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EDITORIAL PREFACE

Jordan Journal of Biological Sciences (JJBS) has had another great year. We have seen a significant increase in articles submission from both regional and international scholars. The editorial board members of JJBS have been very busy throughout the year to maintain excellence in the quality publication of accepted papers. As a result, JJBS has been indexed by CABI's Full-Text Repository, EBSCO and is currently under evaluation to be indexed in National Library of Medicine's MEDLINE\ PubMed system and Elsevier's SciVerse Scopus.

As in the previous two years, this sixth volume of JJBS will include four issues, ten to twelve articles in each issue. In the coming year, it is my vision to have JJBS publishes more outstanding papers and review articles from distinguished scholars in various areas of biological sciences. In addition, I will be working on the inclusion of JJBS in ISI, which will lead to a wider readership and good impact factor. As you read throughout this inaugural volume of JJBS, I would like to remind you that the success of our journal depends directly on the number of quality articles submitted for review. Accordingly, I would like to request your participation by submitting quality manuscripts for review and by encouraging your colleagues to do the same. One of the great benefits we can provide to our prospective authors, regardless of acceptance of their manuscript or not, is the mentoring nature of our review process. JJBS provides authors with high quality, helpful reviews that are shaped to assist authors in improving their manuscripts.

I would like to thank the JJBS International Advisory Board members for their continuous support of JJBS. Furthermore, I would like to thank the JJBS Editorial Board members for their exceptional work and continuous support to JJBS. My thanks are also extended to the Hashemite University and Jordanian Scientific Research Support Fund for their continuous financial and administrative support to JJBS.

Moreover, and as always, I would like to highlight and proudly thank the group of authoritative reviewers, both local and international, who have done an outstanding work. We are honored to have you on our review list and many thanks for your valuable mentorship and contributions you provided to authors. Indeed, we count on your excellent reviews to include only high quality articles worthy of publication in JJBS. Together, we strive to make JJBS reach a remarkable rank among other international journals. I very much appreciate your support to make JJBS one of the most authoritative journals in biological sciences.

Prof. Khaled H. Abu-Elteen
Editor-in-Chief
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Mini-Review

Bionanotechnology: The Novel Nanoparticles Based Approach for Disease Therapy

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Abstract

Bionanotechnology has probably been the most developing field in the last decade as an integration of biotechnology on the molecular level and nanotechnology. It provided new avenues of research and development to produce novel and new nanomaterials of medicinal applications which paved the way to the vibrant field of nanomedicine. This is dependent on combining biological norms with unique characteristics of nano-sized particles to carry out very specific functions. This will hopefully lead to the elucidation of our understanding of disease therapy and the biology of different life forms. The prospects of using nanoparticles in such fields would include drug and gene therapy, tumor control, detection of pathogens and proteins, tissue and cell engineering, DNA probing, pest control in agricultural fields and many other applications. Knowing the expected implications of novel nanoparticles applications in nanoparticle based medicine and research, it became important to compile literature relevant to this subject in a single publication. Thus, this mini review was aimed to be a contribution to this emerging volatile area of bionanotechnology, where efforts are being exerted to apply basic scientific ideas in clinical practice.

Keywords: Bionanotechnology, Nanoparticles, Nanomaterials, Delivery Systems, Disease Therapy.

1. Introduction

Nowadays, global concern has emerged as we are entering the post antibiotic era with reduced capabilities in some areas to combat microbes and disease (Blecher *et al*., 2011). Hence, the development of novel therapeutic approaches to these challenges constitute a focal point of modern research (Carpenter *et al*., 2009; Hentzer and Givskov, 2003; Al-Hussaini and Mahasneh, 2009 a, b; Al-Hussaini and Mahasneh, 2011; Taleb *et al*., 2012; Taleb and Mahasneh, 2010a; Taleb and Mahasneh, 2010b; Taleb and Mahasneh, 2012; Knipe *et al*., 2013). The alternative to traditional therapies would be through investigating new and novel avenues of molecular therapies to include, for example, use of probiotics (Mahasneh and Abbas, 2010; Elbaz and Willner *et al*., 2012), quorum sensing regulation where the virulence of many pathogens is regulated by quorum sensing mechanism (Abudoleh and Mahasneh 2012) and last but not least the combination therapy. In this case, chemotherapy is given in combination with other means such as probiotics and nanoparticles to target specifically the diseased cells or tissues (Taleb and Mahasneh, 2012; Day *et al*., 2009). Such approaches will definitely improve the outcomes of both traditional, as well as, novel bionanotechnology approaches in nanomedicine, stem cell and gene therapies (Lin *et al*., 2013). The nanomedicine arena, if exploited properly, would have far reaching implications on medicinal professions, definition of disease, their diagnosis and treatment. Nanomedicine is based upon the use of nanoparticles at the level of 1-100 nm (Caruthers *et al*., 2007; Pissuwan *et al*., 2011; Knipe *et al*., 2013).

In the last two decades, nanoscaled technologies in health sciences have witnessed ample efforts in research, development and patenting (Caruthers *et al*., 2007). These efforts formed the cornerstone for the transfer of nanotechnological research into clinical settings (Elbaz and Willner, 2012) and it was expanded to shape what is known nowadays as nanomedicine. The aim of this activity was and still is to maximize efficiency in controlling diseases. To achieve this, nanomedicine concepts are used in the early diagnosis which leads to suggesting the treatment program with the least possible side effects and finally to evaluate its efficacy and feasibility (Park *et al*., 2010). Conversely, the European Science Foundation defined nanomedicine as "the science and technology of diagnosing, treating and preventing disease and traumatic injury, of relieving pain, and of preserving and improving human health, using molecular tools and molecular knowledge of the human body" (Bawa *et al*., 2005). Nanodrugs, particularly nano-particulate drugs, offer advantages over their bulk counterparts in one or more properties such as solubility, bioavailability, half life, stability, ability to cross biological barriers, toxicity, dose delivery and specificity

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(Blecher *et al*., 2011; Paulo *et al*., 2010). To elucidate nanomedicine implications in medical practice, such as cancer therapy, development of specific drug delivery system (Figure 1) and nanorobots have attracted a great deal of research where now we know exactly what such systems specifications would be (Arcese *et al*., 2012). The fully functional nanorobot should be able to carry a payload (drug), direct mobility to specific targets in patient body, adhere or attach to tumor cells and finally release the payload. Further researchers' efforts would no doubt be turned to design very specific nanorobots and nanoparticles.

The hopes surrounding nanotechnology in different fields, including medicine, are immense (Salvador-Morales *et al*., 2012). It does not offer improvements to existing techniques only, but provides leads to new tools and applications (del-Pozo-Rodriguez *et al*., 2013). Among these would be drug manipulation at the nanoscale with what impact such manipulation would leave upon the bioactive characteristics such as controlled release, solubility, retention time and probably environmentally triggered controlled release (Douglas *et al*., 2012). It is also realized that nanoparticles' surface area provides increased functionality which lends itself to further biomedical as well as environmental applications (Chunbai *et al*., 2013) and gene therapy (Pissuwan *et al*., 2011).

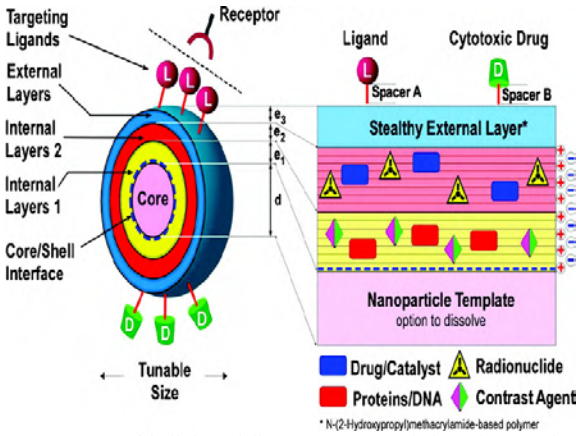

Figure 1. Core/shell drug delivery system consisting of cytotoxic stealth carrier particles based on nanoparticles. Usually the core is surrounded by several layers and chemical groups could be added to the external layer thus allowing attachment of biomolecules.

2. Applications of Biocompatible Nanoparticles

The distinct characteristics of the varying types of nanoparticles presented them for a wide variety of applications in medicine (Sih *et al*., 2013), in biological sciences for the development of biosensors, DNA probes (Tang *et al*., 2006), in medicine (Mukherjee *et al*., 2007), in controlling pathogenic bacteria (Raja *et al*., 2012) and fungi (Min *et al*., 2009), and in ecological applications pertaining to water treatment (Lyon *et al*., 2006) and plant diseases (Jo *et al*., 2009; Aguilar-Mendez, 2010; Kim *et al*., 2008).

Nanoparticles of the size 1-10 nm were capable of readily interacting with HIV virus through selective binding to glycoprotein thus inhibiting viruses from binding to host cells (Elechiguerra *et al*., 2005). Metallic nanoparticles such as silver nanos are being used in medical implants and devices to prevent microbial infections (Geethalakshmi and Sarada, 2010). Furthermore, some nanoparticles have been tested for their probable use in burnt tissues, alternative dental materials and textile fabrics (Duran *et al*., 2007).

Gene therapy is an emerging field where nanoparticles would no doubt play a major role in elucidating means and ways of introducing and delivering genes into target cells or tissues. In here, nucleic acids are delivered through special delivery systems thus controlling gene flow and expression. Pedro *et al*. (2008) recall the great advances in human gene therapy which were driven by interest in understanding of the molecular mechanisms of diseases and what they implicate of vector design in the quest for producing more efficient and safe drug in addition to gene delivery systems thus bridging bottlenecks through new technologies. In this context, it is worthy to mention research efforts in the search for designer bionanoparticles in the form of viral like particles (VLPs). These nanoparticles are unique in their diversity in terms of structure, shapes, architecture and production means (Figure 2). In this direction, new administration routes are being sought through oral and pulmonary means although intravenous route is the one of choice for most current nanoparticles delivery (Uchegbu and Siew, 2013). It should be noted that an array of cell types exist in the human body, which means that higher organisms, including our bodies, are just compilations of multifunctional nanosystems (Suh *et al*., 2009). Research on nanoparticles usually involved organic, inorganic and composite nanos to be produced, characterized and then used in drug delivery, bioimaging and other applications which necessitates further biocompatibility studies (Salvador-Morales, 2012). The shortage of effective and safe *in vitro* delivery systems has been a challenging obstacle in developing new nanoparticle based therapeutics (Troiber and Wagner, 2011). To secure an acceptable level of safety, other aspects of nanoparticles have to be considered. Priority aspects include routes of administration, therapeutic dose and frequency as well as reproducibility (Mohanan, 2010; Azzazy and Mansour,2009).

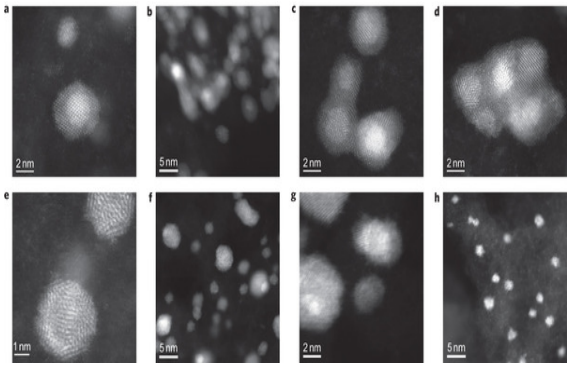

Figure 2. Images of nanoparticles showing diversity in terms of structure, shape, architecture and arrangements.

3. Nanoparticles Preparation Methods

Synthesis of nanoparticles has emerged in the last decade as an intersection between nanotechnology and biotechnology; hence the coining of the new expanding bionanotechnology (Raja *et al*., 2012). Due to the possible wide application of nanoparticles, this emerging science put nanoparticles at the tip of modern material science research. These nanoparticles are expected to exhibit new and/or novel characteristics associated with size, morphology and distribution (Veerasamy *et al*., 2011). Such characteristics are deemed to meet different applications of nanoparticles (Sayed and Ahmad, 2013). To improve the functionality of nanoparticles, it is mandatory to be functionalized accordingly with probably protein or biocompatible surfaces to reduce their toxicity if they are of inorganic origin such as the quantum dots (Sayed and Ahmad, 2013). Recent literature lists several possible means of nanoparticle synthesis ranging from totally chemical methods to fully biological processes (Veerasamy *et al*., 2011) such as reduction in solutions, chemical and photochemical reactions, thermal decomposition, radiation-assisted (Bhuvanasree *et al*., 2013), electrochemical and finally most recently green synthesis (Raja *et al*., 2012; Sayed and Ahmad, 2013; Gopinath and Velusamy, 2013). Nanoparticles synthesis using mostly toxic chemicals with low rates of conversion and laborious purification methods with high energy physical procedures prompted investigators to search for biologically mediated methods including microorganisms such as fungi, bacteria and plant systems including plant extracts (Gopinath and Velusamy, 2013; Sayed and Ahmad, 2013). These biological processes offered great deal in developing new methods to produce nanoparticles. They offer cost-effective methods and they are easily sealed up and economize energy demands and finally they are ecofriendly (Ponarulselvam *et al*., 2012; Song and Kim, 2009; Ahmad *et al*., 2011). Table 1 highlights the most used methods of nanoparticle preparation.

Table 1. Brief description of the most common and emerging nanoparticles preparation methods; (+): produced; (++): experimental optimization.

Method	Advantages	Scale-up
Nanoprecipitation of polymers	Fast; reproducible	++
Polymerization of alkylcyanoacrylates	Easy to produce core-shell nanos	++
Interfacial polycondensations	Modulation of nanos thickness	++
Formation of polyelectrolyte complexes	Positive or negative charge nanos are produced	++
Nanos from neutral nanogels	Organic solvent-free; controlled release of drugs	++
Ionic gelation	Possibility of controlled drug release in response to a stimulus	++
Gelation of emulsion droplets	Hydrophilic and biocompatible	++
Emulsification-reverse salting-out	Less stress upon fragile drugs	++
Emulsification-solvent evaporation	Encapsulation of hydrophilic and lipophilic drugs	++
Colloidal mill	Controlled nano size	++
Natural organisms-Fungi	Simple; non-toxic; reliable; ecofriendly	++
Natural organisms-Bacteria	Well defined size and distinct morphology; biocompatible; ecofriendly	++
Natural products-plant extracts (green synthesis)	Cost-effective; simple; ecofriendly	++

4. Molecular Therapy and Delivery Systems

The ample amount of information pertaining to the understanding of molecular mechanisms of diseases that was gathered in the last two decades coupled with advances at the gene therapy level has prompted research for more efficient, effective and safe delivery systems (Pedro *et al.,* 2008; Bansal *et al.,* 2012; Neves *et al.,* 2012). In cancer treatment, current research include development of delivery systems that allow very specific dosing routes and unusual therapeutic targets (Brannon-Peppas and Blanchette, 2012; Mignani *et al.,* 2013).

In the last few years we observed the emergence of many new and improved intelligent nanoscale platforms which is suitable for drug and gene delivery as well as imaging. It also witnessed the search for smart functionality in delivery systems to include cell and receptor specific targeting in the context of nano-bio interaction (Lehner *et al*., 2013) in nanomedicine (accepted). Nanotechnology-based carriers have shown very successful results in terms of low toxicity and efficient delivery to targets including cells and/or tissues of mammalian systems (Seth *et al*., 2012; Shen *et al*., 2012). Different nanotechnology systems would provide solutions for improved delivery with least toxicity which is the core for translating effectively basic medical sciences into enhanced clinical practice (Liu and Zhang, 2011; Singh *et al*., 2012).

In this context, consideration should be given to the size of delivery vehicle, ease of penetration into the cellular membranes and sustainability in the cytoplasm (Li and Huang 2010). Among the best studied nanocarriers are those synthesized from liposomes, dendrimers, carbon nanotubes and nanoclusters (fullerenes), polymers and metal and metal oxides nanoparticles. Table 2 presents some delivery systems and some of their unique characteristics that make therapy more effective.

From the aforementioned table, it is rather clear that the best fit delivery system is one that exhibits characteristics of both non-viral as well as viral delivery vectors coupled with disarming such systems from undesired properties.

Table 2. Diversity of different delivery systems available and under testing and could be used for the treatment of different diseases.

