70%).

●

Table 3. Prevalence of Anemia among pregnant Women by Number of Pregnancies

Parity	Anemic (%)				Non-Anemic (%)	Hb (Mean ± SD) (g/dL)	No. of Sample
	Severe	Moderate	Mild	Total			
Primigravida	1.6	7.2	40.5	49.3	50.7	11.0 ± 1.4	385
Multipara	2.1	12.4	49.5	64.0	36.0	8.7 ± 1.3	645
Mean	1.85	9.8	45.0	56.7	43.3	9.9 ± 1.4	
Total							1030

Table 4. Prevalence of Anemia among Pregnant women by Pregnancy Age

Pregnancy age (year)	Anemic (%)				Non-Anemic (%)	Hb (Mean ± SD) (g/dL)	No. of Sample
	Severe	Moderate	Mild	Total			
16-19	2.3	12.4	55.3	70.0	30.0	9.5 ± 1.6	130
20-23	1.8	10.2	47.5	59.5	40.5	10.5 ± 1.3	230
21-27	2.6	9.3	38.5	50.4	49.6	9.1 ± 1.4	210
28-31	3.1	10.9	48.5	62.4	37.6	8.9 ± 1.2	220
32-35	1.3	11.5	35.5	48.3	51.7	10.7 ± 1.3	151
36-40	0.0	4.5	44.8	49.3	50.7	11.5 ± 1.3	89
Mean	1.85	9.8	45.0	56.7	43.3	10.0 ± 1.4	
Total							1030

Table 5 shows the effect of pregnancy in its first trimester on alkaline phosphatase activity. Eighty percent of the investigated sample (n=100) showed normal levels of alkaline phosphatase activity (78.3 ± 12.5 U/L), whereas 20.0% showed extremely high levels of alkaline phosphatase activity (146.1 ± 8.8 U/L).

Table 5. Mean Alkaline Phosphatase Activity in Different Types of Early Pregnancy Complications

No. of Sample	Complications	(%)	Alkaline Phosphatase Activity (Mean ± SD) (U/L)
7	Diabetes Mellitus	7	125.0 ± 8.5
3	Pre-eclampsia	3	172.3 ± 8.1
4	Proteinuria	4	155.5 ± 11.2
6	Diabetes Mellitus and Hypertension	6	131.7 ± 7.4
80	No complication	80	78.3 ± 12.5

4. Discussion

The lowest prevalence of anemia among Jordanian pregnant women was observed in the capital Amman (41.4%; mean Hb concentrations 11.4 ± 1.2 g/dL), while it was higher in the Eastern (54.0%; mean Hb concentrations 9.5 ± 1.4 g/dL), Southern (63.4%; mean Hb concentrations 9.1 ± 1.5 g/dL), and Western (67.9%; mean Hb concentrations 9.3 ± 1.3 g/dL). These differences were statistically significant (P <0.01). Eastern region showed the highest prevalence of severe anemia (4.4%). Despite the fact that iron supplementation is given to pregnant women as part of the health management program in Jordan, prevalence of anemia was observed. That is most often due to willfully reduced total intake of food, increased consumption of highly refined food or food fads (Halper, 1987; Dawood *et al.*, 1990; Gharaibeh *et al.*, 2005).

The overall prevalence of anemia by duration of pregnancy was found to be 47.0% during the first trimester (mean Hb concentration 11.0 ± 1.6 g/dL), 56.1 % during the second trimester (mean Hb concentration 10.1 ± 1.3 g/dL) and 66.9% during the last trimester (mean Hb concentration 8.7 ± 1.4 g/dL), which also, showed the highest prevalence of severe anemia.

This trend is consistent with the reports of previous studies (Jackson and Lantham, 1982; Johnson *et al.*, 1982; Simmons *et al*., 1982; Aldallal, 1984; Dawood *et al*., 1990; Charles *et al.*, 2010). A statistically significant increase (P<0.01) was detected between the prevalence of anemia during the second and third trimesters compared to the first trimester.

The overall prevalence of anemia by the number of pregnancies was found to be higher in Multipara women (64.0%) than among primigravida women (49.3%). The difference was statistically significant (P<0.01). This is consistent with previous results (Mirzaie *et al*., 2010; Al-Farsi *et al*., 2011; Taseer *et al.*, 2011). The overall mean hemoglobin concentration was found to be 11.0 ± 1.4 g/dL for primigravida, but it was decreased to 8.7 ± 1.3 g/dL for multipara women.

The prevalence of anemia among pregnant women by pregnancy age was greater among pregnant women of age 16-19 (70%), which is consistent with the data reported by Banerjee *et al.* (2009).

The above-stated findings of the present study showed 56.7% prevalence of anemia among Jordanian pregnant women. Iron deficiency anemia is more frequent in women of high parity. Although iron supplementation is given to pregnant women, it seemed that Jordanian pregnant women were not in a state of excellent nutrition. Therefore, their pregnancy ended with an iron deficit. If the pre-pregnancy nutrition is suboptional, anemia develops that might cause a reduction in iron stores in infant (Halper, 1987; Kilbride *et al*., 2000).

The effect of pregnancy in its first trimester on alkaline phosphatase activity showed that eighty percent of the investigated sample (n=100) had normal levels of alkaline phosphatase activity (78.3 ± 12.5 U/L), whereas 20.0% showed extremely high levels of alkaline phosphatase activity (146.1 ± 8.8 U/L) (1.5 fold increase). High increase in alkaline phosphatase activity may be taken as a complication of pregnancy such as hypertension, pre-eclampsia and eclampsia (Jamjute *et al*., 2009) and linked to preterm delivery (Bashiri *et al*., 2007; Grgic and Bogdanovic, 2009). The 20% of the pregnant women who showed high alkaline phosphatase activity complained of diabetes mellitus (7 cases), pre-eclampsia (3 cases), proteinuria (4 cases), and diabetes mellitus with hypertension (6 cases). The highest increase in alkaline phosphatase activity was among pregnant women with pre-eclampsia (172.3 ± 8.1 U/L) as shown in table 5.

Those findings clearly demonstrate a distinct relation between the existence of pregnancy complications and alkaline phosphatase activity!!! Therefore, it is recommended that alkaline phosphatase activity should be determined not only in the second and third trimesters, but also, in the first trimester of pregnancy.

5. Conclusion

The prevalence of anemia among Jordanian pregnant women was found to be relatively high. The high prevalence of anemia is a warrant for an in-depth study for the determination of the risk factors and for the development of a nutrition intervention action program directed towards pregnant women to combat iron deficiency anemia. Iron supplementation has to be joined with a nutritional education program directed towards pregnant women with special emphasis on:

1. The importance of a balanced diet and of iron-rich food.
2. The use of foods which enhance the absorption of iron.
3. The benefits of the proper use of iron tablets.

Alkaline phosphatase activity is found to be a good indicator for early pregnancy status. Therefore, it is recommended to use alkaline phosphatase as one of the routine clinical tests for pregnant women.

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References

[1] Aldallal ZS. 1984. Some demographic and health information about mothers in Bahrain. In: AO Musaiger. **Studies on nutrition in Bahrain**. Nutrition Unit, Public Health Directorate, Ministry of Public Health, Bahrain. p. 11-12.

[2] Aleem FA. 1972. Total and heat-stable serum alkaline phosphatase in normal and abnormal pregnancies. *Obstet Gynecol,* **40**:163-172.

[3] Al-Farsi YM, Brooks DR, Werler MM, Cabral HJ, Al-Shafei MA and Wallenberg HC. 2011. Effect of high parity on occurrence of anemia in pregnancy: a cohort study. *BMC Pregnancy and Childbirth,* **11**:7-13.

[4] Banerjee B, Pandey GK, Dutt D, Sengupta B, Mondal M and Deb S. 2009. Teenage pregnancy: A socially inflicted health hazard. *Indian J. of Community Medicine,* **34**: 227-231.

[5] Banhidy F, Acs N, Puho EH and Czeizel AE. 2011. Iron deficiency anemia: Pregnancy outcomes with or without iron supplementation. *Nutrition,* **27**: 65-72.

[6] Bashiri A, Katz O, Maor E, Sheiner E, Pack I and Mazor M. 2007. Positive placental staining for alkaline phosphatase corresponding with extreme elevation of serum alkaline phosphatase during pregnancy. *Arch Gynecol Obstet,* **275**: 211-214.

[7] Centers for Disease Control and prevention (CDC). 1998. Recommendations to prevent and control iron deficiency in the United States. *MMWR Morb Mortal Wkly Rep,* **3**: 1-29.

[8] Charles AM, Campbell-Stennett D, Yatich N and Jolly PE. 2010. Predictors of anemia among pregnant women in Westmoreland, Jamaica. *Health Care for Women International,* **31**: 585-598.

[9] Chumak EL and Grjibovski AM. 2010. Anemia in pregnancy and its association with pregnancy outcomes in the Arctic Russian town of Monchegorsk, 1973-2002. *Int J Circumpolar Health,* **69**: 265-277.

[10] Dawood HS, Prakash P and Shubber KMR. 1990. Iron deficiency anemia among Pregnant Arab Women in Kuwait. *The J. of Kuwait Medical Association,* **24**: 167-172.

[11] DeMaeyer EM. 1989. **Preventing and Controlling Iron Deficiency Anemia through Primary Health Care.** World Health Organization, Geneva.

[12] Fenuku RI and Foli AK. 1975. Serum alkaline phosphatase activity during pregnancy and the post-partum period in Ghanaian women. *Trop Geogr Med,* **27**: 371-374.

[13] Gharaibeh M, Al-Ma'aiteh R and Al Jada N. 2005. Lifestyle practices of Jordanian pregnant women. *International Nursing Review,* 52: 92-100.

[14] Grgic G and Bogdanovic G. 2009. Placental alkaline phosphatase in the prediction of preterm delivery. *Acta Medica Academica,* **38**: 16-20.

[15] Halper SL. 1987. **Quick Reference to Clinical Nutrition**, Second ed. J.B. Lippincott Co., Philadelphia.

[16] Jackson RT and Lantham MC. 1982. Anemias of Pregnancy in Liberia. *Am J Clin Nut,* **35**: 710-714.