Delivery system	Main characteristics	Comments
Liposomes	Biocompatible and biodegradable; most suitable for DNA, RNA delivery into mammalian cells; persistence in blood circulation; increased specificity for gene therapy	Pharmaceutical and medical applications; easy to synthesize and easy storage
Dendrimers	Highly symmetric; many functional groups at surfaces; better encapsulation of genes; high payload delivery	May act as multifunctional delivery application; modifiable surface groups thus tuning their activity
Fullerenes	Carbon nanoclusters; possibility of functionalizing it in different ways such as making it water-soluble; less toxic than cationic liposomes	It forms protective sheath over bound DNA expanding chances of incorporation into chromosomes
Carbon nanotubes	Gene and drug delivery; compatibility with aqueous environments; nontoxic in mammalian cells and tissues	Very successful in gene delivery for disease therapy
Quantum dots	Extremely small; mainly of heavy metal origin; very effective in imaging enhancement	Often they undergo leaching in biological environment leading to toxicity in mammalian cells
Gold, silver magnetic nanoparticles	Highly noted in biomedical application, biodiagnostics, imaging, gene and drug delivery for disease and gene therapy	Nontoxic nature of gold nanos; non-opsogenic and of stealth properties; used in magnetic hyperthermia therapy cancer cells
Viral systems – oncoviruses	Specific gene therapy; stability during genome integration; insert large gene segments; infect only dividing cells	During gene therapy trials, possible recombination with endogenous human retroviruses
Viral systems – Adenovirus	Gene therapy; low pathogenicity for humans; infect non-dividing cells	It induces strong immune response; no integration into the host
Viral systems – Adeno-associated-virus	No pathogenicity and toxicity; infect non-dividing cells; long-term transgene expression	No specific integration' may mutagenize cells
Viral systems – Lentivirus	Can carry large gene inserts; stable expression; infect non-dividing cells	Biosafety problems

However, more work is needed to overcome problems such as lack of specific targeting, toxicity, low transfection efficacy and expression in case of gene therapy and specificity, all of which hindered advancement in molecular and regular therapy approaches (Knipe *et al*., 2013).

Finally, it seems that nanotechnology, especially bionanotechnology is expanding at a speedy pace exploring novel methods at both the traditional and the molecular levels of therapy.

It is also clear that progress of the new emerging field of nanomedicine is dependent on exploring bionanotechnology potential in terms of translating the laboratory based trials and observations into the real clinical settings. These necessitate further research on scale-up, inconsistencies from batch to batch, confirmation of stealth properties of nanoparticles *in vivo*, improved specificity of the different nanoparticles and finally improved targeting means through nanorobots development. The concept of smart nanosystems for new and novel biomedical applications is being exploited. Solution of all or part of these concerns would no doubt open new frontiers for the nano-based diagnostics for the detection of tumors, infections and neurological diseases among many others. At this stage of scientific discovery I would dare to say that nanoparticles-based nanomedicine would in the very near future transform the understanding of the human biological structure and function. This transformation will no doubt furnish new nanoparticle based applications to include chemotherapy, activity monitors (blood pressure, glucose monitors), biochips, pacemakers, insulin pumps, needleless injectors, medical flow sensors, drug delivery systems and finally designer gene therapy systems. This would expand the prospective horizons of nanoparticles use as well as the need for further investigations pertaining to the final fate and probable interactions of these bionanoparticles in biological systems.

5. Conclusion

Science has greatly advanced into the area of applied nanotechnology and the internal components of living cells are of the same scale. This lead bionano-technologists to look to cell biology for medicinal applications using biological structures, processes and information. Much of bionanotechnology is molecular biology based applications. Individual molecules, bacteria and viruses can be easily detected. Nano-particles are already in use for drug delivery trials, biological labeling, medical imaging and various other analytical purposes for clinical use. Other new and more complex bionanodevices are being produced and investigated for novel applications.

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Levels of Chromium and Copper in Liver and Muscle Tissues of the Round Sardinella *Sardinella aurita* (Valenciennes) from the Oran Coastline, Algeria

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Abstract

The aim of the present study is to determine the levels of two heavy metals (Chromium and Copper) in the liver and muscle tissues of *Sardinella aurita* from the Oran coastline. Metal levels in fish samples were analyzed by using atomic absorption. 400 readings were carried out between May and October 2007. The order of heavy metal accumulation in the fish were in the order of magnitude as Cu > Cr. The average concentrations recorded in the fish, in this study, ranged, respectively, between 1.9 to 3.97 mg/kg for Copper, and between 0.07 to 0.12mg/kg for Chromium. The levels measured in liver tissue are higher than those reported for muscle tissue. The present study confirms that the round Sardinella *Sardinella aurita* (Valenciennes, 1847), from the Oran coastline, can bioaccumulate heavy metals from a polluted environment. The average concentrations of Cr (0.083±0.01mg/kg) and Cu (2.78±2.92, recorded in our study, were above FAO limiting standards for food fish.

Key words. *Sardinella aurita;* Pollution, Heavy Metals, Chromium, Copper; Oran coastline.

1. Introduction

Heavy metal pollution has become a serious environmental and public health issue (Venkatramreddy *et al*., 2009). Heavy metals are commonly found in natural waters and some are essential to living organisms. Yet they may become highly toxic when present in high concentrations (Ibok *et al.,* 1989). The toxicity for humans is mainly caused by their persistence in the environment. This situation is a result of the rapid growth of population, intense industrialization and other anthropogenic activities, like the exploration and exploitation of natural resources, the extension of irrigation and modern agricultural practices (FAO, 1992). Due to their bioaccumulative and non-biodegradable properties, heavy metals constitute a core group of aquatic pollutants.

The objective of the present study is to determine the level of two heavy metals: chromium and copper in organs of *Sardinella aurita* from gulf of Oran.

Sardinella aurita (Teleosten; Clupeidae) was selected for the present study. It is a small pelagic fish that lives in tropical and subtropical waters of the western and eastern Atlantic Ocean, the Pacific Ocean, the Mediterranean and, occasionally, the Black Sea (Sabate's, 2006). In the Algerian coasts; it is very common and very abundant (Benamar, 2011). It is also considered, in Oran, the most consumed fish after sardine *Sardina pilchardus* and bug *Boops boops* (Benamar, 2011).

For the purposes of the present paper, two metals were detected: copper and chromium. It has been established that copper is one of the most abundant trace metals and for almost all organisms; it is an essential micronutrient (Duffus, 1980). The aquatic toxicology of Cr depends on both biotic and abiotic factors. The biotic factors include the type of species, age and developmental stage. When bioconcentrating in the food chain, heavy metals, including Cr, are potentially cytotoxic to aquatic biota. Therefore, an early detection and ecotoxicologic evaluation of a sensitive biomonitoring system comprising both in vivo and in vitro test systems is essential (Venkatramreddy *et al*., 2009).

The Chromium was also shown to accumulate mainly in metabolically active organs such as liver, gill and kidneys at high concentrations. It was shown that fish go under some behavioral alterations such as suspending feeding, irregular swimming and accelerated operculum movement when first encountered with chromium (Svecevicius, 2009). Chromium also caused structural changes such as hypertrophy and hyperplasia at gill epithelium, degeneration in fin rays and weakening of immune system (Synder and Valle, 1991; Bennani *et al.,* 1996; Arunkumar *et al.,* 2000).

2. Materials and Methods

2.1. Study Area

The gulf of Oran, on the Algerian Mediterranean coast , is located between the industrial gulf of Arzew in the east, and the un spoilt Andalouses Coast in the west (Figure 1). More than 90 million m3 of untreated wastewaters are discharged annually by the Oran metropolis and many industrial units. Generally, these industries shall carry out the evacuation of their waste without any processing. Chemical releases, discharges of hydrocarbons of petrochemical origin (industrial zone of Arzew), and dissolved mineral and metal waste are thrown more heavily in effluents from various industries. The sea constitutes a major receiving area for waste water in Oran, as is the case for the majority of Algerian coastal towns. The sea is also the direct discharge system for effluents from the town of Oran. Total waste-water discharge along the bay of Oran can be estimated for industrial plants (with water consumption of more than 1000 m3/year) and domestic discharge (SOGREAH Engineering, 1998).

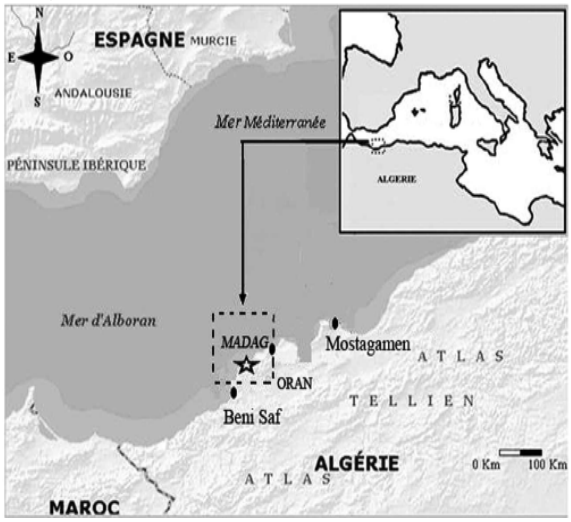

Figure 1. Geographical location of the site bay, Oran, Algeria

2.2. Sample Collection and Treatment

Samples of *Sardinella aurita* were collected between May 2007 and October 2007. Samples were placed in polyethylene bags and transported in a polystyrene ice-chest to the laboratory. The total lengths (cm) and weights (g) of the fish species were measured and after measurements, livers and muscles were removed, weighed and frozen until the time of their chemical analysis. Samples were prepared for trace metal analysis (UNEP, 1984a). Wet mineralization of samples was performed according to the method of Amiard *et al*. (1987) using a mineralizator type VELP. One ml of nitric acid is added to 1g wet weight of fish sample and then adjusted to 4 ml of bidistilled water after one hour at 95°C. The trace metals were determined by flame atomization (UNEP, 1984b) using a Perkin Elmer, Analyst 100 Atomic Absorption Spectrophotometer. To ascertain the accuracy of the results, blanks were included in every batch of nine samples analyzed. Analytical quality control measures adopted by the International Atomic Energy Agency (IAEA-350) – the intercomparison run which uses tuna fish homogenate as a certified reference material was used. The statistical analyses were made by means of the software Statistica version 5.0 (Co Microsoft).

3. Results

In this study, the concentrations of metals copper and chromium were detected in all samples. The average concentration in mg/kg wet weight, in the liver and muscles of the round *Sardinella*, are summarized respectively in figure 2.

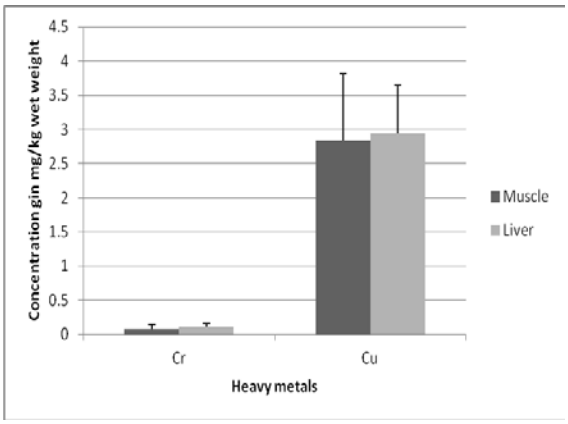

Figure 2. The average concentration of copper and chromium in organs of *Sardinella aurita* (mean ± standard deviation)**.**

The results showed that the values of copper were higher than that of chromium. The distribution patterns of Cr and Cu in the tow organs of the *Sardinella* followed the order: liver > muscles.

The mean concentrations of copper in all examined tissues varied from 1.90±0.4 to 3.96±1.54 mg/kg (figure 3). The higher concentration level in the two organs was observed in August (figure 3). Meanwhile, the chromium concentration ranged from 0.07±0.1 to 0.12 ±0.04 mg/kg. The highest mean concentration level of chromium was observed between June and August, as shown in figure 4.

The mean concentrations of Cr and Cu in liver and muscle of the round *Sardinella* showed that the females accumulate more than the males but without statistical significance (figure 5).

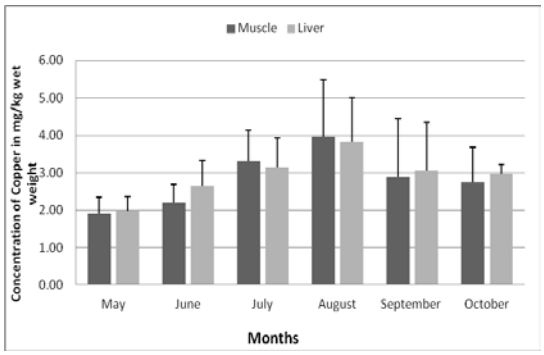

Figure 3. The monthly average concentration of copper in organs of *Sardinella aurita* (mean ± standard deviation)**.**

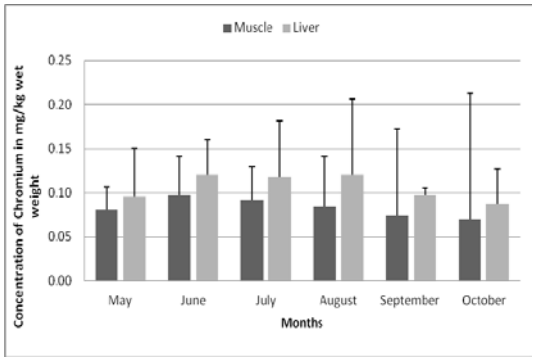

Figure 4. The monthly average concentration of chromium in organs of *Sardinella aurita* (mean ± standard deviation)**.**

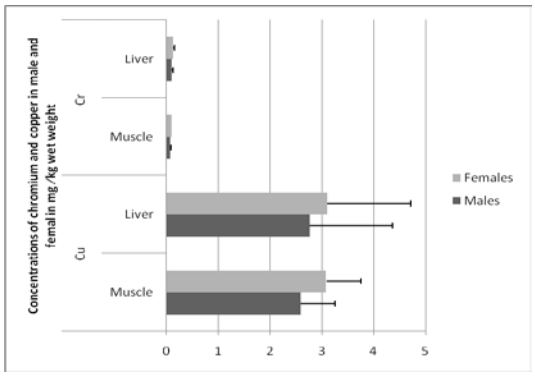

Figure 5. The average concentration between males and females of copper and chromium in organs of *Sardinella aurita* (mean ± standard deviation)**.**

4. Discussion

Concentrations of heavy metal, detected in the muscle and liver samples, showed different capacities for accumulating. The observed variability of heavy metal levels in *Sardinella aurita* depends on the level of exposure (water, food) and physiological factors (age, metabolic activity), together with factors related to environment such as temperature, physical-chemical parameters, presence of other metals (Kim et *al.,* 2004).

Metals accumulate in higher concentrations in the liver of *Sardinella aurita*, compared with muscle tissue. A similar result was found in the sardine *Sardina pichardus* fishing in the Bay of Oran (Merbouh, 1997). In fish, the most toxic metals tend to accumulate in the liver or kidneys (ICES, 1991). It has been established that the liver is a target organ for the accumulation of heavy metals, and the higher levels of these metals in the liver, compared to other organs, may be attributed to the high coordination of metallothionein protein with the metals (Hogstrand and Haux, 1991). In addition, the liver is the principal organ responsible for the detoxification, transportation and storage of toxic substances and an active site of pathological effects induced by contamination. The muscle is not considered an active tissue in accumulating heavy metals (Yilmaz, 2003). However, it has been proven that all trace metals concentration in the muscle tissue of *Saradinella aurita* was positively correlated to lipid content in the muscle tissues (Wafica and Aboul Naga, 1996). As for regulation and guideline levels, the Food and Agriculture Organization (FAO) has set permissible limits for heavy metals in the muscle of fish (because it is the part consumed). The mean levels found in *Sardinella aurita* samples are 2,8 mg/kg for Copper and 0.08 mg/kg for Chromium .

The levels of (Cu) in the fish sampled were higher than those of (Cr). This is because it represents an essential homeostatically regulated metal in all living organisms (Goyer, 1996). Cu as a microelement is essential for the normal growth and metabolism of plants, animals and most microorganisms (Schroeder et *al*., 1996). Additionally, it is the third largest trace element found in a living organism's body after iron and zinc. It is a component of many enzymes like cytochrome-c oxidase, superoxide dismutase, tyrosinase, dopamine beta hydroxylase ferrooxidases and amine oxidase. Copper is involved in neurotransmitter regulation, nutrient metabolism, collagen synthesis, cellular respiration and immune function.

The copper contents in the samples were less than the FAO-permitted level of WHO (3.0mg/kg) (Onianwa *et al*., 2001). Excessive intake of copper may lead to liver cirrhosis, dermatitis and neurological disorders (Onianwa *et al.,* 2001). Cu compounds, such as copper sulphate, are widely used as biocides to control macrophytes, freshwater snails that may harbour schistosomiasis or other disease pathogens, ectoparasites of fish and mammals, marine fouling organisms (Eisler, 1998).

On the other hand, Chromium does not normally accumulate in fish, and hence, low concentrations of Cr were reported even from different industrialized parts of the world (Moore and Ramamoorthy, 1984). Cr bioaccumulation in fish has been reported to cause impaired respiratory and osmoregulatory functions through structural damage to gill epithelium (Heath, 1991). The values of Cr recorded in *Sardinella aurita* in this study were above FAO limiting standards of 0.15mg/kg for food fish. The WHO has proposed that chromium (VI) is a human carcinogen. Several studies have shown that chromium (VI) compounds can increase in risk of lung cancer. Animal studies have also shown an increase in risk of cancer (Moore and Ramamoorthy, 1984).

In this study, higher concentrations of metals were observed in *Sardinella* collected between June and August, which coincide with the summer's months. During this period, peak temperature of the seawater is observed, in the bay of Oran. According to the Algerian National Office of Meteorology, the temperature attained between 26-27 ºC (Benamar, 2011).

The relationship between metal accumulation and sex, found in this study, may be due to the difference in the metabolic activities between the males and the females. According to Mortet (1988), an ovarian sequence for *Sardinella aurita* begins in the Bay of Oran during summer. It is characterized by an intense vitellogenic activity. Vitellogenesis is accompanied by an accumulation of reserves for the growth of eggs, but at the same time, by the metallic pollutants found in the biota (Mortet, 1988).

When comparing the rate of metal traces found in the muscle of *Sardinella aurita* with those found in *Sardinella* from the Strait of Messina (Sicily, Italy) and *Sardinella* from Alexandria waters, we notice that our samples are the least contaminated by chromium. Contrary to the levels of copper which appears to be high in *Sardinella aurita* from Oran coastline (Table 1).

Table 1. Comparative concentrations of copper and chromium in muscles of *Sardinella aurita* from different areas.

Areas studies	Chromium	Copper	Authors
Sardinella aurita from the Strait of Messina (Sicily, Italy)	0,36 ± 0,07	0,373 ±0,012	Lo Turco *et al* ., (2013)
Sardinella aurita from Alexandria waters	0,1± 0,04	0,98 ± 0,3	Wafica and Aboul-Naga (1996)
Sardinella aurita from Oran coastline	0,08± 0,06	2,83 ± 0,9	Present study

5. Conclusion

Compared to the muscle tissue, the liver tissue of *Sardinella aurita* from bay of Oran, contained elevated concentrations of the metals Cu and Cr. This capacity of metal sequestering highlights differences in elemental bioavailability between sites and thereby makes the liver more suitable for biomonitoring purposes than the muscle tissue.

This study shows that the concentration of heavy metals in different fish organs is still below the allowable limits. This means that the measured fish is healthy.

However, accumulation of heavy metal in tissues of fish species, that are consumed as protein sources, passed cumulatively along the food chain and can constitute, after a long time, a public health problem.

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Our gratitude is expressed especially to Professor Boutiba Zitouni, Director of Laboratory network of environmental surveillance, University of Oran, for her invaluable help. Great thanks go to Professor Claude Gimenes, National Institute of Telecommunications, Paris and Madam Anne Chatellier, College of France, Paris for their precious help and advice.

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Evaluation of Antioxidant Properties of *Morus nigra* L. Fruit Extracts [II]

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Abstract

Antioxidant activity for *Morus nigra* L. fruit extracts ethanol (Eth. ext.), flavonoid (F. ext.) and anthocyanin (A. ext.) and the isolated pigment (Pig. a) was evaluated using different methods such as (β-carotene-linoleic acid assay, reducing power assay, scavenging of hydrogen peroxide and total antioxidant capacity). The results proved that Pig. (a) was the most potent antioxidant followed by A. ext. and Eth. ext. Black mulberry fruits contained the highest amount of total phenolic content followed by Eth. ext., whereas flavonoid ext. contained the highest amount of total flavonoid content followed by black mulberry fruits. The effect of pH, temperature and storage had been studied to evaluate the remaining antioxidant capacity of the prepared extracts and Pig. (a). The results showed that the antioxidant capacity was decreased with increasing the time of boiling, storage and extreme pH values.

Key words: *Morus nigra* Extracts, Antioxidant Activity , β-Carotene-linoleic acid, Reducing Power, Scavenging of H_2O_2, Total Antioxidant Capacity, Stability.

1. Introduction

Oxygen is an element obligatory for life where living systems have evolved to survive in the presence of molecular oxygen, which has double-edged properties, being essential for life; it can also aggravate the damage within the cell by oxidative events (Shinde *et al.,* 2006). Oxidative stress results when the balance between the production of ROS exceeds the antioxidant capability of the target cell (Ahmad *et al.,* 2009). The antioxidant defense system in most living cells is composed of two components: antioxidant enzymes (endogenous antioxidants), and small molecule antioxidants (exogenous antioxidant) (Mugwerua and Rusling, 2006). Diet plays a vital role in the production of the antioxidant defense system by providing essential nutrient antioxidants such as vitamin C, vitamin E, and β-carotene. Other antioxidant plant phenols are flavonoids and essential minerals form important antioxidant enzymes. For example, superoxide dismutase (SOD) contains zinc and glutathione peroxidase that contains selenium (Willcox *et al.,* 2004; Kumar *et al.,* 2008). Plants are considered as one of the most important and interesting subjects that should be explored for the discovery and development of newer and safer drug candidates (Hamid *et al.,* 2011).

Under ideal circumstances the body would be in a steady state with free radicals produced and quenched by the endogenous antioxidants. When an endogenous and exogenous antioxidant system can't balance off the free radicals generated in human cells, oxidative stress occurs. Oxidative stress can cause oxidative damage to lipids, proteins and DNA (Jing, 2006). The antioxidants could attenuate this oxidative damage of a tissue indirectly by enhancing natural defenses of cell and/or directly by scavenging the free radical species (Goldberg, 2003).

Antioxidant activity of flavonoid polyphenols and other small molecules is dependent on bioavailability and redox potential which is closely related to its chemical structure (Pokorny *et al.,* 2001).

The antioxidant activity of anthocyanins is attributed to their ability to scavenge free radicals, and the binding with heavy metals such as iron, zinc, and copper. Anthocyanins are inducers of antioxidant enzymes such as glutathione-S-transferase (GST) and SOD (Hosseinian *et al.,* 2008). Besides their color features, anthocyanins have recently attracted even more interest due to their possible health attributes, such as a reducing risk of coronary diseases, reducing risk of stroke,

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anticarcinogen activity, anti-inflammatory effects and improved cognitive behavior (Zhang and Demain, 2005). Laleh *et al.* (2006) s howed that increasing in pH, temperature or exposure to light was able to spoil the anthocyanin molecule. Hurtado *et al.* (2009) proved that the isolated anthocyanins had higher capacity to capture free radicals than ascorbic acid and the hydroxylation degree of the isolated rutinosides had great influence on the antioxidant capacity.

The mulberry belongs to the genus *Morus* of the family *Moraceae.* Mulberry trees have historically been used for leaf yield in sericulture. In addition, their fruit have been used in folk medicine (especially in Chinese medicine) to treat diabetes, hypertension, anemia and arthritis. Also, black mulberry fruits are used for treating mouth lesions in Turkey. Recently, red and black mulberries have gained an important position in the food industry due to the presence of anthocyanins (Ozgen *et al.,* 2009).

The literature survey revealed that no work has been done to assess the antioxidant activity of black mulberry fruits *Morus nigra* L. prevailed in Kurdistan region. Thereby, this study was conducted to determine the antioxidant activity. Moreover, it was deemed of interest to investigate the antioxidant activity of the prepared fruit extracts by comparing various methods including β -carotene-linoleic acid assay, reducing power assay and total antioxidant capacity, besides the determination of total phenolics and flavonoids contents. It was also important to evaluate the effect of heat treatment, pH and storage on the stability of antioxidant activity.

2. Materials and Methods

2.1. Collection of Plant Materials

Black mulberry fruits *Morus nigra* L. were collected in June, 2008 from Duhok-dam, Duhok city- Kurdistan Region of Iraq. The ripe fruits were kept in plastic containers at -10 ºC until the time of use (Pantelidis *et al*., 2007; Spada *et al*., 2008). The plant was botanically authenticated in Agriculture College –Forestery Department, University of Duhok.

2.2. . Chemicals

All chemicals used were of analytical grade. Tannic acid, ammonium molybdate tetrahydrate, potassium ferricyanide and Tween 20 were obtained from Fluka; qurecetin dihydrate were obtained from ROTH; β-carotene from USP; Folin- Ciocalteu reagent from Ajax; linoleic acid from Merck; α-tocopherol from USD and others were obtained either from Fluka or Sigma chemicals.

2.3. Preparation of Plant Extracts

The ethanol extract (Eth. ext.) of black mulberry fruits was prepared according to the method of Laleh *et al*. (2006). The flavonoid extract (F. ext.) of black mulberry fruits was prepared according to the methods of (Peach *et al*., 1955; Harborne, 1984; Andersen and Markham, 2006). Anthocyanin extract (A. ext.) was prepared according to the method of (Harborne, 1984; Schofs, 2004; Andersen and Markham, 2006). Chromatographic techniques were applied for purification and isolation of pigment (a) (Pig.(a)) from anthocyanin extract.

2.4. Antioxidant Activity of Black Mulberry Extracts and Pig. (a)

2.4.1. Total Phenolic Content (TPC)

Total phenolics content of black mulberry fruits and extracts were measured according to the methods that were involving Folin- Ciocalteu reagent and tannic acid as a standard (Slinkard and Singleton, 1997; Kaur and Kapoor, 2002). All samples were assayed in three replications. The same procedure was applied to all standard tannic acid solutions (0–2500 mg/50 ml), and standard curve was obtained. Results were expressed as milligrams of tannic acid equivalent per gram of extract (mg TAE/g).

2.4.2. Total Flavonoid Content (TFC)

Total flavonoid content was determined according to the method described by (Chang *et al.,* 2002; Hsu, 2006). All samples were assayed in three replications. The same procedure was applied to all standard quercetin solutions (12.5–100 µg /ml) in methanol and standard curve was obtained**.** Results were expressed as milligrams of quercetin equivalent per gram of extract (mg QE/g).

2.4.3. Scavenging of Hydrogen Peroxide (H_2O_2)

The ability of the black mulberry fruits extracts and Pig. (a) to scavenge hydrogen peroxide was determined according to the method of (Ruch *et al.,* 1989; Oktaly *et al*., 2003). All samples were assayed in three replications. Results were expressed as milligrams of ascorbic acid equivalent per gram of extracts (mg AAE/g) .

2.4.4. Total Antioxidant Capacity

The phosphomolybdenum assay was performed according to the methods of (Prieto *et al.,* 1999; Delouee *et al.,* 2007). All samples were assayed in three replications. The antioxidant capacity of extracts was expressed as micrograms of α-tocopherol equivalent per milliliter of extracts using extinction coefficient of (4 x 10^3 M^{-1} cm^{-1}) according to Beer's law.

2.4.5. β–Carotene-Linoleic Acid Assay

In β-carotene -linoleic acid assay, antioxidant activity of extracts and of some common antioxidants such as α-tocopherol was determined according to the method of (Marco, 1968; Barriere *et al*., 2001) with slight modifications. Antioxidant activity (AA) was calculated as percentage of inhibition relative to the control (α-tocopherol sample).

2.4.6. Reducing Power Assay

The ability of extracts to reduce iron (III) was assessed by the method of (Oyaizu, 1986; Yildirim *et al*., 2001; Tsasi *et al*., 2006; Su *et al*., 2009). The increase in absorbance of reaction mixture was interpreted as increase in reducing activity of the extract and the results were compared with ascorbic acid as (positive control). All samples were assayed in three replications.

2.5. Effect of Temperature, pH and Storage on the Antioxidant Capacity

The black mulberry fruits extracts and Pig. (a) were heated at 50 and 100 ºC for (60 and 120 min) and the residual antioxidant capacity was determined using total antioxidant capacity method as previously described. For pH stability, the extracts were pre-incubated at different pH values (3, 5, 7, 9 and 11) and the residual antioxidant capacity was evaluated. The extracts were also stored in the dark at 5ºC, and the antioxidant capacity was determined at intervals of 30 days over a period of 90 days. All samples were assayed in three replications. The effect of temperature, pH and storage on the antioxidant capacity of extracts was expressed as micrograms of α- tocopherol equivalent per milliliter of extracts using extinction coefficient of ($4x10^3$ M^{-1} cm^{-1}) (Arabshahi *et al.,* 2007).

2.6. Statistical Analysis

The statistical analysis for all experiments was done by using (SAS, 2000). Means comparison was done by Duncan's Multiple Ranges Test under 1%, and figures were done by Graph Pad Prism 5 program.

3. Results and Discussion

3.1. Antioxidant Activity of Black Mulberry Extracts and Pig. (a)

3.1.1. Total Phenolic Content

Total phenolic content of black mulberry fruits, Eth., F. and A. ext. was determined using the calibration curve between concentration and absorbance at 760 nm of standard tannic acid as shown in Table 1 and Figure 1. Phenolic compounds are one class of antioxidant agents which considered as good proton donors resulting in free radical terminators and contributed to the antioxidant activities of plant (Garzon and Wrolstad, 2009). Mulberries are rich source of phenolics such as rutin, kaempferol, quercetin, isoquercetin and chlorogenic acid (Apak *et al.,* 2007; Turkoglu, *et al.,* 2007). A great variation in terms of total phenolics content was observed among black mulberry fruits and their extracts.

Table 1. Total phenolic content of black mulberry fruits, Eth., F. and A. ext.

Sample	Concentration (mg TAE/g)
Black mulberry fruits	20.19
Eth. ext.	14.26
F. ext.	5.36
A. ext.	3.38

The highest amount of total phenols was observed for black mulberry fruits (20.19 mg TAE/g) which was in agreement with the results that were obtained by Ercisli and Orhan (2007), where they reported that black mulberry fruits were rich in phenols. Also, Eth. extract was high in its phenols content (14.26 mg TAE/g).

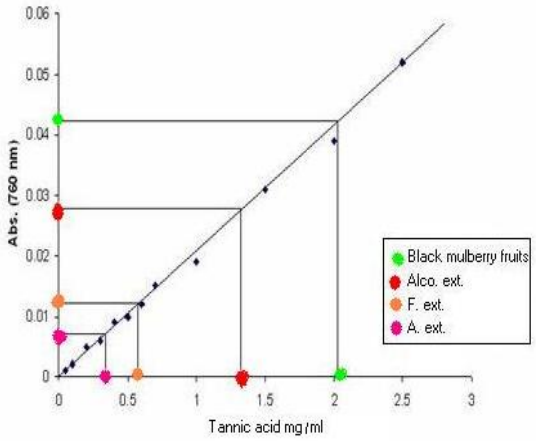


Figure 1. Total phenolic content of black mulberry fruits, Eth., F. and A. ext.

The high amount of phenols content in both black mulberry fruits and Eth. ext. might be due to their flavonoidal and non-flavonoidal phenolic compounds contents. Total phenols content of F.ext. had (5.36 mg TAE/g) due to the presence of flavonoid phenolic compounds only and A. ext. showed the least amount of total phenols content (3.38 mg TAE/g) due to the presence of anthocyanins only.

3.1.2. Total Flavonoid Content

Total flavonoid content of black mulberry fruits, Eth., F., A. ext. and Pig. **(**a**)** were determined using the calibration curve between concentration and absorbance at 415 nm of standard quercetin, as shown in Table 2 and Figure 2. F lavonoids are groups of polyphenolic compounds, which exhibit several biological effects such as antiinflammatory, antihepatotoxic, antiulcer, antiallergic, antiviral, and anticancer activities. They also act as inhibitors enzymes such as reducatase and xanthine oxidase. They are capable of effectively scavenging the reactive oxygen species because of their phenolic hydroxyl groups and are potent antioxidant (Chang *et al.,* 2002; Hsu, 2006).

Table 2. Total flavonoid content of black mulberry fruits, Eth., F. , A. ext. and Pig. (a)

Sample	Concentration (mg QE/g)
Black mulberry fruits	1.11
Eth. ext.	1.17
F. ext.	1.26
A. ext.	0.94
Pig. (a)	0.26

The presence of high phenol and flavonoid contents in black mulberry had contributed directly to the antioxidant activity by neutralizing the free radicals (Umamaheswari and Chatterjee, 2008). Aluminum chloride colorimetric method was used for flavonoids determination. Figure 2 was revealed that F. ext. had the highest total flavonoid content (1.26 mg QE/g), followed by black mulberry fruits and Eth. ext. which contained approximately equal amounts of total flavonoids content (1.11 mg QE/g) and (1.17 mg QE/g), respectively.

This could be due to that ethanolic solvent that can extract most plant components especially phenolic compounds (Harborne, 1984). These results are in agreement with those obtained by Ercisli and Orhan (2007) who reported that total flavonoids content in black mulberry fruits was (2.76 mg QE/g) of extract. Total flavonoids content of A. ext. (0.94 mg QE/g) was lower than the forgoing ones indicating the presence of anthocyanins only. The least amount of total flavonoids content was found in Pig. (a) (0.26 mg QE/g) of extract containing one pigment only.

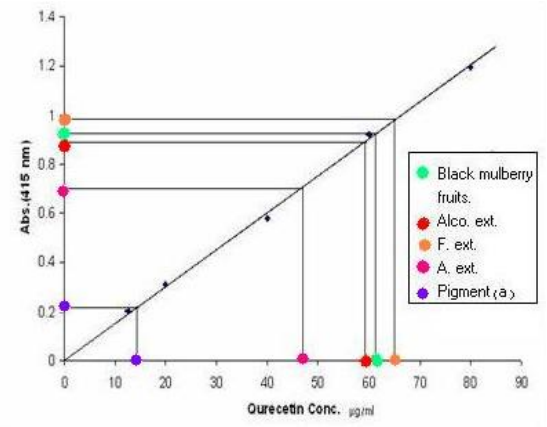


Figure 2. Total flavonoid content of black mulberry fruits, Eth., F., A. ext. and Pig. (a).