[17] Jamjute P, Ahmad A, Ghosh T and Banfield P. 2009. Liver function test and pregnancy. *The J for Maternal-Fetal and Neonatal Medicine,* **22**: 274-283.

[18] Johnson AA, Lantham MC and Roe DA. 1982. The prevalence and etiology of anemia in Guyana. *Am J Clin Nut,* **35**: 309-318.

[19] Khader A, Madi H, Ricardo F and Sabatinelli G. 2009. Anemia among pregnant Palestinian women in the occupied Palestinian territory. *Public Health Nutr,* **12**: 2416-2420.

[20] Kilbride J, Baker TG, Parapia LA and Koury SA. 2000. Iron status, serum folate and B(12) values in pregnancy and postpartum: Report from a study in Jordan. *Ann Saudi Med,* **20**: 371-376.

[21] Mahomed K. 1997. Routine iron supplementation during pregnancy. The Cockrane Library 285-291.

[22] Makarem A. 1974. Clinical chemistry-Principles and techniques, 2nd ed. In: Henry RF, Cannon DC, Winkelman SW(Eeds.), Harper and Row, Hagerstown MD. p. 1128-1135.

[23] Mirzaie F, Eftekhari N, Goldozeian S and Mahdavinia J. 2010. Prevalence of anemia risk factors in pregnant women in Kerman, Iran. *Iranian J of Reproductive Medicine,* **8**: 66-69.

[24] Muller F, Oury JF, Bussiere P, Lewin F and Boue S. 1991. First-trimester diagnosis of hypophosphatasia. Importance of gestational age and purity of CV samples. *Prenat Diagn,* **11**: 725-730.

[25] Okesina AB , Donaldson D, Lascelles PT and Morris P. 1995. Effect of gestational age on levels of serum alkaline phosphatase isoenzymes in healthy pregnant women. *mt S Gynaecol Obstet,* **48:** 25-29.

[26] Passmore R and Eastwood MA. 1986. **Davidson and Passmore Human Nutrition and Dietetics**, 8th ed. Churchill Living Stone, Edinburgh.

[27] Rosenau L, Douay 0, Morel B, Lebouvier B and Grosieux P. 1994. Serum alkaline phosphatase levels in pregnancy. *J Gynecol Obstet Bio Reprod,* **23**: 175-179.

[28] Sanghvi TG, Harvey PWJ and Wainwright E. 2010. Maternal iron-folic acid supplementation programs: Evidence of impact and implementation. *Food and Nutrition Bulletin,* **31(2)**: S100-S107.

[29] Simmons WK , Justum PJ and Fox K. 1982. A survey of the anemia status of pre-school age children and pregnant-acting women in Jamaica. *Am J din Nut,* **35**: 319-326.

[30] Sloan N, Jordan E and Winikoff B. 2002. Effects of iron supplementation on maternal hematologic status in pregnancy. *American J of Public Health,* **92**: 288-293.

[31] Taseer I, Safdar S, Mirbahar A and Awan Z. 2011. Anemia in pregnancy: Related risk factors in under developed area. *Professional Med J,* **18**: 1-4.

[32] Tietz NW. 1983. A reference method for measurement of alkaline phosphatase activity in human serum. Study group on alkaline phosphatase. *Clin Chem,* **29**:751-755.

[33] World Health Organization 1972. **Nutrition Anemias**. WHO technical report series, No. 503. p. 43-89.

[34] Zhang Q, Li Z and Ananth CV. 2009. Prevalence and risk factors for anemia in pregnant women: a population-based prospective cohort study in China. *Paediatric and Perinatal Epidemiology*, **23**: 282-291.

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Effect of Extraction Methods on the Polycyclic Aromatic Hydrocarbons Content Smoked Catfish Species in Niger State of Nigeria.

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Abstract

Catfish species collected from three different fishing zones namely; Katcha/Baro, Shiroro/Sarkin-Pawa and Wushishi/Gwarjiko/Zungeru areas of Niger state smoked with the traditional kilns (the drum and the mud-type smoking kilns) were screened for their polycyclic aromatic hydrocarbons (PAHs) content using three different extraction methods namely; accelerated solvent, Soxhlet and solid-liquid and GC/MS. Results from the study showed that the PAHs content in the studied smoked *Clarias gariepinus* (Catfish), ranged between 0.75-2.25, 0.40-2.00 and 0.25-1.75µg/kg for the accelerated solvent Soxhlet and solid-liquid extraction method irrespective of zone, while the index of PAH contamination, benzo(a)pyrene range between 1.28-1.96, 0.4-1.62 and 0.25 -1.54 µg/kg for the accelerated solvent, Soxhlet and solid-liquid extraction method, respectively. Generally, the PAHs content in the studied smoked fish species from the various zones were comparable and ranged between 0.40-2.25µg/kg and were below the European Union's recommended limit of 5µg/kg for carcinogenicity in smoked meat and fish products. The results also show that the accelerated solvent method was more efficient for the extraction of PAHs in the studied fish species than the Soxhlet and solid-liquid extraction method. The findings also revealed that the variation across zones may have been due to the type of smoking kilns used.

k**eywords:** Smoking kilns, smoked catfish, polycyclic aromatic hydrocarbons, carcinogenicity, extraction, Clarias gariepinus.

1. Introduction

Polycyclic aromatic hydrocarbons (PAH) are formed when complex organic substances are exposed to high temperature or pressure or by the incomplete combustion of woods, coal or oil. They can be found in complex mixtures throughout the environment (Easton *et al*, 2002; Storelli *et al*, 2003; Grova *et al*, 2005; Wretling, *et al* 2010). At ambient temperature, PAH are solids with low volatility. They are relatively insoluble in water and soluble in many organic solvents and are highly lipophilic. They have low vapour pressure, relatively high melting and boiling points due to their high molecular masses. Most PAH can be photo-oxidized and degraded to simpler substances (IPCS, 1998).

Foods can be contaminated by PAHs from environmental sources, industrial food processing and during home food preparation. Industrial food processing represents the major source of human exposure from diet (Zabik *et al*, 1996; Kannapan *et al,* 2000; Wretling *et al*, 2010). As PAHs represent an important class of carcinogens, their presence in foods has been intensively studied. Of the several hundreds of PAHs, sixteen (16) of them have been identified as priority PAHs because they have been considered to be more harmful to man than the others (Andrzej and Zdzislaw, 2005; Chimezie and Hebert, 2006; Wretling *et al*, 2010).

In carrying out analysis of PAHs content in sample matrix, different reagents, extraction methods and instrumental analysis could be used in order to obtain precise information on the extent of contamination in the sample matrix. Extraction is usually the first step in analytical procedures applied to the determination of organic compounds in solid matrices. The use of a convenient type of extraction not only influences the accuracy of results, but also determines the total analysis time and in this way affects sample throughput and analysis costs. Several efficient extraction techniques have been developed and are commonly used for analyte isolation from solid matrices.

Often extraction procedures are non- selective and will extract a broad spectrum of organic samples. The resulting extract has to be purified by the removal of impurities, which may interfere with the analysis of PAHs. The techniques employed for smoked fish vary by the method used to enhance the action of the solvent for the extraction, and ranges from the classic Soxhlet extraction to modern microwave extraction. Different workers have

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reported various methods that could be used for extraction of PAHs from sample matrix (Wretling *et al,* 2010; Khan *et al*, 2005; Juhani *et al*, 2004; Camel, 2001; Bjerkland *et al*, 2000, Hawthorne and Grabanski, 2000; Wang *et al*, 1999; USEPA, 1996; Majors, 1995). Linjinsky and Shubik (1965) first reported the presence of PAHs in smoked meat, since then many studies have confirmed the presence of PAHs in different smoked foods including fish (SCF 2002; EFSA 2008). PAHs concentrations ranging from 0.01-200µg/kg have been reported by different workers in smoked fish and meat products using different extraction and instrumental methods (Wretling *et al.*, 2010; Ajai *et al*., 2010; EFSA 2008; SCF 2002; De Vos *et al*., 1990, Emerole *et al*., 1982, Prinsen and Kennedy, 1977, Steinig and Meyer, 1976).

The PAHs in smoked foods are highly variable. These variations can be attributed in part to the different procedures used to evaluate the PAHs content, but the main reason for such discrepancies is the difference in procedures used for smoking. Such as the type and composition of woods, type of generator, oxygen accessibility, temperature of smoke generation and smoking time (SCF 2002, Vincent *et al*., 2007, Wretling *et al*., 2010) This work is thereof aimed at determining the PAHs content in smoked cat fish species in Niger State using different extraction methods in order to ascertain the best extraction method that will give the highest yield of PAHs from the smoked fish species.

2. Materials and Methods

Agilent gas chromatography (HP 68990GC) manufactured by Agilent Technology, Palo, Alto CA, USA and mass spectrophotometer with flame ionization detector (HP 5973) manufactured by Agilent Technology, Palo, Alto CA, USA. Accelerated solvent extractor ASE 200 by Dionex Corporation, California, USA and Soxhlet extractor B810/428 by Gemini scientific Ltd United Kingdom, were use for this study.

PAH reference standards mixture (500µg/ml) containing the 16 target PAHs obtained from NIST Baltimore, MD, PAH internal standard mixture containing five isotopically labeled PAHS acenaphthene-$_{d10}$, pyrene-$_{d10}$, chrysene-$_{d12,}$ perylene$_{d12}$ and benzo(ghi)perylene-$_{d12}$ obtained from LGC Prochem, Boras, Sweden, dichloromethane, pesticides grade obtained from J. T. Baker, Germany, fluorobenzene-2-fluorobiphenyl, pesticide residue grade obtained from Merck Darmstadt, Germany, silica gel 100/120 mesh obtained from BDH laboratories, and petroleum ether (40-60%), analytical grade obtained from BDH laboratories were used for this study.

Smoked *Clarias gariepinus* species (catfish) were collected from the local fish processors in Katcha and Baro in Katcha, Shiroro in Shiroro, Sarkin Pawa in Munya, Wushishi, Zungeru and Gwarjiko in Wushishi local government areas of Niger state.