3.1.3. Scavenging of Hydrogen Peroxide

The ability of the Eth., F., A. ext. and Pig. **(a)** to scavenge H_2O_2 was determined using the calibration curve between concentration and absorbance at 230 nm of standard ascorbic acid, as shown in Table 3 a nd Figure 3. Since antioxidant compounds present in black mulberry extracts which are good electron donors, they may accelerate the conversion of H_2O_2 to H_2O (Shon *et al.,* 2007). Figure 3 illustrates that the strongest anti - H_2O_2 activity was observed for F. ext. (227.22 mg AAE/g) followed by A. ext. (208.37 mg AAE/g) which may be attributed to the presence of phenolic groups that could donate electrons to hydrogen peroxide and neutralizing it into water (Umamaheswari and Chatterjee, 2008). Eth. ext. and Pig. (a) exhibited weak scavenger H_2O_2 activity (199.10 mg AAE/g) and (191.90 mg AAE/g), respectively. The results showed that there was a strong correlation between total phenol content and scavenging of hydrogen peroxide.

Table 3. The ability of Eth., F., A. ext. and Pig. (a) to scavenge H_2O_2.

Sample	Concentration (mg AAE/g)
Eth. ext.	199.10
F. ext.	227.22
A. ext.	208.37
Pig. (a)	191.90

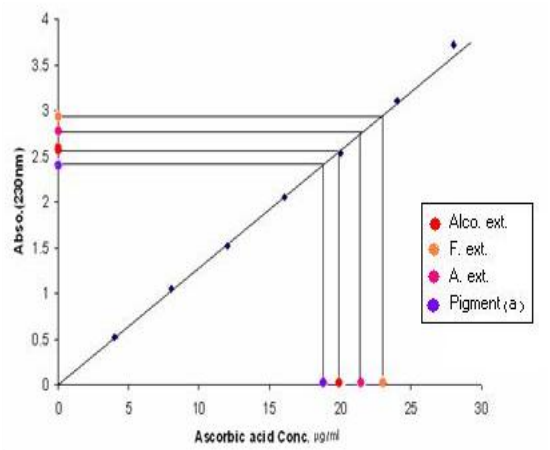
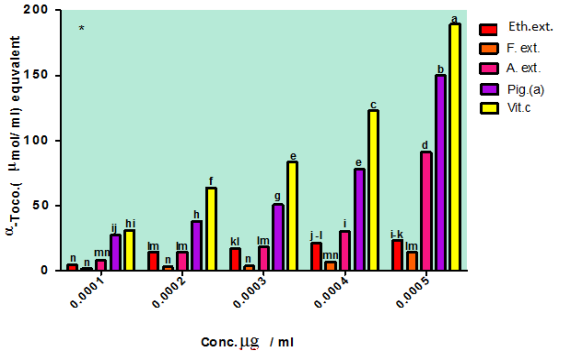


Figure 3. The ability of Eth., F. , A. ext. and Pig. (a) to scavenge H_2O_2.

3.1.4. Total antioxidant capacity

This assay is based on the reduction of Mo (VI) to Mo (V) by the samples of Eth., F., A. ext. and Pig. **(a)**. The antioxidant capacity of the extracts was expressed as micromoles of α-tocopherol equivalent per milliliter of extracts using the calibration curve between micrograms of α-tocopherol equivalent and the concentration determined from Beer's law using extinction coefficient of (4×10^3 M^{-1} cm^{-1}) at 760 nm, as shown in Figure 4. T he extracts were demonstrated electron- donating capacity and, thus they may act as radical chain terminators, transforming reactive free radical species into more stable non-reactive products (Delouee *et al.,* 2007). Figure 4 illustrates that Pig. (a) was significantly exhibited the highest degree of activity at different concentrations especially at 0.0005 µg /ml followed by A. ext. compared to ascorbic acid. This activity might be attributed to the presence of phytochemicals (antioxidant secondary metabolites) (Banso, 2009). Eth. ext. exhibited significant degree of antioxidant capacity followed by F. ext. due to the presence of phenolic compounds which had redox properties allowed them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. They may also had a metal chelating potential (Javanmardi *et al.,* 2003).



* Column followed by the same letter are not significantly different at 1% level based on Duncan's Multiple Rang Test.

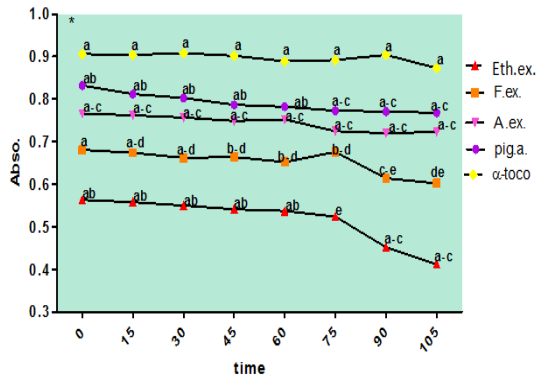
Figure 4. Total antioxidant capacity of Eth., F., A. ext. and Pig. (a).

3.1.5.β–Carotene-Linoleic Acid Assay

In β-carotene-linoleic acid assay, antioxidant activity (AA) of Eth., F. , A. ext. and Pig. **(a)** was compared with antioxidant activity of some common antioxidants such as α-tocopherol when subjected to thermal auto oxidation at 50 °C. The AA was shown in Table 5 and Figure 5.

Table 5. Antioxidant activity of Eth., F. , A. ext. and Pig.(a) as antioxidant compared to α-tocopherol.

Sample	Ai	At	Ai'	At'	AA%
Eth. ext.	0.496	0.412	0.379	0.207	51.20
F. ext.	0.681	0.602	0.379	0.207	54.10
A. ext.	0.766	0.723	0.379	0.207	75.00
Pig. (a)	0.832	0.768	0.379	0.207	62.8
α-tocopherol	0.906	0.873	0.379	0.207	80.9



*The same letters are not significantly different at 1% level based on Duncan's Multiple Rang Test.

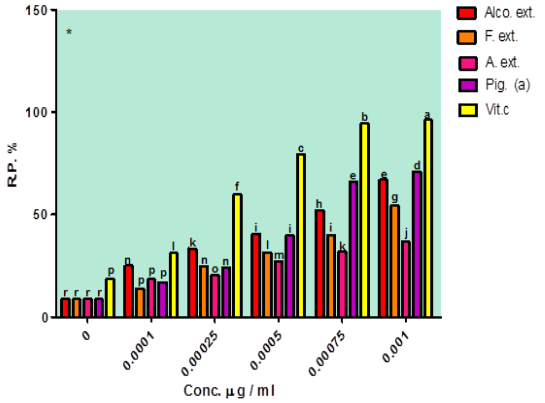
Figure 5. Antioxidant activity of Eth., F., A. ext. and Pig. (a).

The results indicated that the major contributors among individual extracts to AA could be Pig. (a) (62.8%) followed by A. ext. (75 %). Regarding the structure of these compounds, they possess a high degree of hydroxylation and methoxylation on their aromatic rings which suggests their higher contribution to AA of black mulberry antioxidant (Lachman *et al.,* 2009).

Flavonoid ext. showed significant AA (54.10%) especially at zero time which, then decreased when temperature elevated. The obtained results were in agreement with the reports of Zhshen *et al.* (1999) who confirmed the presence of total flavonoids espically rutin, quercetin, isoquercetin and luteolin in mulberry plants. These compounds are the major phytochemicals responsible for antioxidant activity of mulberry plants. Eth. ext. showed a moderate activity (51.20%) due to the presence of polyphenolic compounds in black mulberry fruits especially chlorogenic acid (Lin and Tang, 2007). Positive correlation between total phenol and antioxidant activity was found by many researchers (Brown *et al*., 2005; Yang *et al*., 2009; Garzon and Wrolstand, 2009).

3.1.6. Reducing Power Assay

In this assay, the ability of Eth., F. , A. ext. and Pig. **(a)** to reduce iron (III) to iron (II) was determined and compared to that of ascorbic acid, which is known to be a strong reducing agent. The percentage reduction (R.P. %) of the samples was shown in Figure 6.



* Column followed by the same letters are not significantly different at 1% level based on Duncan's Multiple Rang Test.

Figure 6. Reducing power of Eth., F. , A. ext. and Pig. (a) as a strong reducing agent compared to ascorbic acid.

Kaur and Arora (2008) determined reducing power of Eth., F., A. ext. and Pig. (a) and, compared to ascorbic acid, they considered it as a strong reducing agent. Data were shown that Eth., F., A. ext. and Pig. (a) possessed some degree of hydrogen donation capacity in a concentration dependent manner. The reducing power was increased with increasing concentration, but the capacities were inferior to that of ascorbic acid.

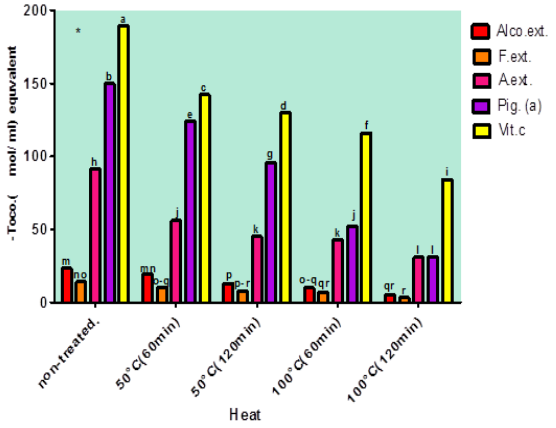
Pigment (a) was the most potent reducing agent followed by Eth. ext., F. and A. ext. due to the highest amount of total phenolics and phenolic acids. Phenols that have more number of hydrolysable groups (OH groups) attached to the ring acting as more powerful reducing agent considered as good pr oton donors resulting in the termination of free radicals chain reactions (Kaur and Arora, 2008). Hence, this may accelerate the conversion of H_2O_2 to H_2O (Ruch *et al.,* 1989). At high concentration, Pig. (a) showed a high potential for being a proton donor, wherever, A. ext. was the weakest in its activity due to the low content of total phenols. Similar relation between iron (III) reducing activity and total phenol content had been reported (Benzi and Szeto, 1999)

3.2. Effect of Temperature, pH and Storage on the Antioxidant Capacity

It is well known that many factors such as antioxidant concentration, temperature and pH of the media, processing treatment and storage are strongly influence the antioxidant capacity (Arabshahi *et al.,* 2007). In this study all extracts and Pig.(a) was subjected to thermal , pH and storage studies. Figure 7 shows the effect of temperature on the antioxidant stability of all extracts and Pig. (a).

The antioxidant capacity was decreased with increasing the time of boiling. This reduction was reached to 20.66% for Pig. (a) and to 34.06% for A. ext. when incubated at 100 ºC for 120 min.. This observation indicated that anthocyanins were not stable at high temperature which reasoned to the speed destruction of anthocyanins at high temperatures (Arabshahi *et al.,* 2007).

Antioxidant capacity of Eth. ext. was reduced to 81.70% followed by F. ext. 72.18% when incubated at 50 ºC for 60 min. The antioxidant capacity was decreased with increasing the time of boiling. The reduction activity was reduced for Eth. ext. and F. ext. when incubated at 100 ºC for 120 min which might be explained to the loss of naturally occurring antioxidants or phytochemicals present in the extract (Arabshahi *et al.,* 2007).

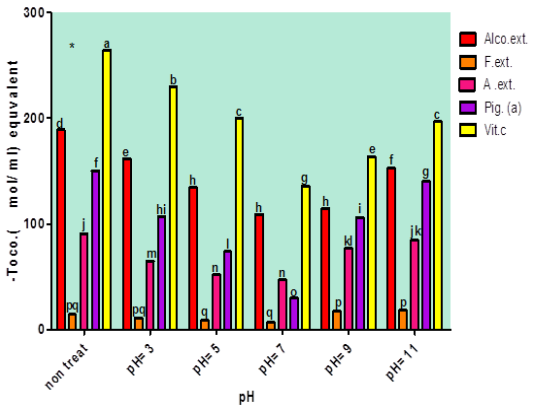


* Column followed by the same letters are not significantly different at 1% level based on Duncan's Multiple Rang Test.

Figure 7. Effect of temperature on the antioxidant capacity of Eth., F., A. ext. and Pig. (a).

The influence of pH on the antioxidant stability of all extracts and Pig. (a) is shown in Figure 8 . The antioxidant capacity of all extracts and Pig. (a) gradually decreased with minimum value at pH 7 followed by continuous increase at alkaline pH, indicating strong dependence of antioxidant capacity of extract to the pH of the system. At pH 3 no significant effect was noticed in Pig. (a) as well as A. ext. comparing to vit. C. Anthocyanin can be found in different chemical forms which depend on the pH of the solution. At low pH 1, the flavylium cation (red color) was the predominant species and contributed to the purple and red colors. The antioxidant capacity of Pig. (a) and A. ext. was reduced at pH 5-7. At pH 7 the antioxidant capacity of Pig. (a) and A. ext. was reduced to 19.80% and 51.86%, respectively.

It had been shown that simple anthocyanins were unstable and quickly decolorized by hydration at the 2-position of the anthocyanidin skeleton. At alkaline pH 11, the antioxidant capacity of Pig. (a) and A. ext. was increased to 93.30% and 93.07%, respectively, due to the increased amount of anhydrous base at alkaline pH (Furtado *et al.,* 1993). The antioxidant capacity of Eth. ext. was least effected by pH (87.23%, at pH 3) followed by F. ext. (75.35%, at pH 3).

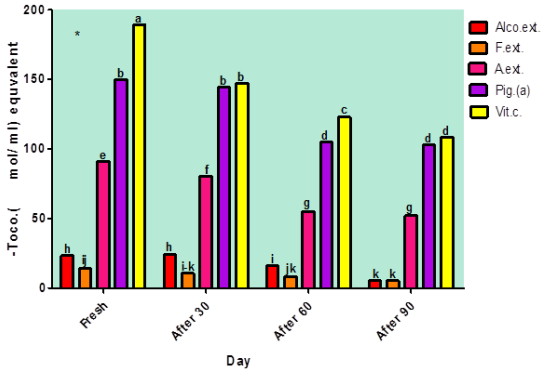


* Column followed by the same letters are not significantly different at 1% level based on Duncan's Multiple Rang Test.

Figure 8. Effect of pH on the antioxidant capacity of Eth., F., A. ext. and Pig. (a).

This observation could be attributed to the presence of antioxidant secondary metabolites other than anthocyanins like polyphenolic compounds in black mulberry fruits, especially chlorogenic acid (Lin and Tang, 2007).

The effect of storage on the antioxidant stability of all extracts and Pig. (a) was also studied up to 3 months at intervals of 30 days as shown in Figure 9. The antioxidant capacity of all extracts and Pig. (a) were gradually reduced with minimum value during 90 days period. The antioxidant capacity of Pig. (a) and A. ext. were unaffected by storage for 30 days and slightly reduced after 60 days only. Their antioxidant capacity after 90 days period were reduced to 68.66% and 57.14%, respectively, which was approximately similar to the antioxidant capacity of vit. C. with a reduction value of 57.25% after 90 days period storage.



* Column followed by the same letter are not significantly different at 1% level based on Duncan's Multiple Rang Test.

Figure 9. Effect of storage on the antioxidant capacity of Eth., F., A. ext. and Pig. (a).

Antioxidant capacity of Eth. and F. ext. was significantly affected by storage for 30 days and reduced after 60 days and their antioxidant capacity after 90 days period were reduced to 23.40% and 36.97%, respectively. These results were in agreement with the reports of Arabshahi *et al.,* (2007) who noticed that the

remaining activity of anthocyanin black mulberry extracts was about 65% after three months storage indicating that it could be still considered as a source of natural antioxidants.

4. Conclusions

Based on the obtained results, it may be concluded that *Morus nigra* fruits are a well recognized source of secondary metabolites like flavonoids and anthocyanins which considered as an excellent antioxidants. The antioxidant activities of *Morus nigra* fruits extracts varied with the test method . Pigment (a) showed a strong total antioxidant capacity , reducing power ability and inhibition of ß-carotene bleaching. Flavonoid ext. exhibit a suitable H_2O_2 scavenging activity which may be correlated with the high amount of polyphenolic content especially flovonoids and anthocyanins. Increasing the time of boiling, storage , and extreme pH values decreased total antioxidant capacity of all extracts.

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Antimicrobial Activity of Xerophytic Plant (*Cotula cinerea* Delile) Extracts Against Some Pathogenic Bacteria and Fungi

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Abstract

In the present investigation, an Algerian commonly available plant namely *Cotula cinerea*, found throughout sandy desert grounds, was screening for antimicrobial activity against five different human pathogenic microbes namely, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans*. The antimicrobial activity was evaluated using the agar disc diffusion method. Aerial parts of *C. cinerea* were subjected to extraction using four solvents of different polarity (70% ethanol, *n*-butanol, ethyl acetate and petroleum ether). Petroleum ether and *n*-butanol extracts had the most effective antimicrobial activity with Gram-negative *K. pneumoniae* demonstrating the highest susceptibility. Linear regression analysis was performed to find correlations between extract concentrations and inhibition activity. Results showed a significant increase in mean diameter of inhibition zone with increasing extract concentrations of all solvents except *n*-butanol. Two-way ANOVA test was used to compare the effect of *C. cinerea* extracts on the antimicrobial properties. All plant extracts have shown significant differences in their actions as antimicrobial agents. Indeed, the *n*-butanol extract at a low concentration of 0.25 mg mL^{-1} indicated a potent antimicrobial activity of *C. cinerea* extracts.