2.1. Preparation of standard solutions

Five standard solutions each containing the 16 target compounds were prepared by diluting 1.0, 2.0, 4.0, 10.0 and 20.0 cm^3 of 500µg/mL of each standard PAH with 100 cm^3 of dichloromethane. To all of these solutions were added 0.5µg each of the five internal standards namely acenaphthene-$_{d10}$, pyrene-$_{d10}$, chrysene-$_{d12,}$ Perylene$_{d12}$ and benzo(ghi) perylene-$_{d12}$. The solutions were transferred into capped and sealed vials until ready for analysis.

2.2. Recovery studies

Prior to extraction 0.5µg of each of the five surrogate standards were added to the sample to monitor the recovery of different target compounds. This was used to monitor unusual matrix effect and gross sample processing error. The surrogate standards used include acenaphthene-$_{d10}$, pyrene-$_{d10}$, chrysene-$_{d12}$, perylene$_{d12}$ and benzo(ghi)perylene-$_{d12}$. Those standards serve as surrogates the different sets of target PAHs because they have molecular masses and chemical characteristics close to those of the surrogates. The surrogate standards were subjected to the same extractions procedures as described above. The surrogate percentage recovery was calculated using the expression:

$$\% \text{ Recovery} = \frac{\text{Quantity determined} - \text{Quantity added}}{\text{Quantity added}}$$

2.3. Extraction procedures

In this work three extractions methods, the accelerated solvent, Soxhlet and solid-liquid extraction methods were used to extract the PAHs content in the different fish species studied.

2.3.1. Accelerated solvent extraction method

Prior to extraction, silica gel was activated by oven-drying for 24 hours at 130^{o}C. Concentrated H_2SO_4 acid was then added to the silica gel (1:1v/v) and the mixture shaken vigorously. The mixture was then stored at room temperature prior to use.

The extraction cell was prepared and then tightly packed with 0.5g of sand and 6.5g of activated silica gel. The cap of the extraction cell was temporally removed and 50cm^3 of dichloromethane was passed over the column for conditioning. Then the cell was packed with 5g of dried, ground and well homogenized fish sample followed by 0.5g sand and finally with cellulose filter before capping the cell. The cell was placed into the carousel for extraction. 20cm^3 dichloromethane was then introduced into the extraction cell in the carousel to extract the PAHs in the fish sample. The operating temperature and pressure of the setup was then programmed to 160^{o}C and 2000psi respectively, and the sample heated by direct contact with the oven.

The extraction was achieved by direct contact of the sample with the hot solvent in both static and dynamic modes. The static extraction time used in this study was 5 minutes. Compressed nitrogen gas was finally used to purge the extract into a collector vial, capped and stored in a refrigerator prior to clean up. Same procedure was used for other fish samples.

2.3.2. Soxhlet extraction method

Five gram of the pounded fish sample was weighed and homogenized with 5g of anhydrous sodium sulphate in a laboratory mortar until a complete homogenate was obtained. The extraction was carried out using a Soxhlet extractor apparatus consisting of a 250cm^3 round bottomed flask, condenser and an extractor tube, seated in a

temperature-controlled heating mantle. A Fischer brand rotary evaporator was used to evaporate the extract to the desired concentration. The homogenate was carefully transferred into the extraction thimble placed in the extraction chamber of a Soxhlet extraction unit. The extraction was carried out as recommended by USEPA 3540 method (USEPA, 1994), using 150 cm^3 dichloromethane for 16 hours. The extract was concentrated to 2 cm^3 using a rotary evaporator in a water bath that was pre-set to a temperature of 35^oC and was stored in an amber bottle and kept in a refrigerator to avoid oxidation of the extract prior to clean up. Also, same procedure was used for all fresh fish samples collected.

2.3.3. Solid-liquid extraction method

Five gram of anhydrous Na_2SO_4 and 5 pre-cleaned glass beads were added into a pre-cleaned extraction flask. 5g of well ground homogenized fish sample was placed inside the separatory funnel. 20ml of dichloromethane was then added and the separatory funnel was capped tightly. The flask was shaken vigorously until a slurry was formed. More Na_2SO_4 was added and shaken vigorously to produce free flowing finely divided slurry. The samples were extracted by the use of a centrifuge.

The solvent layer was pipetted into a collecting vial through a small glass funnel containing a layer of anhydrous sodium sulphate (Na_2SO_4) over a plug of glass wool. The extract was then filtered into a 25 cm^3 concentrator flask using a glass funnel packed with plug of glass wool. The sample was extracted twice more using 5 cm^3 of dichloromethane and the extracts combined.

The combined extracts were transferred into a concentrator flask. Boiling chips were added to the concentrator flask and the extract was evaporated in a constant temperature hot water bath until the volume was reduced to approximately 1cm^3, then removed and allowed to cool. The extract was collected, and concentrated using a Kuderna-Danish concentrator. The extract was transferred into a vial fitted with a screw cap and stored in a refrigerator prior to clean up. Same procedure was used for extracting fresh fish samples.

2.4. Sample purification

The extracted samples were purified by passing them through a silica gel column prepared by loading 10g of activated silica gel onto a chromatographic column (1cm internal diameter) to about 5cm. This was topped with 1cm of anhydrous Na_2SO_4. It was then conditioned with dichloromethane. 2 cm^3 of the concentrated extract was loaded and eluted with 20 cm^3 of dichloromethane. This method is able to remove the very polar lipids off the extract. Prior to analysis with GC/MS, the extracts obtained were preserved in an amber bottle to avoid oxidation.

2.5. GC/MS Analysis

An Agilent 6850 gas chromatograph equipped with auto sampler connected to an Agilent FID mass selective detector was used. 1µl of sample solution was injected in the pulsed spilt-less mode onto a 30m x 0.25 mm id DB-12 ms coated fused silica column with a film thickness of 0.15µm. Helium was used as the carrier gas and the column head pressure was maintained at 35 psi to give constant flow 1.1ml/min. Other operating conditions were pre-set, pulse time 0.90mins, purge flow 50 cm^3, purge time 1min, and injection temperatures 300^oC. The column temperature was initially held at 70^oC for 3mins, increased to160^oC at a rate of 20^oC/min, then to 210^oC at a rate of 3^oC/min and to a final temperature of 310^oC at a rate of 5^oC/min and held for 10mins and transfer line of 320^oC. The mass spectrometer (MS) condition was electron impact positive ion mode. The retention time and quantifying ions of PAHs and internal standards are shown in table1. The PAHs identification time was based on retention time since each of the PAHs has its separate retention time in the column. Those with lower retention times were identified first followed by those with longer retention time.

2.6. Calibration

A calibration curve was obtained by analysing each of the standard PAHs solutions prepared with the GC/MS. The target PAH compound/internal standard peak heights were plotted against the PAH concentration to obtain a linear graph Y= mx + b, with an intercept (b) on the y-axis. The concentration of PAH in each sample was calculated using the formula

$$\text{PAH (µg/kg)} = \frac{\text{RP/RIS-b} \times \text{MIS (µg)}}{\text{X (g)} \times 1000}$$

Where

RP = Response PAH peak height

RIS = Response internal standard peak height

b = intercept on the y-axis of the standard calibration curve

MIS = Mass of added internal standard

X = weight of sample used

The limit of detection (LOD) and the limit of quantification (LOQ) for each PAH were calculated from the standard deviations of results obtained from the analysis of the several dilutions of the analyte, table1. The LOD for individual PAH in the fish samples were calculated as 3 times the standard deviation of the mean and the LOQ as 10 times the standard deviation of the mean.

3. Results and Discussion

The results obtained from the study are shown in tables 1-4 with table 1 showing the chromatographic characteristics of the target compounds, table 2 gives the percentage recovery, table 3 shows PAHs profiles in the studied fish species and table 4 summarizes the statistical analysis of the obtained results using one way Anova and Tukey's Multi range comparison test.

Table 1. Chromatographic characteristics of the target compounds

	Retention time (Minutes)	Major Peak ion		Mean conc.	SD	LOD	LOQ	RSD (%)
PAHs	GC	m/z	Internal Standard	µg/kg		µg/kg	µg/kg	
Naphthalene	8.93	128	$Acenaphthene_{d10}$	0.362	0.014	0.04	0.14	3.87
Acenaphthylene	13.03	152	$Acenaphthene_{d10}$	0.830	0.012	0.04	0.12	1.45
Acenaphthene	13.61	154	$Acenaphthene_{d10}$	0.845	0.011	0.03	0.11	1.30
Fluorene	15.03	166	$Acenaphthene_{d10}$	0.125	0.005	0.02	0.05	4.00
Phenathrene	17.55	178	$Pyrene_{d10}$	0.186	0.010	0.03	0.10	5.38
Anthracene	17.72	178	$Pyrene_{d10}$	0.423	0.014	0.04	0.14	3.31
Fluoranthene	20.77	202	$Pyrene_{d10}$	0.537	0.014	0.04	0.14	2.61
Pyrene	21.41	202	$Pyrene_{d10}$	0.412	0.015	0.05	0.15	3.64
Benz(a)anthracene	24.78	228	$Chrysene_{d12}$	0.506	0.020	0.06	0.20	3.95
Chrysene	24.99	228	$Chrysene_{d12}$	0.796	0.021	0.06	0.21	2.64
Benzo(b)fluoranthene	27.64	252	$Perylene_{d12}$	2.320	0.035	0.01	0.35	1.52
Benzo(k)fluoranthene	27.72	252	$Perylene_{d12}$	1.465	0.042	0.01	0.42	2.87
Benzo(a)pyrene	28.33	252	$Perylene_{d12}$	1.180	0.045	0.01	0.45	3.79
Indeno(1,2,3-cd)pyrene	30.92	276	$Benzo(ghi)perylene_{d12}$	1.120	0.062	0.02	0.62	5.54
Dibenz(a,h)anthracene	31.01	276	$Benzo(ghi)perylene_{d12}$	1.968	0.114	0.34	1.14	5.79
Benzo(ghi)perylene	31.49	278	$Benzo(ghi)perylene_{d12}$	1.850	0.155	0.05	1.55	8.38

SD = Standard Deviation LOD = Limit of Detection

LOQ = Limit of Quantification, RSD = Relative Standard Deviation

3.1. Chromatographic characteristics of the target compounds

Table 1 shows the chromatographic characteristics of the target compounds. The retention time increases with increasing m/z ratio of the target compounds. The retention time obtained was within the limit reported by other workers of not more than 32 minutes (Andrzej and Zdzislaw, 2005). The mean PAHs concentration range between 0.125-2.320µg/kg with benzo(b)fluoranthene having the highest of 2.320µg/kg and fluorene the lowest of 0.125 µg/kg. The LOD range between 0.01-0.34 µg/kg, with dibenz(a,h)anthracene having the highest (0.34µg/kg) and benzo(b)fluoranthene, benzo(k)fluoranthene and benzo(a)pyrene the lowest. This signifies that any of these parameters that fall below these values in the course of analysis cannot be detected by the instrument and will therefore fall below non-detectable limit. The LOQ, range between 0.05-1.55µg/kg with benzo(ghi)perylene having the highest of 1.55µg/kg and fluorene the least with 0.05µg/kg respectively. The (RSD) range between 1.30-8.97% with benzo(ghi)perylene having the highest 8.38% and acenaphthene the lowest with 1.30% respectively.