Keywords: Antimicrobial activity, *Cotula cinerea*, Pathogenic microbes, Medicinal plant.

1. Introduction

Medicinal plants, which form the backbone of traditional medicine, have been in the last few decades the subject of very intense pharmacological studies. This has been brought by the acknowledgement of the value of medicinal plants as potential sources of new therapeutic compounds and drug development (Matu and van Staden, 2003). According to the World Health Organization, about 80% of the world's population living in developing countries rely mostly on plants for primary health care (McKay and Blumberg, 2007).

In recent years, pathogenic microorganisms have developed multiple drug resistance due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases (Katsumi *et al*., 2005). An increase in the emergence of multidrug-resistant bacteria is worrying the world population. Infection rates have greatly increased and antibiotics resistance has become an ever-increasing therapeutic problem (Shahid *et al*., 2008).Therefore, there is a need to develop alternative antimicrobial drugs from various sources such as medicinal plants (Cordell, 2000).

Plants with antimicrobial activities have become more interesting because many people are aware of problems associated with the over-prescription and misuse of traditional antibiotics. However, only about 20% of the plants found in the world have been subjected to pharmacological or biological testing (Mothana and Lindequist, 2005). Plants in the environment are exposed to a range of abiotic stresses such as osmotic stress, salinity, and temperature variations. These, in-turn, affect their growth and the metabolic processes involved in the synthesis of a wide range of secondary metabolites, such as polyphenols, tannins, terpenoids, alkaloids etc, which may have remarkable antimicrobial properties (Cowan, 1999; Marzouk *et al*., 2010; Daglia, 2012).

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The Algerian flora plays a key role in supporting traditional medicine, which is widely practiced over the country. This flora holds a rich diversity of medicinal and endemic plants (Beloued, 2005). Many plants used in the Algerian traditional medicine have the potential to provide pharmacologically active natural products (Maiza *et al*., 1993; Hammiche and Maiza, 2006). Ethnopharmacological interest in the sources of these compounds has increased nationally and worldwide, particularly in the search for drugs to counter multi-drug resistant microorganisms.

Cotula cinerea L., syn. *Brocchia cinerea* Del. (Asteraceae), is a xerophytic plant widely distributed in sandy and desert grounds (Markouk *et al*., 1999a). This medicinal plant popularly known as (Gartoufa or Chouihiya), is commonly used in Algerian folk medicine, as well in the rest of the Maghreb region, as an anti-inflammatory, analgesic, antipyretic, antiseptic, and for treatment of various other diseases, including digestive problems (constipation and colic), rheumatism, and urinary and pulmonary infections. It is much appreciated in green tea or mixed with food to enhance the flavour (Maiza *et al*., 1993; Markouk *et al*., 1999a, 1999b; Larhsini *et al*., 2002; Hammiche and Maiza, 2006). Several compounds have been isolated from *C. cinerea*, including flavonoids, sesquiterpene lactones, sesquiterpene coumarins and tannins (Ahmed *et al*., 1987; Markouk *et al*., 1999b). The objective of this work is to evaluate the antimicrobial activity of *C. cinerea* extracts, obtained using various solvents, against some pathogenic bacteria and fungi.

2. Materials and Methods

2.1. Plant Material

Cotula cinerea samples were collected from its natural range of distribution in El-Oued (Algerian Sahara Desert) (about 4 km Southeast of El-Oued city, 33°20'N to 33°19'N, 6°52'E to 6°53'E) in March 2011.

2.2. Extraction Protocol

2.2.1. Extracting Solvents

The extraction was carried out by using four solvents of increasing polarity: (i) petroleum ether (non-polar), (ii) ethanol, (iii) *n*-butanol, (iv) ethyl acetate (later three are moderately polar).

2.2.2. Preparation of Plant

The freshly picked aerial parts of the plant used in the screening were air-dried at room temperature for 2 weeks, with no direct sunlight. Once dry, plant was ground into fine powder and stored at 4 °C until time of extraction.

2.2.3. Preparation of the Extracts

The powdered plant material (20 g) was macerated for 24 h three times (3×24 h) in a mixture of ethanol/water (70:30; v/v) with frequent agitation at room temperature (25 ± 1 °C). Then the mixture was filtered using filter paper (Whatman No. 1) under the vacuum of a water pump and the ethanol was evaporated under low pressure using a rotary evaporator at 50 °C. The residue was taken as the hydro alcohol extract (Dall'Agnol *et al*., 2003). The remaining aqueous extract was fractioned with petroleum ether, ethyl acetate and *n*-butanol (3×100 mL for each solvent). These extracts were dried under reduced pressure using a rotatory evaporator at 40 °C. The residues were taken as the petroleum ether, ethyl acetate and *n*-butanol extracts of the plant (Boligon *et al*., 2011).

2.3. Antimicrobial Activity

2.3.1. Microbial Strains and Growth Conditions

Five clinical isolates of microorganisms were used for assessing the plant antimicrobial properties, including the Gram-positive *Staphylococcus aureus*, the Gram-negative *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*, and the yeast *Candida albicans*. Table 1 lists the strains and their resistance phenotypes. All strains were obtained from the Microbiology Laboratory of Hospital Benamor Djilani (El-Oued, Algeria) and were maintained at 4 °C on slants of Nutrient Agar (NA) for bacteria and Sabouraud Dextrose Agar (SDA) for the yeast. Active cultures were prepared by transferring a loop of cells from the agar slant to a test tube containing 5 mL of Nutrient Broth for bacteria and Sabouraud Dextrose broth for the yeast. They were then incubated overnight to reach the logarithmic phase of growth; for about 6–10 hours at 37 °C for bacteria and 12–16 h at 30°C for *C. albicans*.

Table 1. Resistance pattern of target organisms to antibiotics.

Strain	Phenotype of resistance	Phenotype of sensibility
Staphylococcus aureus	AMC, AMX, CE, CF, OX, PEN	CHL, CIP, GEN, PEF
Escherichia coli	AMC, AMX, CIP, PEF, SXT	CE, CF, CEF, C, GEN, IMI, STR
Pseudomonas aeruginosa	AMC, AMX, ATM, CF, CIP, PEF, SXT	GEN, IMI, PEF, PIP
Klebsiella pneumoniae	IMI	C, SXT
Candida albicans	ND	ND

(AMC: amoxicillin + acid clavulanic, AMX: amoxicillin, ATM: aztreonam, C: chloramphenicol, CE: cefatxin, CF: cefalotin, CEF: cefazolin, CIP: ciprofloxacin, GEN: gentamicin, IMI: impipenem, ND: not determined, OX: oxacillin, PEF: pefloxacin, PEN: penicillin, PIP: piperacillin, STR: streptomycin, SXT: trimethoprim/sulfamethoxazol)

2.3.2. Antimicrobial Assay (Disk Diffusion Assay)

The disc-diffusion assay (Qaralleh *et al*., 2010) was used to determine growth inhibition caused by plant extracts. Inoculums, containing 10^6–10^8 CFU per milliliter, was spread on Mueller–Hinton (MH) agar plates for the four bacteria and 10^4–10^5 CFU per milliliter was poured over the base plates forming a homogenous top layer on SDA for the yeast. Using sterile forceps, Whatman's filter discs (Ø = 5 mm), impregnated with different dilutions of extracts (25, 50, 75 and 100%) from the initial concentration of 1 mg mL^{-1}, were placed on inoculated plates and left at 4 °C for 2 h before being incubated to allow the diffusion of the extract. Discs saturated with solvents (ethanol, petroleum ether, ethyl acetate, and *n*-butanol) were air-dried and used as negative controls. The plates were incubated at 37 °C for

24 h for bacteria and 48 h at 30 ℃ for the yeast, after which, inhibition zones around each disc (> 5 mm) were measured (disc diameter included).

2.4. Statistical Analysis

Linear regression analysis (LRA) was carried out to find statistically significant correlation between the different concentrations of each extract and their overall antimicrobial activity, assessed as diameter of inhibition with disregard to the tested strains. The data pertaining to antimicrobial activity of different *C. cinerea* extracts were also analysed with two-way ANOVAs, to test the effect of "tested strains" and "extract dilutions" on the levels of antimicrobial activity. Interaction between tested microbe-species and extract concentration was also included in the analysis for each plant extract. Besides the explanatory ability of LRA, it could be used, supported by ANOVA outputs, to detect potency "effectiveness" of the extract itself disregarding its concentration. Both LRA and ANOVA were considered statistically significant when P-value < 0.05.

3. Results

Hydro-alcohol extract of *C. cinerea* had a much higher extraction yield (w/w %) than the other extracts, whereas, the *n*-butanol extract had the lowest yield. Since extract yield increases with extracting solvent polarity. As a result, 70% ethanol, which was the most polar of all solvents and which was used for fractionation, has afforded the maximum yield (11.0%) compared to petroleum ether (1.0%), *n*–butanol (6.0%) and ethyl acetate (1.2%).

The inhibition zone, referring to antimicrobial activity of *C. cinerea* extracts, was measured after incubation of the plates. Each of the extracts was tested three times and the average (±SD) of three values was determined. Generally, the results showed that the inhibitory effect of extracts increased with increasing of concentrations (Figure 1).

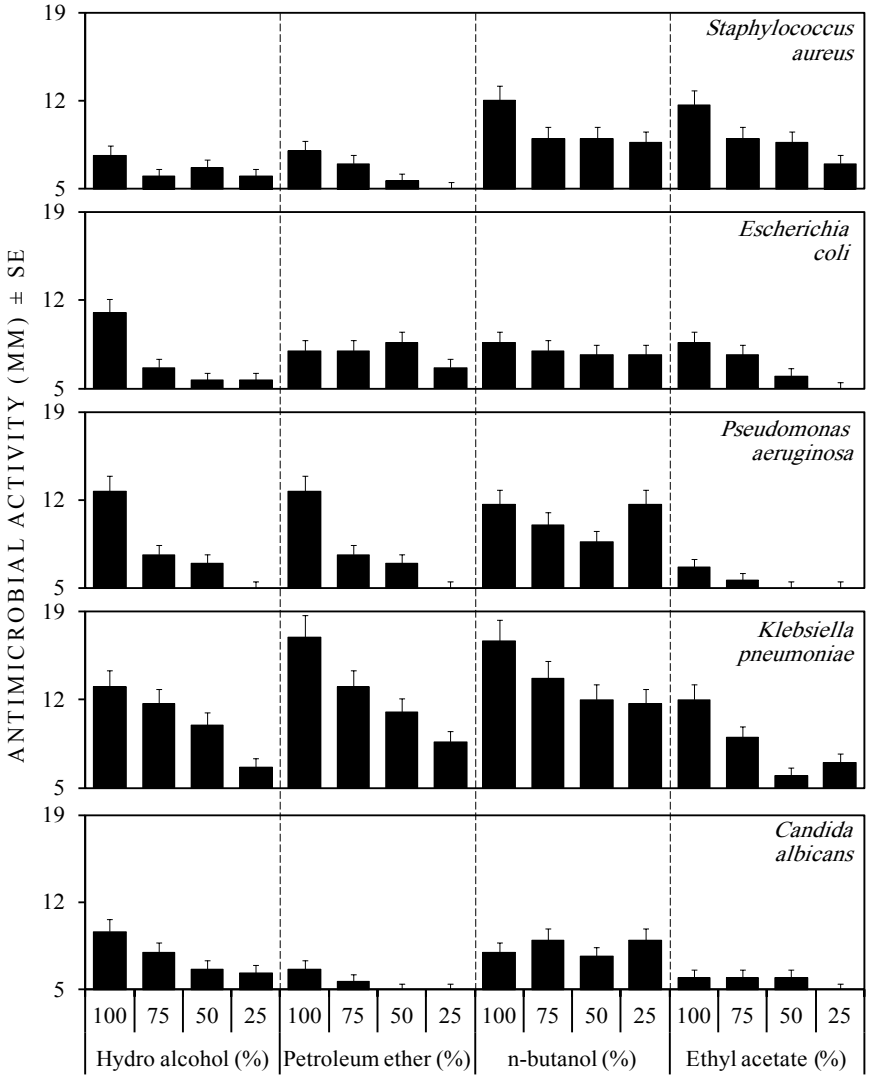

Figure 1. Antimicrobial activity of *Cotula cinerea* extracts (Extract initial concentration = 1 mg mL^{-1}, disc diameter = 5 mm).

The antimicrobial activity assayed for *C. cinerea* extracts showed an overall inhibitory effect against *K. pneumoniae* (16.67 ± 5.77 mm) with the *n*-butanol extract, and 17 ± 1.73 mm with the petroleum ether extract. Little activity was observed against *E. coli*, *P. aeruginosa*, *S. aureus* and *C. albicans* at concentrations of 0.25 mg mL^{-1}. High activity against *S. aureus* was found with *n*-butanol and ethyl acetate extracts where inhibition zones equalled 12 ± 5.20 mm and 11.67 ± 3.79 mm, respectively. Moreover, the hydro alcohol extract was the most active extract against *E. coli*. Similarly, only the hydro alcohol extract of *C. cinerea* had antimicrobial activity against *C. albicans* (Figure 1). Comparing results of growth inhibition zones for the four extracts, it is evident that the petroleum ether extract possesses moderate antimicrobial properties as compared to the most active extract (*n*-butanol) and less active extracts (ethyl acetate and hydro alcohol).

The *n*-butanol extract at 25% of concentration demonstrated moderate to high antimicrobial activity against *K. pneumoniae*, *P. aeruginosa* and *S. Aureus*, whereas *C. albicans*, *E. coli and P. aeruginosa* remained uninhibited at an equal concentration of ethyl acetate extract. The hydro alcohol extract at 25% of concentration also demonstrated low antimicrobial activity against all tested species.

The susceptibility of microbial species to crude *C. cinerea* extracts was in the following descending order: For hydro-alcohol extract, *K. pneumoniae* > *P. aeruginosa* > *E. coli* > *C. albicans* > *S. aureus*. For petroleum ether extract, *K. pneumoniae* > *P. aeruginosa* > *E. coli* > *S. aureus* > *C. albicans*. For *n*-butanol extract, *K. pneumoniae* > *S. aureus* > *P. aeruginosa* > *E. coli* > *C. albicans*. For ethyl acetate extract, *K. pneumoniae* > *S. aureus* > *E. coli* > *P. aeruginosa* > *C. albicans*.

LRA determined that the slopes were statistically significantly non-zero, i.e. one can assume that a relationship exists between inhibition activity and extract concentrations of hydro alcohol ($P < 0.001$), petroleum ether ($P < 0.001$), and ethyl acetate ($P < 0.001$). However, the linear regression slope for *n*-butanol was not statistically significantly different from zero ($P = 0.187$), indicating there was no significant trend between *n*-butanol extract concentrations and inhibition zone (Table 2). Additionally, comparison of regressions showed that slopes were not significantly different ($P = 0.232$) while the intercepts were highly significantly different ($P < 0.001$).

Table 2. Linear regression analysis applied for extract dilutions and antimicrobial activity.

Extracts	Slope	Intercept	R^2	S(C/I)	SS(I)	*F*	*P*
Hydro alcohol	6.16	4.13	0.304	28.88	584.98	25.34	<0.001
Petroleum ether	5.60	4.57	0.224	26.25	655.73	16.76	<0.001
n-butanol	2.37	8.53	0.030	11.13	886.98	1.78	0.187
Ethyl acetate	4.29	4.47	0.192	20.13	449.65	13.80	<0.001

(C/I: Concentrations/Inhibition, SS: Sum square).

Two-way ANOVA revealed that all plant extracts had significant differences in their actions as antimicrobial agents with either tested strains or extract concentrations or even their interaction. Fisher-values in "Strain test" and "Extract dilutions" factors showed all highly or very highly significant effects for the four extracts (except for dilutions of *n*-butanol). Thus antimicrobial activity of *C. cinerea* varied significantly between tested strains (in particularly for the petroleum ether extract, $P < 0.001$) and according to dilutions (especially within the petroleum ether extract, $P < 0.001$). In general, the interaction effect of the two factors (Strain test * Extract concentration) had no s tatistical significance on t he variation of antimicrobial activity in all extracts except that of petroleum ether ($P = 0.002$) (Table 3).

Table 3. Two-way analysis of variance (ANOVA).