Table 2. Percentage recovery by each extraction method (%)

Compounds	ASE	SOX	SLE	Internal Standard
Naphthalene	87.34	72.30	69.25	$Acenaphthene_{d10}$
Acenaphthylene	87.34	72.30	69.25	$Acenaphthene_{d10}$
Acenaphthene	87.34	72.30	69.25	$Acenaphthene_{d10}$
Fluorene	87.34	72.30	69.25	$Acenaphthene_{d10}$
Phenanthrene	91.58	89.60	64.34	$Pyrene_{d10}$
Anthracene	91.58	89.60	64.34	$Pyrene_{d10}$
Fluoranthene	91.58	89.60	64.34	$Pyrene_{d10}$
Pyrene	91.58	89.60	64.34	$Pyrene_{d10}$
Benz(a)anthracene	71.25	67.24	58.72	$Chrysene_{d12}$
Chrysene	71.25	67.24	58.72	$Chrysene_{d12}$
Benzo(b)fluoranthene	82.43	80.32	55.50	$Perylene_{d12}$
Benzo(k)fluorathene	82.43	80.32	55.50	$Perylene_{d12}$
Benzo(a)pyrene	82.43	80.32	55.50	$Perylene_{d12}$
Benzo(ghi)perylene	75.55	68.26	52.24	$Benzo(ghi)perylene_{d12}$
Indeno(1,2,3-cd)pyrene	75.55	68.26	52.24	$Benzo(ghi)perylene_{d12}$
Dibenz(ah)anthracene	75.55	68.26	52.24	$Benzo(ghi)perylene_{d12}$

ASE=Accelerated solvent extraction, SOX= Soxhlet,
SLE = Solid liquid extraction

3.2. Extraction Efficiency

The result of percentage recovery which measures the efficiency of each extraction method is shown in table 2. According to the European Commission (2005), PAHs recovery of 50-120% is an indication that an analytical procedure adopted for PAHs analysis is an acceptable procedure. The results show that that accelerated solvent extraction method gave the highest extraction efficiency and range between 71.25-91.58 %. The solid-liquid extraction method gave the lowest extraction efficiency of 52.24-69.25% respectively. In using the accelerated solvent extraction method, phenanthrane, anthracene, fluoranthene and pyrene gave the highest percentage recovery of 91.58%, while benz(a)anthracene and chrysene the lowest 71.25% respectively. The Soxhlet extraction also had the highest extraction efficiency of 89.60% in phenanthrane, anthracene, fluoranthene and pyrene, while benz(a)anthracene and chrysene the lowest 67.24%. The solid liquid extraction method gave the least extraction efficiency of all the parameters and had its highest extraction efficiency of 69.25% in naphthalenee, acenaphthylene, acenaphthene and fluorene and the lowest of 52.24% in benzo(ghi)perylene, indeno(1,2,3-cd)pyrene and dibenz(ah)anthracene respectively. It should be noted that groups of PAHs that have similar percentage recovery along column were extracted using same internal standard as indicated on the table.

The PAHs content in *Clarias gariepinus* species from zone A using accelerated solvent method (Table3), ranged between 0.84-2.25µg/kg, the PAHs profiles using Soxhlet extraction method ranged between 0.40-2.00µg/kg.

The solid-liquid extraction method ranged between 0.36-1.85µg/kg. From this zone, accelerated solvent extraction method has the highest cumulative PAHs burden of 24.08µg/kg followed by Soxhlet extraction method (20.41µg/kg) and solid-liquid extraction method (15.90µg/kg) in the studied fish species.

The PAHs profile in samples from zone B table 3 ranged between 0.75-2.02µg/kg using accelerated solvent extraction method, PAHs profiles using Soxhlet extraction method range between 0.40-1.94µg/kg. PAHs profile using solid-liquid extraction method range between 0.25-1.75µg/kg. Accelerated solvent extraction yielded the highest cumulative PAHs burden (21.53µg/kg) followed by Soxhlet (20.33µg/kg) and solid- liquid extraction method with 17.13µg/kg respectively.

The PAHs profiles in *Clarias gariepinus* species from zone C using GC/MS (Table 3), ranged between 0.70-1.94µg/kg using accelerated solvent extraction method, also using Soxhlet extraction method, the PAHs profiles in *Clarias gariepinus* species range between 0.59-1.88µg/kg, the PAHs profiles using solid-liquid extraction method range between 0.30-1.33µg/kg. The Soxhlet extraction method yielded the highest cumulative PAHs burden of 19.86µg/kg followed by accelerated solvent extraction method (19.31µg/kg) and by solid-liquid extraction method (13.55µg/kg). The PAHs content in the studied samples compared favourably with those reported by Karl and Leinamann (1996); Simko, (2000), Ajai *et al*., (2010) and Wretling *et al*., (2010) in smoked and non-smoked fish.

Table 3. PAHs Profiles in smoked *Clarias gariepinus* species using different extraction method (µg/kg).

Zones	A			B			C		
PAHs/Extraction methods	**X**	**Y**	**Z**	**X**	**Y**	**Z**	**X**	**Y**	**Z**
Naphthalene	0.84	0.45	0.50	1.36	1.25	1.03	0.96	0.65	0.30
Acenaphthylene	1.14	1.25	0.86	2.00	0.40	1.20	1.94	1.34	1.30
Acenaphthene	2.14	0.75	1.25	1.00	1.65	1.25	1.46	1.78	0.79
Fluorene	ND	1.15	1.00	1.28	1.30	1.12	1.40	1.30	0.87
Phenathrene	1.75	1.50	ND	1.92	1.83	ND	0.96	0.59	0.67
Anthracene	2.20	1.58	1.20	2.02	1.94	1.75	1.50	1.88	1.25
Fluoranthene	1.88	1.00	0.95	1.29	1.15	0.73	0.80	1.20	1.00
Pyrene	2.05	2.00	ND	1.90	1.50	ND	ND	1.34	0.62
Benz(a)anthracene	1.95	1.60	1.12	1.45	1.69	1.10	0.95	0.85	0.72
Chrysene	1.25	1.10	0.86	1.87	1.55	1.42	1.20	1.62	0.86
Benzo(b)fluoranthene	2.25	2.00	1.60	0.87	ND	0.25	1.25	1.60	1.33
Benzo(k)fluoranthene	ND	0.96	0.84	0.75	0.96	0.64	1.50	0.60	1.04
Benzo(a)pyrene	1.86	0.40	0.36	1.57	1.62	1.54	1.28	1.20	ND
Indeno(1,2,3-cd)pyrene	1.53	1.44	1.20	1.27	0.62	1.10	1.91	1.25	0.94
Dibenz(a,h)anthracene	1.60	1.38	1.26	1.25	1.17	1.35	1.50	1.75	1.23
Benzo(ghi)perylene	1.69	1.75	1.05	1.63	1.30	1.15	0.70	0.91	0.63
Cumulative PAHs	24.08	20.41	15.90	21.53	20.33	17.13	19.31	19.86	13.55

X =Accelerated solvent extraction method, Y = Soxhlet extraction method, Z = solid-liquid extraction method. A,B,C are the different zones. ND= Not derected

The PAHs content in *Clarias gariepinus* species from zone A using accelerated solvent method (Table3), ranged between 0.84-2.25µg/kg, the PAHs profiles using Soxhlet extraction method ranged between 0.40-2.00µg/kg. The solid-liquid extraction method ranged between 0.36-1.85µg/kg. From this zone, accelerated solvent extraction method has the highest cumulative PAHs burden of 24.08µg/kg followed by Soxhlet extraction method (20.41µg/kg) and solid-liquid extraction method (15.90µg/kg) in the studied fish species.

The PAHs profile in samples from zone B table 3 ranged between 0.75-2.02µg/kg using accelerated solvent extraction method, PAHs profiles using Soxhlet extraction method range between 0.40-1.94µg/kg. PAHs profile using solid-liquid extraction method range between 0.25-1.75µg/kg. Accelerated solvent extraction yielded the highest cumulative PAHs burden (21.53µg/kg) followed by Soxhlet (20.33µg/kg) and solid- liquid extraction method with 17.13µg/kg respectively.

The PAHs profiles in *Clarias gariepinus* species from zone C using GC/MS (Table 3), ranged between 0.70-1.94µg/kg using accelerated solvent extraction method, also using Soxhlet extraction method, the PAHs profiles in *Clarias gariepinus* species range between 0.59-1.88µg/kg, the PAHs profiles using solid-liquid extraction method range between 0.30-1.33µg/kg. The Soxhlet extraction method yielded the highest cumulative PAHs burden of 19.86µg/kg followed by accelerated solvent extraction method (19.31µg/kg) and by solid-liquid extraction method (13.55µg/kg). The PAHs content in the studied samples compared favourably with those reported by Karl and Leinamann (1996); Simko, (2000), Ajai *et al*., (2010) and Wretling *et al*., (2010) in smoked and non-smoked fish.