Extracts	Effect	*SS*	*Df*	*MS*	*F*	*P*
Hydro alcohol	Strain test	97.6	4	24.4	4.1	0.007
	Extract dilutions	191.3	3	63.8	10.6	<0.001
	Interaction	56.2	12	4.7	0.8	0.667
	Residuals	240.0	40	6.0		
	Total	585.0	59	9.9		
Petroleum ether	Strain test	334.4	4	83.6	39.2	<0.001
	Extract dilutions	152.4	3	50.8	23.8	<0.001
	Interaction	83.6	12	7.0	3.3	0.002
	Residuals	85.3	40	2.1		
	Total	655.7	59	11.1		
n-butanol	Strain test	229.4	4	57.4	4.1	0.007
	Extract dilutions	45.5	3	15.2	1.1	0.370
	Interaction	48.7	12	4.1	0.3	0.988
	Residuals	563.3	40	14.1		
	Total	887.0	59	15.0		
Ethyl acetate	Strain test	120.9	4	30.2	6.0	<0.001
	Extract dilutions	90.2	3	30.1	6.0	0.002
	Interaction	38.6	12	3.2	0.6	0.793
	Residuals	200.0	40	5.0		
	Total	449.7	59	7.6		

(SS: Sum of squares, MS: Mean of the Sum of Squares, *Df*: Degrees of freedom).

4. Discussion

Infectious diseases represent a serious public health problem and remain the major cause of death throughout the world. Alternative natural products of plants could be of high interest to attenuate the increasing incidence of antibiotic resistance. Some phytochemicals and plant extracts are known to have antimicrobial properties, which could be of great importance in the therapy of microbial infections. Recently, various studies have been conducted over different countries, and have demonstrated the efficacy of this type of treatment (e.g. Coutinho *et al*., 2008; Habbu *et al*., 2009). Algeria has also recently increased research on traditional herbal medicines following scientific findings that verified their effectiveness in healing several health issues.

The present investigation explored the use of one such plant, *C. cinerea* Del., endemic in North Africa, for treating infectious diseases. The assay of antimicrobial activity of *C. cinerea* extracts showed that the hydro

alcohol extract was least active against the tested strains, and only the relatively polar fraction (*n*-butanol) had high activity against the strains. ANOVA analysis confirmed that *n*-butanol had significant variation in its antimicrobial activity against the tested strains, however there was no significant variation in activity for its tested concentrations, which was also demonstrated by LRA. These findings support the conclusion that the active antimicrobial compounds are highly concentrated in this fraction. The other extracts (hydro alcohol, petroleum ether and ethyl acetate) had the best antimicrobial activity when used at high concentrations. This was clearly revealed by both LRA and ANOVA. The wide range of antimicrobial activity shown by *C. cinerea* extracts might reflect the differences in chemical concentrations and composition obtained by each solvent. Indeed, the successful extraction of active botanical compounds from plant materials is dependent on the type of solvent used in the extraction procedure (Parekh *et al*., 2005; Hayouni *et al*., 2007).

The plant extracts were active against both Gram-positive and Gram-negative bacteria, though they were more active against the later. Concerning the antimicrobial activity against *C. albicans*, the present study revealed low to no activity by the plant extracts. In our study, the highest activity was recorded against the Gram-negative bacteria: *K. pneumoniea*, which was the most susceptible bacterium of all the tested strains. These results may be of great importance in infection therapy since *K. pneumoniae* can be commonly involved in urinary, intra-abdominal, and respiratory infections (Lavender *et al*., 2005; Keynan and Rubinstein, 2007; Ahmad *et al*., 2012).

Markouk *et al*. (1999a) reported that the acetate extract of *C. cinerea* collected from Zagora (Sothern Morocco) exhibited an antibacterial effect with a minimum inhibitory concentration (MIC) of 200 µg/mL against all tested bacteria, and that the *n*-butanol extract was highly effective too, especially against *Pseudomonas fluorescens* and *Bacillus* sp., with an MIC of 12 µg/mL. In the same study, the ethyl ether extracts of *C. cinerea* were found to be inactive against all tested bacteria. These results are in agreement with ours, and confirm that bioactive components of any plant may differ in their solubility based on: (i) the extracting solvents (Hayouni *et al*., 2007; Hassan *et al*., 2009), (ii) the nature of biologically active components such as alkaloids, saponins, tannins, phenols, etc. (Hassan *et al*., 2009; Marzouk *et al*., 2010), and (iii) the geographical origin of the plant material (Seidel *et al*., 2008), because ecological conditions in general (including abiotic factors "edaphic, climatic, water stress…" or biological interactions "such as intra and/or interspecific competitions…") may have a large impact on growth and fitness of vegetation species (Cordell, 2000; Seidel *et al*., 2008), particularly by affecting their metabolism and secondary metabolites production (Cowan, 1999). Moreover, Ahmed *et al*. (1987) reported that *C. cinerea* is particularly rich with flavonic compounds besides sesquiterpene-lactone and sesquiterpene coumarins, which have been also isolated of this plant.

The results reported here can be considered as the first report on the antimicrobial properties of *Cotula cinerea*, an endemic species of the Algerian Saharan flora. Our findings also contribute to the knowledge of antimicrobial properties reported elsewhere for other *Cotula* species. Based on these antimicrobial results obtained using the disk diffusion method, it appears that this technique could not always be a reliable and sure method for screening the antimicrobial activity of plant extracts. As indicated by Moreno *et al*. (2006), the absence of an inhibition zone did not necessarily mean the compound was inactive, especially for the less polar compounds, which diffuse more slowly in the culture medium.

Consequently the analysis of the present results offers a simple scientific basis for traditional use of *C. cinerea* against microbial pathogen. However, *in vivo* studies on this medicinal plant are necessary and should seek to determine toxicity of different active compounds, their side effects, pharmacokinetic properties and reach their required minimum inhibitory concentration (MIC) in tissues and organs having the infection. The antimicrobial activities could be enhanced if active components are purified and adequate dosage determined for proper administration.

5. Conclusion

In the light of this study, *C. cinerea* is a prospective wild plant for the isolation of new antimicrobial substances. Although further investigations are clearly necessary to clarify and identify the bioactive constituents, we believe that our results presented herein represent a solid stepping-stone for other researchers in the field. Moreover, our antimicrobial assays results has justified and supported, at least in part, the Algerian common usage of the plant. The screening of some medicinal plant crude extracts has shown that some of those were potentially rich sources of antimicrobial agents. Finally, promoting human well-being deserves joining efforts in considering and valorising Saharan natural patrimony, as well as carrying out more scientific research on plants living in drylands by conducting chemical, biological, toxicological and pharmacological investigations as well as investigating therapeutic potential.

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Correlation Between Numerical Profiles Generated for Soil Spore Forming Bacilli and Their Inhibitory Potential Against *Staphylococcus aureus* ATCC 6538

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Abstract

Numerical profiling using conventional tests for studying bacterial diversity of soil have probably never been employed. The main objective of this work was to describe the heterogeneity of spore forming bacilli in a vegetative soil using numerical profiling and to establish the possible application of this approach for the prediction of the antimicrobial potentials of soil dwelling *Bacillus* species. A total of 100 spore forming Gram positive bacilli were recovered from soil samples and their characters were determined using 9 conventional tests. A 3 digit numerical profile was constructed for each isolate. This labelling system resulted in the generation of 12 different profiles which indicated the diversity of soil *Bacillus* isolates. The highest frequency of occurrence was detected among the isolates labelled with the profile 4.1.5 and the lowest among those labelled with 4.7.5; 4.6.6 and 1.0.3 profiles. Out of 13 isolates labelled with 4.4.3, the cell free extract of 12 exhibited no antimicrobial activity against *Staphylococcus aureus* ATCC 6538 whereas, extracts obtained from 15 out of 18 isolates labelled with the profile 4.1.5 were inhibitory to the test organism. It is concluded that the numerical profiling system is a significant tool for the establishment of diversity among soil spore forming bacilli which can also be used for the prediction of the potentials of isolates to produce antimicrobial metabolites.

Keywords: *Bacillus* species, Vegetative soil, Diversity, Antimicrobial effect, Numerical profiles.

1. Introduction

Bacillus species are soil dwelling organisms that produce metabolites with diverse chemical structures and potentials to inhibit a wide spectrum of bacteria (Morsi and Atef, 2010). Antibiotics produced by Bacillus species include bacitracin, colistin, circulin, gramycidin, polymyxin, and tyrotricidin. However, these antibiotics were discovered many decades ago, they are still used in medical practice (Fickers, 2012).

The isolation of *Bacillus* species from soil is usually carried out by traditional techniques which involve the treatment of soil with heat to inhibit vegetative microorganisms and to allow the selection of spore forming bacteria (Kuta *et al*, 2009). Isolates may be characterized by a range of methods. Morphological and physiological characteristics were used for classification and identification. However, such techniques can provide the basis for bacterial, and *Bacillus* taxonomy, additional tests were found to be necessary for confirmation and for fine-scale resolution (Maughan and Van der Auwera, 2011).

Identification of spore forming bacteria is a difficult task, it was suggested that for the accurate identification of these Bacilli, several standard schemes should be used together for the establishment of different phenotypic characteristics of all known species (Morsi *et al*, 2010). In recent years, molecular techniques were introduced for the characterization of isolates at a genetic level (Kadyan *et al*, 2013). Although, this characterization approach is valuable in discriminating and grouping *Bacillus* strains, it provides little ecological information. For example, 16S rRNA gene based methods provides good phylogenetic information to the genus level, but in themselves, give little information on function (Mandic-Mulec and Prosser, 2011; Maughan and Van der Auwera, 2011). On the other hand, traditional approaches that group isolates on the basis of common metabolic properties

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may be limited in terms of phylogenetic power but provide clues to environmental factors and can be strong indicators of potential ecosystem function (Koeppel *et al*, 2008). Kim *et al.* (2003) indicated that for the accurate identification and classification of spore forming Bacilli from soil, a multiphasic approach may be required.

Antimicrobial activity is usually established by screening cell free extract derived from soil isolates against a panel of bacteria using the agar diffusion method (Manga and Oyeleke, 2008). Therefore, it is evident that identification procedures in addition to preliminary screening are tedious and time consuming. If a system or a model can be devised to label the isolates and simultaneously predict their potential to produce antimicrobial substances, then definitely; time, efforts and money can be saved. The effective labeling system should be capable of grouping isolates according to their biochemical properties and should detect variations among isolates. These stipulations were taken into considerations by (Griffiths and Lovitt, 1980), who suggested that numerical profiling can be used as a significant tool for the study of bacterial diversity in different ecosystems. The advantage of this system over traditional and modern identifications methods is that all isolates are labeled and none is left unidentified. Depending on the tests included in the characterization of isolates, it can provide an idea about the function of the isolate from the respective environment. The objectives of this communication were to establish the diversity of endospore forming *Bacillus* species recovered from rich vegetative Jordanian soil using the numerical profiling system and to investigate the possible application of this system for the prediction of antimicrobial potential of these isolates.

2. Materials and Methods

2.1. Collection of Soil Samples

A total of 10 soil samples were collected from highly vegetative locations in Ajloon Mountains (near the castle). Samples were derived from the depth of 5 cm below the surface, placed in sterile containers and processed in the laboratory within 2 days of collection.

2.2. Isolation of Bacillus Species

One gram of each soil sample was separately suspended in a sterile tube containing 9 ml of sterile distilled water and shaken vigorously for 2 minutes using a vortex mixer. The samples were heat treated at 80 ºC for 10 minutes in a water bath. Upon cooling to room temperature, each soil suspension was 10 fold serially diluted in sterile distilled water. Aliquots of 0.1 ml of the appropriate dilution were plated on Nutrient Agar Medium (Difco- USA) using the spread plate technique. All plates were then incubated at 37 °C for 2 days. From plates that contained counts between 30 to 250 colonies, 10 colonies with apparently different colonial morphology were isolated and further purified by repeated subculture.

2.3. Preparation of Cell Free Extracts

Each purified isolate was inoculated into a sterile tube containing 10 ml of Nutrient Broth (Difco- USA) and then incubated at 30 °C for 72 hours. The tubes were centrifuged and supernatant was then filtered through 0.22 bacteriological filters and stored at 4°C until used as described in the next experiment.

2.4. Antimicrobial Activity Test

Cell suspension of *Staphylococcus aureus* ATCC 6538 was prepared in accordance with the method described by Abu Shaqra and Al Groom (2012) using 0.5 McFarland standard. The suspension prepared, contained 10^6 Colony Forming Unit /ml and was employed to streak plates of Mueller Hinton Agar (Difco- USA). After drying, wells were bored using sterilized cork borer of 6 mm diameter and each well received 50 µl of the extract of a single isolate. Plates were incubated for 24-48 h at 37 ºC before measuring the diameter of inhibition zones developed.

2.5. Numerical Profiles

Tests used to characterize each soil isolate are presented in table 1. These tests were performed as described by Baron *et al* (1994). Results were arranged in groups of 3 and scores of 1, 2, or 4 were assigned to label the isolates according to their position in the table. In this context, attention should be drawn to two important points; first, all tests carried equal weights as they were randomly positioned in the table and second, scoring numbers were chosen to avoid overlap between tests in case of positive results when the total score is calculated. For example, if the first digit was 3, this would indicate that the organism was xylose fermentor, grew in 10% concentration of NaCl and was unable to grow under anaerobic conditions. If the score of the third test in the profile was 3, then one would be confused wither the organism was positive for the first two tests or merely positive for the third test.

The percentage diversity of the *Bacillus* isolates in relation to the profiles generated was calculated according to the equation given by Abu Shaqra and Mashni (2006) as follows:

$$\frac{\text{Number of profiles}}{\text{Total number of isolates}} \times 100$$

The work was concluded by relating the antimicrobial activity of each isolate with its generated numerical profile to determine if the profiles constructed can provide indications regarding the inhibitory activity of the cell free extract derived from the isolates.

Table 1. Characters used to generate numerical profiles for spore forming bacilli recovered from soil samples.

Score	First digit	Second digit	Third digit
0 or 1	Xylose fermentation	Mannose fermentation	Nitrate reduction
0 or 2	Growth in 10% NaCl	Raffinose fermentation	Hipurate hydrolysis
0 or 4	Anaerobic growth	Voges Proskauer	Starch hydrolysis

2.6. Tabulation of Results

Each numerical profile generated has taken a separate row in a table and the numbers of isolates labelled with the respective profile were tabulated next to it. Upon measurement of the antibacterial effect against *Staphylococcus aureus* ATCC 6538, zones of inhibitions were categorized into 3 groups; no zone of inhibition, ≤ 10 mm and > 11 mm. Isolates that belonged to a specific profile generated were grouped according to the size of inhibitory zone determined and were also placed in the table next to their profiles (Table 2). Therefore, it was possible to correlate the number of isolates in each profile with their antimicrobial activity and this is discussed in more details in the results section.

2.7. Statistical Analysis

The system of Microsiris statistical analysis and data management was employed. Student t test was performed to establish significance of difference between the number of isolates grouped in each numerical profile and the zone of inhibition measured for the cell free extract derived from each isolate against *S. aureus* ATCC 6538. The test was performed to profiles that contained equal or more than 7 isolates. The difference was considered as significant when Probability (*p*) value was less than 0.05.

3. Results

A total of 100 pure isolates of bacteria were obtained from 10 soil samples collected from mountainous and highly vegetative region in Jordan. All recovered organisms were spore forming, Gram positive bacilli in pairs or longer chains. Most of the isolated colonies were white, opaque or translucent and they were rough, granular, or wispy. These characteristics were considered as presumptive for the identification of the isolates as *Bacillus* species.

Using the numerical profiling system and the 9 identification tests given in table 1, it was possible to generate 12 different profiles and this indicated the diversity of the *Bacillus* species recovered (12% diversity). Figure 1 illustrates the percentage occurrence of positive characters which constituted the back bone of this diversity. It is evident that 83% of the isolates were capable of nitrate reduction while 67% were starch hydrolysers.

Table 2 demonstrates the frequency of occurrence of each profile among the isolates. This table clearly shows that profile 4.1.5 was the label for the most common isolates whereas; 4.6.6; 4.7.5 and 1.0.3 were the least encountered.

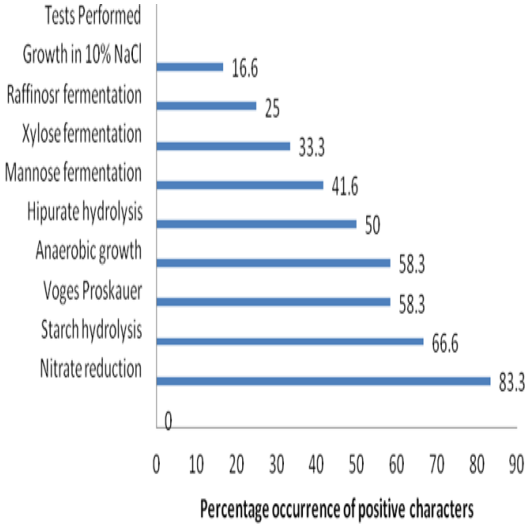

Figure 1. Percentage occurrence of positive characters among spore forming isolates recovered from soil samples.

The diameter of the inhibition zone produced by cell free extract derived from each isolate was taken as a criterion to mark its antimicrobial activity. Table 2 also shows the number of isolates in each profile generated in relation to the zone of inhibition measured. Two examples are given below to explain the content of this table; out of 9 isolates labeled with the profile 2.5.2, 5 produced zones of inhibition > 11 mm, while 3 exhibited zones ≤ 10 mm and only 1 isolate was none inhibitory. On the other hand, 13 isolates were labelled with the profile 4.4.3, 12 of these isolates were inhibitory and none showed the formation of any inhibitory zone.