The benzo(a)pyrene irrespective of zones ranged between 1.28-1.86, 0.40- 1.62 and nd-1.54µg/kg for the accelerated solvent, Soxhlet extraction and solid-liquid extraction methods respectively, with the accelerated solvent extraction method having the highest yield and the solid-liquid extraction method the least. These values fell within the limit reported by Karl and Lienemann (1996) and Steinig (1976) for smoked fish and below the maximum of 5.0µg/kg recommended by European Union for smoked fish and meat products respectively.

Table 4. One way Anova of the effect of extraction methods on the PAHs content in the studied smoke SEM) (µg/kg) n=2

Fish Species	A			B			C		
PAHs/Extraction methods	**X**	**Y**	**Z**	**X**	**Y**	**Z**	**X**	**Y**	**Z**
Naphthalene	0.84 ± 0.01^{b}	0.45 ± 0.03^{a}	0.50 ± 0.03^{a}	1.36 ± 0.04^{b}	1.25 ± 0.04^{b}	1.03 ± 0.01^{a}	0.96 ±	0.65 ± 0.01^{b}	0.30 ± 0.03^{a}
Acenaphthylene	1.14 ± 0.10^{b}	1.25 ± 0.01^{b}	0.86 ± 0.01^{a}	2.00 ± 0.01^{c}	0.40 ± 0.03^{a}	1.20 ± 0.01^{b}	1.94 ± 0.06^{b}	1.34 ± 0.03^{a}	1.30 ± 0.04^{a}
Acenaphthene	2.14 ± 0.01^{c}	0.75 ± 0.04^{a}	1.25 ± 0.04^{b}	1.00 ± 0.04^{a}	1.65 ± 0.04^{c}	1.25 ± 0.03^{b}	1.46 ± 0.03^{b}	1.78 ± 0.04^{c}	0.79 ± 0.03^{a}
Fluorene	0^{a}	1.15 ± 0.03^{c}	1.00 ± 0.03^{a}	1.28 ± 0.03^{b}	1.30 ± 0.01^{b}	1.12 ± 0.03^{a}	1.40 ± 0.04^{b}	1.30 ± 0.03^{b}	0.87 ± 0.01^{a}
Phenathrene	1.75 ± 0.04^{c}	1.50 ± 0.03^{b}	0^{a}	1.92 ± 0.03^{b}	1.83 ± 0.04^{b}	0^{a}	0.96 ± 0.01^{b}	0.59 ± 0.04^{a}	0.67 ± 0.03^{a}
Anthracene	2.20 ± 0.04^{c}	1.58 ± 0.01^{b}	1.20 ± 0.03^{a}	2.02 ± 0.03^{b}	1.94 ± 0.06^{b}	1.75 ± 0.01^{a}	1.50 ± 0.02^{b}	1.88 ± 0.01^{c}	1.25 ± 0.03^{a}
Fluoranthene	1.88 ± 0.03^{b}	1.00 ± 0.01^{a}	0.95 ± 0.03^{a}	1.29 ± 0.01^{c}	1.15 ± 0.01^{b}	0.73 ± 0.03^{a}	0.80 ± 0.01^{a}	1.20 ± 0.03^{c}	1.00 ± 0.04^{b}
Pyrene	2.05 ± 0.03^{b}	2.00 ± 0.03^{b}	0^{a}	1.90 ± 0.06^{c}	1.50 ± 0.04^{b}	0^{a}	0^{a}	1.34 ± 0.06^{c}	0.62 ± 0.03^{b}
Benz(a)anthracene	1.95 ± 0.03^{c}	1.60 ± 0.03^{b}	1.12 ± 0.01^{a}	1.45 ± 0.01^{b}	1.69 ± 0.04^{c}	1.10 ± 0.01^{a}	0.95 ± 0.01^{b}	0.85 ± 0.04^{b}	0.72 ± 0.03^{a}
Chrysene	1.25 ± 0.01^{c}	1.10 ± 0.01^{b}	0.86 ± 0.03^{a}	1.87 ± 0.06^{b}	1.55 ± 0.04^{a}	1.42 ± 0.03^{a}	1.20 ± 0.01^{b}	1.62 ± 0.04^{c}	0.86 ± 0.04^{a}
Benzo(b)fluoranthene	2.25 ± 0.01^{c}	2.00 ± 0.03^{b}	1.60 ± 0.01^{a}	0.87 ± 0.06^{c}	0^{a}	0.25 ± 0.03^{b}	1.25 ± 0.01^{a}	1.60 ± 0.03^{b}	1.33 ± 0.04^{a}
Benzo(k)fluoranthene	0^{a}	0.96 ± 0.04^{b}	0.84 ± 0.04^{b}	0.75 ± 0.03^{b}	0.96 ± 0.01^{c}	0.64 ± 0.03^{a}	1.50 ± 0.04^{c}	0.60 ± 0.03^{a}	1.04 ± 0.06^{b}
Benzo(a)pyrene	1.86 ± 0.01^{b}	0.40 ± 0.03^{a}	0.36 ± 0.04^{a}	1.57 ± 0.04^{a}	1.62 ± 0.03^{a}	1.54 ± 0.06^{a}	1.28 ± 0.03^{c}	1.20 ± 0.01^{b}	0^{a}
Indeno(1,2,3-cd)pyrene	1.53 ± 0.03^{b}	1.44 ± 0.06^{b}	1.20 ± 0.03^{a}	1.27 ± 0.01^{c}	0.62 ± 0.03^{a}	1.10 ± 0.01^{b}	1.91 ± 0.01^{c}	1.25 ± 0.03^{b}	0.94 ± 0.06^{a}
Dibenz(a,h)anthracene	1.60 ± 0.02^{b}	1.38 ± 0.06^{b}	1.26 ± 0.01^{a}	1.25 ± 0.03^{b}	1.17 ± 0.04^{a}	1.35 ± 0.04^{b}	1.50 ± 0.04^{b}	1.75 ± 0.03^{c}	1.23 ± 0.04^{a}
Benzo(ghi)perylene	1.69 ± 0.01^{b}	1.75 ± 0.01^{b}	1.05 ± 0.04^{a}	1.63 ± 0.04^{c}	1.30 ± 0.01^{b}	1.15 ± 0.04^{a}	0.70 ± 0.03^{a}	0.91 ± 0.01^{b}	0.63 ± 0.03^{a}
Cumulative PAHs	24.08	20.41	15.90	21.53	20.33	17.13	19.31	19.86	13.55

Those with different superscripts across rows within zones are significantly different from each other (P<0.05), where c>b>a. X =Accelerated solvent extraction method, Y=Soxhlet extraction method, Z= liquid-solid extraction method. A,B,C are the different zones

To validate the extraction methods, the obtained results were subjected to statistical analysis using one way Anova at 95% confidence level (Table 4). The results of statistical analysis show significant differences between the different extraction methods in their extraction of PAHs from the sample matrix as represented by different superscripts letters across rows P<0.05 (Table 4). There are variations in their extraction efficiency of the different PAHs in the smoked fish samples. Of the sixteen PAHs determined, the accelerated solvent extraction method yielded higher amount of PAHs in thirteen of them (indicated by superscript c), indicating better extraction method (c>b>a), while the Soxhlet three (3) of the parameters and solid-liquid extraction method the least. 4.Conclusion

From the study it is obvious that smoked catfish contained some PAHs as contaminants which could be as a result of the deposition of some of the by-products of the pyrolysis of woods on the fish during smoking. Also, based on the different extraction methods used in this study, the accelerated solvent extraction method seems to be more efficient than the Soxhlet and the solid-liquid extraction methods, while the solid-liquid extraction method which was least efficient was also most tedious in its experimental approach.

References

Ajai AI, Suleiman MAT, Dauda BEN, Sadiku SOE and Aberuagba F. 2010.

Determination of polycyclic aromatic hydrocarbons in some selected smoked fish species in Gwarjiko area of Niger state, Nigeria. *International J of Chemical Science,* **3 (2):** 2006-3350.

Andrzej S and Zdzislaw E S. 2005. Polycyclic aromatic hydrocarbons in smoked fish: critical review. *J. of Food Chemistry*,**91**: 303-311.

Bjorklund E, Nilsson BT, Bowadt S, Pilorz K, Mathiasson L and Hawthorne S B. 2000. Introducing supercritical fluid extraction as a new tool for determining the sorption/ desorption behaviour and bioavailability of organic pollutants in sediments. *J of Biochemistry. Biophysical. Methods,* **43**: 295-3111.

Camel V. 2001. Recent extraction techniques for solid matrices supercritical fluid extraction, pressurized fluid extraction and microwave assisted extraction: their potential and pitfalls. *Analyst*, **126**: 1182-1193.

Chimezie A and Herbert C. 2006. Determination of polynuclear aromatic hydrocarbons (PAHs) in selected water bodies in the Niger Delta. *African J of Biotechnology,* **5 (21)**: 2024-2031.

De Vos R H, Van Dokkum W, Schouten A and Dejong-Berkhout P. 1990. Polycylic aromatic hydrocarbons in Dutsch, total diet samples. (1984 – 1986). *Food Chemisty and Toxicology.* **28**: 263 – 268.

Easton MDL, Luszniak D and Von der Geest E. 2002. Preliminary examination of contaminant loadings in farmed salmon, wild salmon and commercial salmon feed. *Chemosphere,* **46**: 1053-1074.

European Food Safety Authority (EFSA). 2008. Scientific opinion of the panel on contaminants in the food chain on request from the European Commission on polycyclic aromatic hydrocarbons in food. *The EFSA Journal*. **724**.

Emerole G O, Uwaifo A O, Thabarew M I and Baba Bunmi E A 1982. The presence of afflatoxin and some polycyclic aromatic hydrocarbons in human foods. *Cancer Letter.* **15**: 123 – 129.

European Commission. 2005. Commission Recommendation No. 2005/108 of 4 February 2005 on the further investigation into the levels of polycyclic aromatic hydrocarbons in certain foods. *Official J of the European Union.* **34**: 3-5.