According to results presented in table 2, 43% of isolates demonstrated no inhibitory zone, while 21% and 36% of the isolates showed inhibitory zone equal or less than 10 and > 11 mm in diameter, respectively. It is worth noting that the majority of *Bacillus* isolates labeled with profiles such as 4.1.5 and 3.4.5 were effective inhibitors to the test organism whereas, the majority of species labelled with 2.1.7, 4.4.3 and 0.0.1 were none inhibitors. Although it was not the intention of this work to identify isolates to species level, it was possible using the few tests performed and the identification tables given by (Barrow and Feltham, 1993) to predict closely the *Bacillus* species to which many of the isolates belonged. For instance, profile 4.1.5 fitted *B. licheniformis* while profiles 4.4.3, 2.1.7 and 3.7.7 most probably denoted *B. laterosporus, B. firmus and B. subtilis*, respectively.

Table 2. Frequency of numerical profiles occurrence in relation to the inhibition zone measured for their cell free extract against *S. aureus* ATCC 6538

Profiles generated	occurrence Frequency of each profile	No. of isolates in relation to inhibition zone diameter		
		None	≤ 10 mm.	> 11 mm.
4.1.5	18	3	5	10
3.7.7	6	0	2	4
2.5.2	9	1	3	5
4.4.3	13	12	1	0
2.1.7	12	10	2	0
4.4.5	9	6	1	2
5.3.4	7	4	2	1
0.0.1	7	7	0	0
3.4.5	7	0	2	5
4.7.5	4	0	0	4
4.6.6	4	0	1	3
1.0.3	4	0	2	2
Total	100	43	21	36

4. Discussion

Numerical profiling is not a new approach for the study of bacterial diversity. Griffiths and Lovitt (1980) were the first to use this system while investigating the bacterial diversity of oil storage tanks water bottom. These authors suggested that the system could be used to study bacterial diversity in different ecosystems. Abu Shaqra and Mashni (2006) used the numerical profiling for studying the bacterial diversity of hard gelatine capsules contaminants and proposed the system as a significant tool for establishing the diversity of contaminants in cosmetic and pharmaceutical products. However, methods for studying bacterial diversity in soil are available (Kirk *et al*, 2004), as far as is known, numerical profiling as a mean for establishing the diversity of spore forming bacilli in soil was never used.

Tests used for the characterization of isolates were chosen on the basis of their convenience and ability to establish diversity. For instance, spore formation test and reaction to Gram stain though were carried out, they were excluded because they were of no significance in determining the diversity of *Bacillus* as the vast majority of species gave positive reaction to Gram stain and by most produced endospores (Sneath, 1986).

The best test to be selected for the detection of bacterial diversity in a particular habitat is the one which can differentiate between isolates on equal bases. For clarification, the ideal test should give positive results for 50% of isolates and negative test results for the remaining 50 % (Griffiths and Lovitt, 1980). Figure 1 demonstrates the percentage occurrence of positive characters among our isolates and indicates that none of the tests used was ideal as no single test was capable of 50% differentiation. It is evident that this method relies on stressing the characters of the organism rather than focussing on their names, although in occasions as the case is in this investigation, assigning names was possible.

The diversity of *Bacillus* species in soil varies considerably in terms of numbers and types of isolates. Nishijima *et al.,* (2005) have studied soil samples collected from different locations in Japan and found that number of spore forming bacteria did not differ a lot from one place to another but the types of isolates did. They were able to recover up to 18 different species from one location whereas; only one species was isolated from another. In this work 12 different numerical profiles were generated for the isolates. Had more tests been used in the generation of profiles, diversity could have been higher. In fact addition of tests for the construction of the numerical profiling system merely requires the increase in the number of digits; instead of 3 digits, 4 or 5 digits could be constructed.

The frequency of occurrence of each profile generated is illustrated in table 2. It is clear from this table that profile 4.1.5 was the most frequently encountered, followed by 4.4.3 while isolates labeled with profiles 5.7.5, 4.6.6 and 1.0.3 were the least encountered. These observations are consistent with those made by Kuta *et al.,* (2009) who found that frequency of occurrence among their *Bacillus* soil isolates (identified to species level by conventional methods) varied between 1.9% and 30.8%. The close similarity of results reported herein and those of Kuta *et al.,* (2009) indicated the effectiveness of the numerical profiling system in dealing with soil bacterial diversity as compared to traditional approaches.

Soil is a well known source of microorganisms capable of producing a variety of biologically active metabolites including antibiotics. Table 2 shows that 36% of *Bacillus* isolates recovered from Ajloon soil exhibited effective antagonistic activity against *S. aureus* ATCC 6538. This percentage is not far away from that determined by Kuta *et al.,* (2009) who found that 41.7% of *Bacillus* species isolated from soil samples obtained from 16 refuse dump sites in Minna – Niger were inhibitory against a strain of *S. aureus.*

It is of profound importance to note that isolates with similar profiles did not give 100% exact results in the antimicrobial activity test. This particular observation is of dual significance. First, had isolates with the same profile been with absolute phylogenetic relation and this is clearly not the case, all would have given the same inhibitory activity against the test organism. The lack of this absolute relatedness was expected as tests used were dependent on the biochemical activity and no tests were performed to identify the genotype of isolates. However, simple and limited number of tests was used, numerical profiling system proved to be as good as any conventional approach employed for the study of bacterial diversity. Second, not all isolates with similar profiles gave the same spectrum of activity against *S. aureus*. Out of 18 isolates labeled with the profile 4.1.5, 10 were effective inhibitors while, 5 and 3 isolates were with limited or no antimicrobial activity, respectively.

Statistical significance was found to exist ($p < 0.05$) between each profile and the number of isolates grouped in that profile with respect to the zone of inhibition measured. Table 2 shows that isolates with profiles 4.4.3 and 2.1.7 catered for 13 as well as 12 isolates, respectively, with the majority of these isolates being none inhibitory to the test organism.

Based on the outcome of this work, future study may exempt isolates labeled with profiles that were found to be of poor antimicrobial effect from being screened for inhibitory activity as they are unlikely to exhibit antimicrobial property. It might be thought that the use of a single bacterial species in the antimicrobial study is a draw back in the set up of this investigation, the fact is, this work was not designed to isolate a strain or strains of *Bacillus* with broad spectrum of antimicrobial activity but to demonstrate the usefulness of the profiling system in predicting the antimicrobial potentials of isolates and this was achieved.

5. Conclusion

This investigation has shown for the first time that numerical profiling is a useful technique for studying the diversity of soil spore forming bacteria. It also points out to the possibility of exploiting the same system in the prediction of antibacterial activity of isolates recovered from this extremely diverse habitat.

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Nitrate Reductase Assay Using Sodium Nitrate for Rapid Drug Susceptibility Testing of *Mycobacterium tuberculosis* Directly on Sputum Samples

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Abstract

Multidrug-resistant tuberculosis is an increasing public health concern in many parts of the world, especially in low-income countries, where most cases occur. Traditional drug susceptibility testing is either time-consuming, such the proportion method on solid media, or expensive, such as the BACTEC 960 System. The aim of this study was to evaluate a nitrate reductase assay (NRA) using sodium nitrate ($NaNO_3$) on smear- positive sputum for the detection of multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB) and compared it with the nitrate reductase assay using potassium nitrate (KNO_3) and Proportion Method (PM) or Direct Proportion Method (DPM). The NRA-$NaNO_3$ results were compared with other methods for 91 sputum samples for which comparable results were available. The sensitivity (ability to detect true drug resistance) and specificity (ability to detect true drug susceptibility) of the NRA- $NaNO_3$, were 100% and 96%, 93% and 100%, 85% and 98%, and 76% and 97% for Rifampin, Isoniazid, Streptomycin and Ethambutol, respectively. The results were in most cases available in 10 days. NRA-$NaNO_3$ is simple to perform and provides a rapid, accurate, especially in low-income countries and might become alternative to traditional methods.

Keyword: Nitrate reductase assay, Sodium nitrate, *Mycobacterium tuberculosis.*

1. Introduction

Tuberculosis (TB) remains one of the major causes of morbidity and mortality from infection in humans. The World Health Organization (WHO) estimates that one third of the world population is infected with Mycobacterium tuberculosis, 9.4 million new cases of tuberculosis and 1.3 million deaths from tuberculosis occurring worldwide. The worldwide incidence is 140 cases per 100.000 population (WHO, 2010).

The emergence of multidrug-resistant (MDR) tuberculosis, defined as tuberculosis caused by strains resistant to the two first-line drugs (Isoniazid and Rifampin), and extensively drug-resistant (XDR) tuberculosis, defined as tuberculosis caused by strains resistant to the two above mentioned drugs, to at least one fluoroquinolone, and to at least one of three injectable second-line drugs (Amikacin, Kanamycin and Capreomycin) (Bwanga *et al*., 2009).

Data from more than 100 countries collected during the last decade show that 5% of all TB cases have MDR-TB. There were an estimated 500,000 new MDR-TB cases in 2007. Twenty- seven countries accounted for 85% of all MDR-TB cases. The top five countries with the largest number of MDR-TB cases are India, China, the Russian Federation, South Africa and Bangladesh, while XDR-TB has been found only in 58 countries to date (WHO, 2010).

Tuberculosis is one of the most important health problems worldwide. For this reason, the rapid diagnosis of TB drug resistance is a priority to avoid the spread of resistant strains (Palomino, 2005). There are different methods for detection of TB drug-resistance.

The BACTEC radiometric system has the advantage of being more rapid (5-10 days), but requires the use of radio-isotopes and can be costly to be performed routinely. Commercial tests (MGIT, E-Test) and molecular tools (INNO –LIPA) have been proposed, but are expensive and also impractical for routine use (Lemus *et al.,* 2004; Palomino, 2005).

For developing countries, it would be useful to have a simple and inexpensive test that could rapidly detect drug-resistant *M. tuberculosis* strains. Several methods have been reported, including colorimetric methods that use redox indicators (MTT and resazurin) and phage amplification technology (Martin *et al.,* 2003; Simboli *et al.,* 2005).

Conventional tests for the detection of drug resistance require several weeks to yield results. Recently, alternative rapid methods have been developed (Solis *et al.,* 2010). Among them, the colorimetric nitrate reductase assays (NRA), based on the ability of *M. tuberculosis* to reduce nitrate to nitrite,

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has been successfully applied on solid medium. This indirect method result in less than 14 days but requires an initial 3 to 4 weeks for cultivation of the isolate (Coban *et al.,* 2004). Another conventional method is proportion method (PM) or Direct Proportion Method (DST) for mycobacterial drug susceptibility testing requires several weeks of incubation to give results (Canetti *et al.,* 1989).

The aim of the present study was to comprise performance of a direct NRA, PM and using sodium nitrate ($NaNO_3$) with clinical sputum samples instead of bacterial isolates in determining the susceptibilities to rifampin (RIF), isoniazid (INH), streptomycin (STR), and ethambutol (EMB) of *M. tuberculosis* strains in microscopy-positive clinical samples from patients with pulmonary tuberculosis.

2. Materials and Methods

2.1. Specimen Processing

From February to August 2012, a total of 100 smear-positive sputum samples from new and treated patients, with positive score of 1 + or more (>1 acid-fast bacillus-AFB) per field (WHO, 1998), were collected at the tuberculosis chest disease clinic in Basrah city. The samples (one per patient) were processed using the Modified Petroff Digestion Decontamination (WHO, 1999). The sediment was re-suspended in 1ml of sterile distilled water, and portions were plated onto NRA drug susceptibility testing medium and into a Lowenstein Jensen (LJ) tube without nitrate, which was later used for the Indirect Proportion Method (IPM)

2.2. Direct NRA Drug Susceptibility test (by using KNO_3)

The NRA was performed as described previously by Musa *et al.*(2005). We used standard LJ medium with 1.000 µg of KNO_3/ml and with or without Rifampin (RIF). For LJ medium with RIF, the critical concentration of 40µg/ml was used. Before NRA, part of the decontaminated suspension was diluted 1:10 in sterile distilled water. For each specimen, 0.2 ml of the undiluted suspension was inoculated into LJ medium containing KNO_3 and RIF, and 0.2 ml of the 1:10 dilution was inoculated into four drug-free LJ medium tubes containing KNO_3. The tubes were incubated at 37ºC.

The assay was performed as described previously by Angeby *et al.* (2002). After 10 days of incubation, 0.5 ml of freshly prepared reagent mixture (1 part 50% concentrated hydrochloric acid, 2 parts 0.2% sulfanilamide, and 2 parts 0.1% n-1-naphtyl-ethylenediamine dihydrochloride) was added to one drug-free tube. If any color appeared, the tube with RMP was developed with the reagent mixture. Otherwise, the other tubes were re-incubated, and the procedure was repeated at day 14 , day 18, and finally at day 28. An isolate was considered to be resistant if there was a color change in the RMP tube equal or greater than that in the 1:10-diluted growth control. An isolate was considered to be susceptible if there was no color change or a color change less than that in the 1:10-diluted growth control. NRA was considered to be invalid if the nitrate reaction was negative in the drug-free medium at day 28 despite the presence of colonies.

2.3. Direct Proportion Method (DST) or Proportion Method (PM)

The technique was carried out on normal LJ medium according to the laboratory standard procedure (Canetti, 1993). The medium was prepared in 7-ml portions in 150-by-155 mm glass tubes with rubber plugs, with or without antimicrobial agents incorporated. Critical concentrations of antituberculosis drugs were the same as were used for NRA. The critical proportion values were 10% for RIF and STR and 1% for INH and EMB. For each strain, part of the suspension was diluted 1:100, and 0.2ml of the dilution was inoculated into two tubes of LJ medium without antibiotics. Then, 0.2ml of the undiluted suspension was inoculated into the tubes containing LJ medium with antibiotics. The tubes were incubated at 37ºC. Final susceptibility results were reported after 40 days following the laboratory standard procedure, but preliminary results could be reported earlier for resistant strains, sometimes as early as after 20 days.

2.4. Direct NRA by using Sodium Nitrate (NaNO3)

The method is similar of direct NRA drug susceptibity test but using sodium nitrate ($NaNO_3$) in replacement of potassium nitrate (KNO_3) (Maira *et al.*, 2012).

2.5. Quality

Internal quality control was done using the fully susceptible *M. tuberculosis* H37Rv and Known MDR *M. tuberculosis* isolate.

2.6. Statistical Analysis

In the present study, the term sensitivity reflects the ability to detect a true drug resistance in a strain, whereas specificity reflects the ability to detect a true drug susceptibility. Statistical analysis of data was carried out by using SPSS analysis (Moore, 2000).

3. Results

One hundred sputum samples of *M. tuberculosis* were analyzed by the Direct NRA-KNO_3, Direct NRA-$NaNO_3$ and DST methods. Table 1 shows the results obtained with Direct NRA-KNO_3 compared to DST method using sputum samples. The smear results for AFB were positive with more than 10 AFB per field (+++). Of the 100 smear microscopy-positive results, 9 had negative growth control as determined by the NRA method and could thus not be used in the comparison. Then, 91 sputum samples could be used for the comparison between three methods.

In table 1, for RIF, 60 isolates were found resistant and 24 susceptible by both methods. For INH, 64 isolates were resistant and 22 susceptible by both methods; four strain gave a discordant result being susceptible by DST method. For STR , 67 isolates were resistant and 20 susceptible by both methods; three isolates were susceptible by DST but resistant by NRA-KNO_3. In other hand, for EMB, 67 isolates were

resistant and 18 susceptible by both methods; 4 isolates were susceptible by DST but 2 isolates susceptible by NRA-KNO_3. The results were available in 10 days for 11 samples, in 14 days for 45 samples, and in 18 days for 35 samples.

Table 2 shows the sensitivity and specificity obtained with NRA using $NaNO_3$ and KNO_3 compared to the DST method. Drug susceptibility testing for RIF showed a sensitivity of 93% with KNO_3 and 100% with $NaNO_3$, but specificity was 96% for both nitrate sources. For INH the sensitivity was 90% with KNO_3 and 93% with $NaNO_3$ while the specificity was 97% and 100%. For STR the sensitivity was 80% with KNO_3 and 85% with $NaNO_3$ while the specificity was 94% and 98%. In addition, for EMB the sensitivity was 71% with KNO_3 and 76% with $NaNO_3$ while the specificity was 90% and 97%.

Figure 1 shows the comparison of three methods are NRA- $NaNO_3$, NRA-KNO_3 and DST.

Table 1. Comparison of the susceptibility results, sensitivityand specificity to the Direct NRA method by using KNO_3,$NaNO_3$ and DST for *M. tuberculosis* in sputum samples.

Drug	Direct proportion method (DST) determination	Direct NRA by using KNO_3			
		NO.		%	
		R	S	Sensitivity	Specificity
RIF	R	60	5	93	-
	S	2	24	-	96
INH	R	64	4	90	-
	S	1	22	-	97
STR	R	67	3	80	-
	S	1	20	-	94
EMB	R	67	4	71	-
	S	2	18	-	90
Total	R	258	16	80	-
	S	6	84	-	97

R= Resistant ; S= Susceptible

Sensitivity = reflects the ability to detect (true resistant).