Groova N, Monteau F, Le Bizec B, Feidt C, Andre F and Rychen G. 2005. Determination of phenanthrene and hydoxyphenanthrenes in various biological matrices at trace levels using gas chromatography coupled to mass spectrometry. *J of Analytical Toxicology,* **29**: 175-181.

Hawthorne S B and Grabanski C B. 2000. Correlating selected super critical fluid extraction with bioremediation behaviour of PAHS in a field treatment plot. *Environmental Science Technology*. **34**: 4103-4110.

International Programme on Chemical Safety. (1PCS) 1998. Selected non – heterocyclic polycyclic aromatic hydrocarbons. Environmental Health Criteria 202. World Health Organization, Geneva.

Juhani K, Kettunen J, Hartonen K and Marja-Lissa R. 2004. Pressurized hot water extraction of n-alkanes and PAHs in soil and sediment from the oil shale industry district in Estonia. *J of Soils Sediments*. **1**:8.

Kannappan S, Indra Jasmine G, Jeyachandran P and Tamil Selvi A. 2000. Polyaromatic hydrocarbons in processed fishery products. *J of Food Science and Technology* **37**: 596-601

Karl H and Leinemann M. 1996. Determination of polycyclic aromatic hydrocarbons in smoked fishery products from different smoking kilns. *Z. Lebensen Unters, Forsch.* **202**: 458 – 464.

Khan Z, Troquet J and Vachelard C. 2005. Sample preparation and analytical techniques for determination of polycyclic aromatic hydrocarbons in soils. *International J of Environmental Science and Technology,* **2(3):** 272-286

Lijinsky W and Shubik P. 1965. Polynuclear hydrocarbons carcinogens in cooked meat and smoked foods. *Industrial Medical Surgery.* **34**: 152 – 154.

Majors R E. 1995. Current trends and developments in sample preparation. *Liquid Chromatography-Gas Chromatography International,* **8**: 128.

Prinsen A J and Kennedy W B H 1977. Quantity of alpha – benzopyrene in smoked food aroma and flavouring substances Bilthoven, National Institute of Public Health and Environmental Protection. Report No 11: 1 – 11.

Scientific Committee on Food. 2002. Opinion of the Scientific Committee on food on the risk to human health of polycyclic aromatic hydrocarbons in food. SCF/CS/CNTM/PAH/29 Final. Brussels: European Commission, Health and Consumer Protection Directorate General.

Shewan J M. 1977. The bacteriology of fresh and spoiling fish and the biochemical changes induced by bacterial action. In handling, processing and marketing of tropical fish. London, Tropical Products Institute, 185.

Simko P. 2002. Determination of polycyclic aromatic hydrocarbons in smoked meat products and smoked flavouring additives. *J of Chromatographica* B. **770**: 3-18

Steinig J and Meyer V. 1976. 3, 4 – Benzo(a)pyrene contents in smoked fish depending on smoking procedure,. *Lebensmittel unters forsch*, **162**: 235 – 242.

Storelli MM, Stuffer RG and Marcotrigiano GO. 2003. Polycyclic aromatic hydrocarbons, polychlorinated biphenyls, chlorinated pesticides (DDTs), hexachlorocyclohexane and hexachlorobenzene residues in smoked sea food. *J of Food Protection* **66**: 1095- 1099.

United State Environmental Protection Agency (USEPA), 1996. Method 3540C, Soxhlet Extraction test method for evaluating solid waste. EPA Washington DC. US

US EPA 1994. Soxhlet extraction Method, 354.

Vincent V, Thierry S, Fabrice M, Bruno LB and Carole P. 2007. Determination of PAHs profiles by GC-MS/MS in salmon processed by four cold- smoking techniques. *Food Additives and Contaminants.* **24(7):** 744-756

Wang G, Leo SA, Lewis M, Kamath B and Archer RK 1999. Accelerated solvent extraction and gas chromatography/mass spectrometry for determination of polycyclic aromatic hydrocarbons in smoked food samples. *J of Agriculture and Food Chemistry,* **47**: 1062-1066.

Wretling S, Eriksson A, Eskhult G A and Larson B. 2010. Polycyclic aromatic hydrocarbons in Swedish smoked meats and fish. *J of Food Composition and Analysis,* **23(1):** 264-272.

Zabik M E, Booren A, Zabik M J, Welch R S and Humphrey H. 1996. Pesticides residues, PCBs and PAHs in broiled, charbroiled, salt broiled and smoked Great lake Trout. *Food Chemistry*, **55 (3):** 231-239.

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Jordan Journal of Biological Sciences **Case Report**

Isolation of Methicillin Resistant *Staphylococcus aureus* (module 2011) in Taif Area, Saudi Arabia

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a bacterium responsible for several difficult-to-treat infections in humans. It may be named multidrug-resistant *S. aureus* or Oxacillin-resistant *S. aureus* (ORSA). MRSA is a strain of *S. aureus* that developed resistance to β-Lactam antibiotics, including Penicillins, which include (Methicillin, Dicloxacillin, Nafcillin, Oxacillin, etc.) and Cephalosporine. The aim of this studt was to investigate the new MRSA strain from patient in Taif area who complained from chronic prostatitis and clinically uncured with treatment by Amoxicillin antibiotics for two months; after that the patient was treated by Quinolones antibiotics for three weeks and he was clinically cured. The causative strain isolated was reinvestigated with antibiotics groups to confirm that it is a new module of MRSA that has a different antibiotics sensitivity characterestics in Taif area. The results revealed that the MRSA tested was resistant to antibiotics groups β-Lactam (Penicillin, Oxacillin, Ampicillin and Augmentin) and Glycopeptides (Vancomycin) and of intermediate sensitivity to Macrolides (Erythromycin, Clindamycin and Azithromycin), Aminoglycosides (Gentamycin) and sensitive to Nitrofurantoin from Macrolides, Amikacin from Aminoglycosides and (Ciprofloxacin, Ofloxacin and Norfloxacin) from Quinolones. The study revealed the resistance of new isolated MRSA strain and its sensitivity to little groups of antibiotics that lead to serious health problems if the sensitive antibiotics turned to be resistant or unavailable treated antibiotics in the area. MRSA, widely dangerous for human health as well resistant to antibiotics, is used increasing by time due to fast changes in the genome of *S.aureus* and the misuse of antibiotics by patients. Further studies are required to improve the interaction between human infections by MRSA, prevention, slowering resistant of MRSA and new strong sensitive antibiotics production.

k**eywords:** *S. aureus*, MRSA, ORSA, GISA, VISA, VRSA, CA-MRSA.

Abbreviations:

MRSA	Methicillin Resistant *S.aureus.*
ORSA	Oxacillin Resistant *S.aureus*.
GISA	Glycopeptide Intermediate *S.aureus*.
VISA	Vancomycin Intermediate *S.aureus*.
VRSA	Vancomycin Resistant *S.aureus*.
CA-MRSA	Community Associated MRSA infections.

1. Introduction

Staphylococcus aureus is one of the major resistant pathogens, found on the mucous membranes and the human skin of around a third of the population, extremely adaptable to antibiotic pressure. It was one of the earlier bacteria in which Penicillin resistance was found in 1947, just four years after the drug started being mass-produced (Bulent *et al*., 2003). Methicillin was then the antibiotic of choice, but has since been replaced by Oxacillin antibiotic due to significant kidney toxicity. MRSA strain was first detected in Britain in 1961 and is now in hospitals (Bulent *et al*., 2003). MRSA was responsible for 37% of fatal cases of sepsis in the UK in 1999, up from 4% in 1991. More than half of *S.aureus* infections in USA are resistant to Penicillin, Methicillin, Tetracycline and Erythromycin (Bulent *et al*., 2003). Vancomycin is the only effective agent available against MRSA at the time. However, strains with intermediate levels of resistance, termed GISA (Glycopeptides intermediate *S.aureus*) or VISA (Vancomycin intermediate *S.aureus*), began appearing in the late 1990s (Bulent *et al*., 2003). The first identified case was in Japan in 1996 and strains had since been found in hospitals in England, France and USA. The first documented strain with complete resistance to Vancomycin termed VRSA (Vancomycin-

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resistant *S.aureus*) appeared in United States in 2002 (Bozdogan *et al*., 2003).

Infections caused by *S.aureus* with high-level resistance to Vancomycin (VRSA), as only isolated cases have been reported. VRSA developed by the present of the *vanA* gene, which was transferred from *Enterococci* with Vancomycin resistance. On the other hand, infections caused with intermediate resistance to glycopeptides (VISA), or heterogeneously expressed intermediate level Glycopeptides resistance (hVISA), are more common. These infections were associated with clinical failure of Glycopeptides therapy. While the biochemical and phenotypic features, including a thickened cell wall of hVISA and VISA, are well known, the genetic basis of these phenotypes remains unknown. Certain genetic regulatory elements such as *agr* II are associated with reduced susceptibility of *S.aureus* to Glycopeptides. Available data suggested that certain infections might be successfully treated using higher doses of Vancomycin (Ruef, 2004).

CA-MRSA (Community-acquired MRSA) had emerged as an epidemic that is responsible for rapidly progressive (Boyle-Vavra and Daum 2007). The epidemiology of infections caused by MRSA is rapidly changing, in the past 10 years; infections caused by this organism had emerged in the community (Cynthial *et al*., 2007). The two MRSA clones in United States most closely associated with community outbreaks, USA400 (MW2 strain, ST1 lineage) and USA300, often contain Panton-Valentine leukocidin genes and more frequently had been associated with skin and soft tissue infections (Susan and Robert, 2007). Outbreaks of community-associated infections (CA-MRSA) had reported in correctional facilities among athletic teams and in newborn nurseries. CA-MRSA infections appeared to be endemic in many urban regions and caused most infections (Maree *et al*., 2007).

The Macrolides-resistance due to the effect of gene*ermA* or *ermC* was detected in 67.6% of MRSA and 71.4% of MSSA. These results suggested that SCC*mec* types IV or V have spread, particularly in MSSA carrying *etb* in the community (Hidemasa *et al*., 2008).

MRSA is a bacterium responsible for several difficult-to-treat infections in humans. It may also be called multidrug-resistant *S. aureus* or Oxacillin-resistant *S. aureus* (ORSA). MRSA is, by definition, any strain of *S. aureus* that had developed resistance to β-Lactam antibiotics which include the Penicillins and the Cephalosporins (Holten and Onusko, 2000). MRSA is a major cause of hospital-acquired infections that are becoming increasingly difficult to combat because of emerging resistance to all current antibiotic classes. The evolutionary origins of MRSA was poorly understood, no rational nomenclature exists (Mark *et al*., 2007). A new type of MRSA, designated community-acquired MRSA, became increasingly noticeable in the community, some strains of which caused fatal infections in otherwise healthy individuals. By contrast with hospital-acquired MRSA, community-acquired MRSA was more susceptible to non β-lactam antibiotics. An investigation of high virulence potential shows certain strains of this bacterium (Christopher, 2002). Species ST8:USA300 was a strain of community-associated MRSA that emerged as a particularly, antibiotic resistant epidemic that was responsible for rapidly progressive infections. The epidemiology of infections caused by MRSA was rapidly changing: in the past 10 years, infections caused by this organism had emerged in the community (Boyle-Vavra and Daum 2007). As two tested MRSA clones in United States that are most closely associated with community outbreaks, USA400 and USA300 often contained Panton-Valentine leukocidin (PVL) genes and, more frequently, CA-MRSA infections that appeared to be endemic in many urban regions, causing most MRSA infections (Diep *et al*., 2008).

Susceptibility profile of 19 *S.aureus* isolates were acquired from two teaching hospitals and ATCC towards 16 selected antibiotics was analyzed and an antibiogram was generated. Findings also indicated resistance against many of the available antibiotics and thus an urgent need to search for alternative antibiotics (Saiful *et al*., 2006).

MRSA was determined by *mecA* gene based PCR., *mecA* is composed of 50 kb or more of DNA founded, *mec* contained *mecA*, the gene for Penicillin-binding protein 2a (PBP2a); *mecI* and *mecR1*, regulatory genes controlling *mecA* expression and numerous other elements and resistance determinants (Diep *et al*., 2006). A distinctive feature of Methicillin resistance, its heterogeneous expression, borderline resistance, a low-level type of resistance to Methicillin exhibited by strains lacking *mecA*, was associated with modifications in native PBPs, β-lactamase hyperproduction, or possibly a methicillinase (Diep *et al*., 2006). The resistance phenotype was influenced by numerous factors, including *mec*A and β-lactamase regulatory elements, *fem* factors and yet to be identified chromosomal loci. The heterogeneous nature of Methicillin resistance confounds susceptibility testing. Methodologies based on the detection of *mecA* are the most accurate (Diep *et al*., 2006). Vancomycin was the drug of choice for treatment of infections caused by Methicillin-resistant strains. PBP 2a confers cross-resistance to most currently available β-Lactam antibiotics. Investigational agents that bind PBP-2a at low concentrations appear promising but had not been tested in humans. Alternatives to Vancomycin are few due to the multiple drug resistances typical of MRSA (Chambers 1997).

In Schleswig-Holstein, a resident was diagnosed with furuncle caused by a Panton-Valentine leukocidine (PVL)-positive (CA-MRSA). As a result of active cased finding, 54% of all residents were screened for MRSA and two further PVL-positive CA-MRSA cases were identified (Dudareva *et al*., 2011).

A Reported case of empyema caused by MRSA sequence type ST398 was in a 79-year-old Spanish man who had severe chronic obstructive pulmonary disease; he was treated by intravenous Levofloxacin, but he did not clinically improve. The isolates were MRSA from his specimens and nasal swab. Antimicrobial drug therapy was changed to intravenous Linezolid, but the patient's clinical situation rapidly worsened and he died of multiorgan failure (Lozano *et al*., 2011).

MRSA caused high-throughput genomics approach that provides a high-resolution view of the epidemiology and microevolution of a dominant MRSA strain (Simon *et al*., 2010). This approach revealed the global geographic

structure within the lineage, its intercontinental transmitted through four decades, the potential to trace person-to-person transmission within a hospital environment. The ability to interrogated and resolved bacterial populations was applicable to a range of infectious diseases, as well as microbial ecology (Simon *et al*., 2010).

Antibiotic resistance was with 40 *S..aureus* strains clinical isolated from 110 diabetic patients 36% was evaluated to 80% of the isolates showed multidrug-resistance to more than eight antibiotics and 35% isolates were found to be MRSA. All *S-.aureus* strains 100% screened from diabetic clinical specimens were resistant to Penicillin, 63% to Ampicillin, 55% to Streptomycin, 50% to Tetracycline and 50% to Gentamicin. However, low resistance rate was observed to Ciprofloxacin 20% and Rifampicin 8%. In contrast, all 100% *S.aureus* strains recorded susceptibility to Teicoplanin, which was followed by Vancomycin 95%. Genotypical examination revealed that 80% of the Aminoglycoside resistant *S.aureus* (ARSA) had Aminoglycoside modifying enzyme (*AME*) coding genes; however, 20% of ARSA which showed non-*AME* mediated (adaptive) Aminoglycoside resistance lacked these genes in their genome. In contrast all MRSA isolates possessed *mecA*, *femA* genetic determinants in their genome (Raju *et al*., 2010).

Therefore, the aim of this study was to investigate the new isolated MRSA strain from a clinically uncured patient (from Taif area) with chronic prostatitis and failed treatment with Amoxicillin for two month. He was treated by Quinolones antibiotics for three weeks and thus he clinically cured. The strain was re-investigated with available antibiotics groups as a means of reaching the confirmation that it is a new module of MRSA (resistant to more antibiotics than before) in Taif area.

Case report

A 50-year-old male who had chronic prostatitis was treated for two months by antibiotic Amoxicillin, but no clinical improvement was seen. He was then treated by antibiotic Quinolones group for three weeks. He was treated by Quinolones which improved his clinical conditions more rapily than before, leading to his cure. We also re-investigated and identified the causative micro organism.

2. Materials and Methods

This study was carried out in the Microbiology section of Bioneers Lab in Al-Taif area. All clinical specimens were collected in sterile containers, taking care of all the aseptic measures. The specimens were inoculated on sheep blood agar, Mac-Conkey agar and blood chocolate agar. All inoculated media were kept at 37°C in incubator for 24 hours. ATCC strain was used as a control *(S. aureus* 25923) (CDC 2003). *S. aureus* isolate, recovered from the patient specimens, was identified by conventional method and conformed by colony morphology, Grams staining, Catalase, Coagulase, DNase latex agglutination and Mannitol fermentation test (National standard method, 2007).

Pastorex *Staph*-Plus latex agglutination kit (BIO-RAD) and API-*Staph* test system (Bio-Merieux Vitek, Hazelwood, Mo.) were used. Tryptic soy broth and Tryptic soy agar for purify the strain (Difco, Detroit) (CDC 2003) were also used.

The isolate was then tested by the modified Kirby Bauer disc diffusion technique and the results were interpreted as outlined by National Committee for Clinical Laboratory Standards criteria NCCLS. The data were confirmed by Micro-scan and API (Brown *et al*., 2005). Standardized methods for disc diffusion had been defined by the BSAC51,67 and the NCCLS were used for the cultivation and analysis of bacterial cultures, grown at 37°C with vigorous aeration (CDC 2003).

The antibiotics discs tested were differentiated into groups: β-Lactam group (Penicillin 10µg, Oxacillin 1µg, Ampicillin 10µg and Augmentin 20/10µg), Macrolides group (Nitrofurantoin 300µg, Erythromycin 15µg, Clindamycin 2µg and Azithromycin 15µg), Aminoglucosides group (Amikacin 30µg and Gentamycin 10µg), Quinolones group (Ciprofloxacin 5µg, Ofloxacin 5µg and Norfloxacin 10µg) and Glycopeptides group (Vancomycin 30µg). Antibiotics discs were obtained from various manufacturers using a disc diffusion method on Mueller-Hinton agar (Oxoid) according to the CLSI (Clinical and Laboratory Standards Institute) directives (M100-S17M2-A9). MICs of Moxifloxacin and Clindamycin were determined by the E-test method (AB BIODISK) (CDC 2003).

3. Results

The strain of *S.aureus* (MRSA) was isolated from the infected patient was tested for antimicrobial susceptibility test. The MRSA strain was resistant to antibiotics groups as follow β-Lactam including (Penicillin, Oxacillin, Ampicillin and Augmentin) as the patient was treated by the same group, and Glycopeptides including (Vancomycin) (Table 1). The strain was intermediate sensitive for Macrolides (Erythromycin, Clindamycin and Azithromycin) and Aminoglucosides (Gentamycin). The greatest prevalence of resistance was found to all β-Lactam, Macrolides, Glycopeptides groups and unfortunately resistant to Vancomycin. Fortunately, great Sensitive to Nitrofurantoin from Macrolides group, Amikacin from Aminoglucosides group and (Ciprofloxacin, Ofloxacin and Norfloxacin) from Quinolones group as the patient was cured with the same group.

4. Discussion

The *S. aureus* strain isolated from the patient was called MRSA because of multi-resistance to antibiotics *in-vivo* (i.e., during the infection period of patient), and *in-vitro* (i.e., during the search). The isolated MRSA passed as in infection through the search; it was resistant to all β-Lactam group, tested as in the first period of the infection of the patient, that he was treated by Amoxicillin and thus failed to get clinically cured; it was however sensitive to all Quinolones group, tested as in the second period, which was used to treat the patient with, and thus lead to his cure.

MRSA had become an enormous problem for health care providers, because it hardly treats and it is sometimes called super bug. Multiple studies had been carried out on the growing concern over multidrug resistance.

Table 1 Antibiotics Sensitivity Tests for Isolated MRSA Strain

Antibiotics groups	Concentration	Sensitivity range	Results mm	Degree
β-Lactam:				
Penicillin	10 µg	>29 mm	18 mm***	Resistant
Oxacillin	1 µg	>18 mm	10 mm***	Resistant
Ampicillin	10 µg	>27 mm	15 mm***	Resistant
Augmentin	20/10 µg	>28 mm	17 mm***	Resistant
Macrolides:				
Nitrofurantoin	300 µg	>17 mm	18 mm*	Sensitive
Erythromycin	15 µg	>23 mm	16 mm**	Intermediate
Clindamycin	2 µg	>30 mm	25 mm**	Intermediate
Azithromycin	15 µg	>17 mm	12 mm**	Intermediate
Aminoglucosides:				
Amikacin	30 µg	>17 mm	19 mm*	Sensitive
Gentamycin	10 µg	>15 mm	13 mm**	Intermediate
Quinolones:				
Ciprofloxacin	5 µg	>21 mm	22 mm*	Sensitive
Ofloxacin	5 µg	>22 mm	24 mm*	Sensitive
Norfloxacin	10 µg	>17 mm	19 mm*	Sensitive
Glycopeptides:				
Vancomycin	30 µg	>17 mm	14mm***	Resistant

* The measure of inhibition zone by mm for sensitive antibiotics.

** The measure of inhibition zone by mm for intermediate antibiotics.

*** The measure of inhibition zone by mm for resistant antibiotics.

MRSA was becoming a problem involving hospital setting and community transferring. The rate of the prevalence of MRSA isolates had increased over the years (Bozdogan *et al*., 2003; Boyle-Vavra and Daum 2007; Maree *et al*., 2007). More than half of MRSA was positive for Panton-Valentine Leuocidine PVL which CA-MRSA (Dudareva *et al*., 2011). The transmission of MRSA as dominant was very easy to trace person-to-person (Simon *et al*., 2010). The old Spanish man died with MRSA sequence type ST398 which caused empyema (Lozano *et al*., 2011). MRSA was responsible for 37% of fatal cases of sepsis in the UK in 1999, up from 4% in 1991. More than half of *S. aureus* infections in USA were resistant to Penicillin, Methicillin, Tetracycline and Erythromycin (Bulent *et al*., 2003). Vancomycin was the only effective agent available at the time against MRSA. However, MRSA strains had intermediate levels of resistance (Bulent *et al*., 2003). The first identified case was in Japan in 1996, and strains have since been found in hospitals in England, France and USA. The first documented strain with complete resistance to Vancomycin appeared in the USA in 2002 (Bozdogan *et al*., 2003). In this study, the pattern of antibiotics sensitivity revealed highly resistant to β-Lactam group (Mark *et al*., 2007). Thus, more resistance appeared in the tested MRSA strain for β-Lactam group and Vancomycin also (Raju *et al*., 2010). The MRSA tested acquired strain from community was very resistant to antibiotics including Penicillin, Oxacillin, Ampicillin, Augmentin and Vancomycin (Christopher, 2002). This was due to the changing in the genome of MRSA leading to the increase of the antibiotic resistance contrast; MRSA isolates possessed *mecA*, *femA* genetic determinants in their genome (Diep *et al*., 2008; Raju *et al*., 2010). The tested MRSA strain was intermediate to Erythromycin, Clindamycin, Azithromycin and Gentamycin due to cross-resistance to most currently available antibiotics related to β-Lactam group (Chambers, 1997). On the other hand, the MRSA strain tested appeared sensitive to some antibiotics Macrolides group (Nitrofurantoin), Aminoglycosides group (Amikacin) and Quinolones group (Ciprofloxacin, Ofloxacin and Norfloxacin) (Raju *et al*., 2010). So, it showed that the antibiotics currently of choice for the treatment of life-threatening infection caused by MRSA decreased.

5. Conclusion

This study showed high resistance of new MRSA strain to β-Lactam, Glycopeptides groups and some intermediate from other groups (Macrolides and Aminoglucosides). The

study also showed a restricted choice for treatment because the sensitive antibiotics for isolated MRSA strain decreased in number. So the treatment of new MRSA will be difficult, good hygiene practices, infection control and emphasis on hand washing, control misuses of antibiotics, etc. may slow down the process of resistance.

Though the prevalence of MRSA is alarming, the rise of CA-MRSA in recent times has been almost troublesome. Genetic elements allow CA-MRSA to rapidly spread and infection. This has led to poor prevention methods on sporting teams, in prisons and in training centers. The rapid transmission of MRSA infections and the potential for them to progress into life-threatening conditions make these situations quite dangerous. While antibiotic resistance in MRSA increases, the most effective methods of dealing with this threat remain effective preventions of infection.

The next step for confirmation of new MRSA strain will use the molecular methods PCR, including Real-time PCR and Quantitative PCR; they are being increasingly employed in clinical laboratories. This protocol includes detection of nuclease, coagulase, protein A (*spa*), *femA* and *femB*, *Sa442*, 16S rRNA and surface-associated fibrinogen-binding protein genes, which detects a specific sequence within the internal transcribed spacer (ITS) region of MRSA, have proved to be a successful and a novel molecular approach utilizing isothermal signal-mediated amplification of mRNA transcribed from the *coa* gene.

Another common laboratory test is a rapid latex agglutination test that detects the PBP2a protein. PBP2a is a variant penicillin-binding protein that imparts the ability of *Staph.aureus* resistant to Oxacillin. It is important to mention that those protocol control strains, both positive and negative for the target genes, are essential.

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References

Boyle-Vavra S and Daum RS. 2007. Community-acquired Methicillin-resistant *Staph. aureus*: the role of Panton-Valentine leukocidin. *Lab. Invest*., **87(1):** 3–9.

Bozdogan BüL, Esel D, Whitener C, Browne FAand Appelbaum PC. 2003. Antibacterial susceptibility of a Vancomycin-resistant *Staph.aureus* strain isolated at the Hershey Medical Center. *J. Antimicrobial Chemother*, **52(5)**: 864.

Brown DFJ, Edwards DI, Hawkey PM, Morrison D, Ridgway GL, Towner KJ and Wren MWD. 2005. Guidelines for the laboratory diagnosis and susceptibility testing of MRSA. *J. Antimicrob Chemother.*, **56**: 1000-1018.

Bulent B, Duygu E, Cynthia W, Frederick AB and Peter CA. 2003. Antibacterial susceptibility of Vancomycin-resistant *Staph.aureus* strain isolated at the Hershey Medical Center. *J. Antimicrobial Chemother.*, **52(5):**864-868.

Center for Disease Control and Prevention(CDC). 2003. Standard safety practices in the Microbiology. Appendix 1. 163-235.

Chambers HF .1997. Methicillin resistance in *Staph.aureus*: molecular and biochemical basis and clinical implications. *Clin. Microbial. Rev*., **10**:781-791.

Christopher TW .2002. MRSA in Boston. Harvard Medical School, *Lancet*, **359** (9320): 1819 – 1827.

Cynthial LM, Robert SD, Susan BV, Kelli M and Loren GM. 2007. Community-associated Methicillin-resistant *Staph.aureus* isolates causing healthcare-associated infections. *Emerging Infectious Dis.*, **13(2):**236-242.

Diep BA, Chambers HFand Graber CJ. 2008. Emergence of multidrug-resistant, community-associated, Methicillin-resistant *Staph.aureus* clone USA300 in men who have sex with men. *Ann. Internal Med.*, **148(4):** 249–57.

Diep BCH, Carleton H, Chang R, Sensabaugh G and Perdreau RF. 2006. Virulence genes in the evolution of hospital- and community- associated strains of MRSA. *Infect Dis.*, **193(11):**1495-503.

Dudareva S, Barth A, Paeth K, Krenz-Weinreich A, Layer F, Delere yand Eckmanns T. 2011.Cases of community-acquired MRSA in an Asylum Seekers Center in Germany, November 2010. *Euro surveillance* **16(4):**165-173.

Hidemasa N, Norihisa N, Masami I, Mikiko H, Minoru S, Shinji Y, Tomoko Y, Tqkaski A, Mitsura S and Masanori S.2008. Molecular epidemiology and antimicrobial susceptibilities of 273 exfoliative toxin-encoding-gene-positive *Staph.aureus* isolates from patients with impetigo in Japan. *J. Med. Micorbiol*., **57(10):**1251-1257.

Holten KB and Onusko EM. 2000. Appropriate prescribing of oral β-lactam antibiotics. *American Family Physician*, **62(3):**611-620.

Lozano C, Gomez-Sanz E, Zarazaga M and Torres C. 2011. Empyema caused by MRSA ST398 with atypical resistance profile, Spain. *Emerging Infect. Dis.*, **17(1):**138-140.

Maree CL, Daum RS, Boyle-Vavra S, Matayoshi K and Miller LG. 2007. Community-associated Methicillin-resistant *Staph.aureus* isolates causing health care-associated infections. *Emerging Infect. Dis.*, **13(2)**: 236–42.

Mark CE, Ashley R, Gaynor R, Edward JF, Hajo G and Brian G.2007. The evolutionary history of Methicillin-resistant *Staph.aureus* (MRSA). *J. Lab. Invest.*, **87(1)**:15-20.

National Standard Method.2007. Standards Unit, Evaluations and Standards Lab., *Center for Infections*. 1-17.

Raju S, Ajay KO, Patil SA and Kelmani CR. 2010. Prevalence of multidrug-resistant *Staph.aureus* in diabetics clinical samples. *World J. Microbiol. Biotechnol*., **26(1):**171-176.

Ruef C .2004. Epidemiology and clinical impact of Glycopeptides resistance in *Staph.aureus*. *Infection*, **32(6):**315-327

Saiful AJ, Mastura M, Zarizal S, Mazurah MI, Shuhaimi M and Ali AM. 2006. Detection of *Staph.aureus* using mecA/nuc genes and antibiotic susceptibility profile of Malaysian clinical isolates. *World J. Microbial. Biotechnol*., **22(12):**1289-1294.

Simon RH, Edward JF, Mathew TGH, Micheal AQ, Emma KN, Narisara C, Susana G, Ana T, Nick D, Jodi AL, Jonathan DE, Herminia DL, Julian P, Sharon JP and Stephen DB. 2010. Evolution of MRSA during hospital transmission and intercontinental spread. *Science J.*, **327**(5964):469-474.

Susan BV and Robert SD. 2007. Community-acquired methicillin-resistant *Staph. aureus*: the role of Panton-Valentine leukocidin. *Lab. Invest.*, **87**:3-9.

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