Specificity = reflects the ability to detect (true susceptibility).

Table 2. Sensitivity and specificity of the NRA using KNO_3 and $NaNO_3$ compared to the DST method for *M. tuberculosis* in sputum samples.

Drug	Direct proportion method (DST) determination	NRA – KNO_3				NRA- $NaNO_3$			
		No		%		NO		%	
		R	S	Sensitivity	Specificity	R	S	Sensitivity	Specificity
RIF	**R**	60	5	93	-	61	3	100	-
	S	2	24	-	96	2	25	-	96
INH	**R**	64	4	90	-	65	5	93	-
	S	1	22	-	97	1	20	-	100
STR	**R**	67	3	80	-	63	4	85	-
	S	1	20	-	94	2	22	-	98
EMB	**R**	67	4	71	-	60	6	76	-
	S	2	18	-	90	1	24	-	97
Total	**R**	258	16	80	-	250	18	86	-
	S	6	84	-	97	6	91	-	98

R = Resistant ; S = Susceptible

Sensitivity = reflects the ability to detect true resistant.

Specificity = reflects the ability to detect true susceptibility.

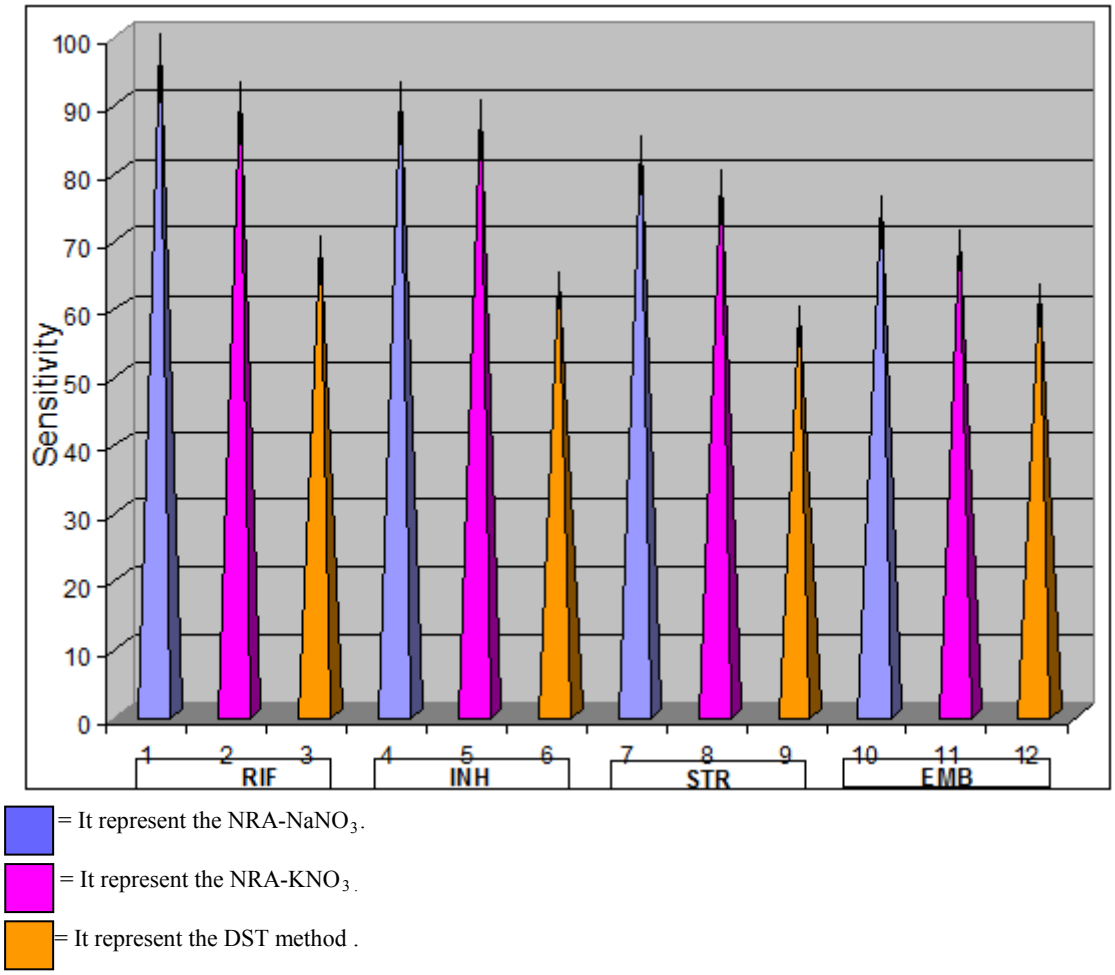

Figure 1. Results the sensitivity of NRA using KNO_3, $NaNO_3$ compared to DST method.

4. Discussion

To our knowledge, this is the first evaluation of the direct NRA in Iraq. The most worrisome trend during recent years is an increase in multidrug-resistant MDR (for example resistant to RIF and INH) TB strains. Rapid detection of MDR strains is very important to restrict their spread in the population. Current method for DST of MDR-TB are either costly or very slow. So, a cost-effective and rapid drug-susceptibility method is required to guide the treatment of TB (Coban *et al.*, 2004 ; Mishra *et al.*, 2009). Complete agreement between the results of the direct NRA and DST method was found for RIF, which is important since RIF, together with INH, is the most important antituberculosis drug. Resistance to RIF is also almost always associated with multidrug resistance (Vareldzis *et al.*,1994) and can thus serve as a marker of MDR of *M. tuberculosis* strains if resources are limited.

The direct NRA was comparable to the direct DST method regarding susceptibility testing of INH (sensitivity to detect resistant was 90% with specificity was 97%). In addition, the sensitivity to detect resistance to STR and EMB were low to be acceptable (80% and 71%), but the specificity were 94% and 90% respectively. Results for RIF and INH susceptibility were similar to indirect NRA method (Sethi *et al.*, 2004 ; Musa *et al.*, 2005). These results may be need to adjusting the critical drug concentrations used in the NRA test, although the susceptibility of *M. tuberculosis* to STR and EMB is more complicated to determine the antibiotic sensitivity (Maira *et al.*, 2012) .

The NRA method utilizes the detection of nitrate reduction as an indication of growth, and therefore, results can be obtained faster than by visual detection of colonies. The ability to reduce nitrate is typical for *M. tuberculosis*, although some other mycobacterial species, like *Mycobacterium kansasii*, and most rapid growers share this characteristic, nitrate reductase-negative strains of *M. tuberculosis* are rare (< 1%)(Rosales *et al.*, 2009).

In the other hand, this study showed that the NRA gave similar results using KNO_3 or $NaNO_3$ as nitrate source. NRA using $NaNO_3$ showed high sensitivity and specificity for RIF (100% and 96%) and INH (93% and 100%). These results are in agreement with previous studies presented in a meta-analysis that evaluated the accuracy of the NRA for the detection of MDR .

According to that meta-analysis; the sensitivity and specificity were more than 94% and 92% for RIF and INH (Maire *et al.*, 2012). Another important finding in this study was that 97% of the isolates showed results in 10 days with NRA using $NaNO_3$ whereas 88% of the isolates gave results in 10 days with NRA using KNO_3 in the previous studies (Coban *et al.*, 2004). Our study suggests the use of $NaNO_3$ as the source of nitrate for NRA.

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Protective Effects of *Enantia chlorantha* Stem Bark Extracts on Acetaminophen Induced Liver Damage in Rats

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Abstract

The study was designed to evaluate the hepatoprotective activity of different solvent extracts (hexane, chloroform, ethyl acetate and methanol) of *Enantia chlorantha* stem bark in acute experimental liver injury induced by acetaminophen. The effects observed were compared with a known hepatoprotective agent, silymarin (100 mg/kg p.o.). Preliminary phytochemical tests and acute toxicity study were done. The degree of hepatoprotection was measured using serum transaminases (AST and ALT), alkaline phosphatase, bilirubin, albumin, and total protein levels. In the acute liver damage induced by acetaminophen, *E. chlorantha* stem bark extracts (200 mg/kg, p.o.) significantly reduced the elevated serum levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and bilirubin in acetaminophen induced hepatotoxicity. The total serum protein was significantly increased ($P<0.05$) by all the solvent extracts. Histological examination of the liver tissues supported the hepatoprotection. Our findings suggested that *E. chlorantha* stem bark extracts possessed hepatoprotective activity; the hexane extract of stem bark of *E. chlorantha* plant possesses better hepatoprotective activity compared to other extracts.

Keywords: Hepatoprotection; Acetaminophen; Liver; Stem Bark Extracts; *Enantia chlorantha*

1. Introduction

The liver is the most important organ in the body. The liver plays a pivotal role in regulating various physiological processes (Rajib *et al.,* 2009). It is the centre of metabolism of nutrients such as carbohydrates, proteins and lipids. It is also involved in the metabolism and excretion of waste metabolites, drugs and other xenobiotics from the body thereby providing protection against foreign substances by detoxifying and eliminating them (Mohamed *et al.,* 2010). As a result of this, the liver is exposed to all types of toxic abuse from both endogenous and exogenous substances which may produce liver degeneration.

Liver diseases have become one of the major causes of morbidity and mortality in man and animals and hepatotoxicity due to drugs appears to be the most common contributing factor (Russmann *et al.,* 2009). For instance, drug-induced liver injury accounts for at least 13% of acute liver failure cases in the United States (Au *et al.,* 2011). The manifestations of drug-induced hepatotoxicity are highly variable, ranging from asymptomatic elevation of liver enzymes to fulminant hepatic failure. Acetaminophen also known as paracetamol, taken in overdose can cause severe hepatotoxicity and nephrotoxicity (Yakubu *et al.,* 2008). In spite of the tremendous advances in modern medicine, there is no effective drug available that stimulates liver function, offers protection to the liver from damage or helps to regenerate hepatic cells (Chaudhary, 2010).

Medicinal plants play a key role in human and animal health care. About 80% of the world population relies on the use of traditional medicine, which is predominantly based on plant material (WHO, 1993). Despite the significant popularity of several herbal medicines in general, and for liver diseases in particular, they are still unacceptable treatment modalities for liver diseases due to lack of standardization of the herbal drugs, lack of identification of active ingredient(s)/principles(s), lack of randomized controlled clinical trials (RCTs) and lack of toxicological evaluation (Radha & Yogesh, 2005). Therefore, due importance has been given globally to develop plant-based hepatoprotective drugs effective against a variety of liver disorders.

There are numerous plants and traditional formulations available for the treatment of liver diseases. *Silybum marianum, Orthosiphon stamineus* and *Foeniculum*

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vulgare are amongst natural products whose hepatoprotective effects have been investigated and documented (Hanefi *et al.,* 2004; Pradhan and Girish, 2006; Subramanian *et al.,* 2006).

Enantia chlorantha Oliv (family-*Annonaceae*) common name-African Yellow Wood is widely distributed along the coasts of West and Central Africa and also very common in the forest regions of Nigeria (Adesokan *et al.,* 2007).

Several studies have shown that the stem bark of *E. chlorantha* possesses wide spectrum antimicrobial and antimalarial (Adesokan *et al.,* 2007; Odugbemi *et al.,* 2007) activities. In Cameroon, the stem bark extract is also used to treat jaundice, urinary tract infections (Adjanohoun *et al.,* 1996), hypoglycaemia, typhoid fever (FAO, 2001). The stem bark is also used for treating leprosy spots, as haemostatic agent and uterus stimulant (Gill, 1992). An anti-sickling compound has also been isolated from the ethanolic extract of the plant (Ejele *et al.,* 2012).

Despite its numerous medicinal uses and importance in the treatment of many illness and diseases in Africa, to our knowledge, no c oncrete scientific study has been reported to prove the folklore claim of the utility of *E. chlorantha* in the treatment of liver diseases and hence one of the objectives of the present study was to correlate the ethnobotanical evidence with scientific study. Further, the study also attempts to evaluate *in vivo* hepatoprotective and curative effects of stem bark extracts of *E.chlorantha* on acetaminophen induced hepatotoxicity models in rats using solvents of various polarities.

2. Materials and Methods

2.1. Plant Material and Authentication

The plant samples were collected from local region between September and October (rainy season), 2012. The plant was identified and authenticated at the Forestry Research Institute of Nigeria, Ibadan and voucher specimen (FHI. 109950) was preserved at the herbarium.

2.2. Plant Materials Extraction

Stem barks of *E. chlorantha* were dried under shade for 7 days until a constant weight was obtained. This was ground into powder using an electric blender (Blender/Miller III, model MS-223, Taiwan, China). The powder was packed into Soxhlet column and extracted with hexane. The same material was successively extracted with chloroform, ethyl acetate and methanol. The solvents were filtered, squeezed off and evaporated off under reduced pressure in a rotary evaporator to obtain the crude extract. After concentrated preparation, the dried powder extract was stored at 4ºC (Prakash *et al.,* 2008).

2.3. Experimental Animals

Thirty five wistar albino rats (*Rattus norvegicus*) consisting of both male and female with average weight of 150-200 g were obtained from the Animal Holding Unit of the Department of Veterinary Physiology, Biochemistry and Pharmacology, Faculty of Veterinary Medicine, University of Ibadan, Nigeria. The animals were allowed free access to feed and fresh water *ad libitum*. All the animals were acclimatized to laboratory conditions for two weeks before commencement of the experiment. The study was approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, University of Ibadan.

2.4. Drugs and Chemicals

Silymarin (Micro labs, Tamilnadu, India), ethanol, hexane, chloroform, ethyl acetate, methanol and the rest of the chemicals utilized were of analytical grade and were prepared in all glass distilled water. Acetaminophen (Emzor Paracetamol®) was purchased over the counter.

2.5. Phytochemical Screening

The qualitative methods already established to test for classes of compounds in plant extracts by Ciulei (1964) and Chitravadivu *et al.* (2009) were used. The substances that were tested for included: phenolics, alkaloids, steroids, tannins, flavonoids, saponins, glycosides and phlebotanins. The hexane, chloroform, ethyl acetate and methanol extracts of *Enantia chlorantha* stem bark were used to determine the compounds.

2.6. Acute Toxicity Study

This study was conducted according to the Organisation for Economic Cooperation and Development's (OECD) revised up and down procedure for acute toxicity testing (OECD, 2001). Animals were divided into eight groups of five rats each. The control group received distilled water (10 ml/kg) groups II-VIII received 100, 200, 400, 800, 1000, 2000 or 3000 mg/kg of ethanol extract of *Enantia chlorantha* stem bark orally in a single dose. Immediately after dosing, the rats were observed for mortality and clinical signs for the first hour, then hourly for three hours and then periodically for 72hours and then kept for up to 14 days post-treatment in order to observe for any toxic symptoms and mortality.

2.7. Experimental Design

Animals were randomly divided into seven groups (I-VII) of five animals per group. Group I (normal control) received neither the plant extract nor acetaminophen for 8 days. Group II (negative control group) Induction of hepatotoxicity using acetaminophen: The animals received distilled water for 7 days and were administered acetaminophen (500 mg/kg) orally on day 8. Group III (positive control group) – pre-treatment with silymarin (100 mg/kg) for 7 days (p.o) followed by a single dose of acetaminophen on day 8. Groups IV, V, VI and VII – Pre-treatment with hexane, chloroform, ethyl acetate and methanol extract of *Enantia chlorantha* stem bark respectively at 200 mg/kg for 7 days (p.o) followed by a single dose of acetamionophen on day 8.

During the period of drug treatment the rats were fed *ad libitum* with standard pellet diet and had free access to water. The biochemical parameters were estimated after 24 hours following the administration of acetaminophen.

2.7.1. Serum Biochemical Analyses

Blood was obtained from all animals by puncturing retro-orbital plexus**.** The blood samples were allowed to coagulate and then serum was separated by centrifuging at 3000 rpm for 20 min, collected into sterilized tubes and

stored at -20 °C. Serum biochemical parameters were analyzed: Aspartate aminotransferase (AST) (Reitman and Frankel, 1957), alanine aminotransferase (ALT) (Reitman and Frankel, 1957), alkaline phosphatase (ALP) (Kind and King, 1954), serum bilirubin (Mallay and Evelyn, 1937), total protein, albumin, blood urea nitrogen (BUN) using RANDOX® laboratory reagent kits (RANDOX® Laboratories Ltd., Ardmore, United Kingdom).

2.7.2. Histopathological Examination

After collection of blood samples the rats in different groups were sacrificed. 3-5 mm samples of the liver tissue were collected and placed in 10% formaldehyde solution for histopathological study. The pieces of liver were processed and embedded in paraffin wax and sections were made about 4-6 µm in thickness. After staining with haematoxylin and eosin (H&E), slides were examined under microscope (Olympus, Japan) for histopathological changes and photographed.

2.8. Statistical Analysis

All data were expressed as mean ± standard error of mean (SEM), comparison was by the student t test using Graphpad Prism version 4.00 for Windows, Graphpad Software. Significance was reported at $P < 0.05$.

3. Results

Phytochemical screening of the extracts of *E. chlorantha* stem bark revealed the presence of phenolics, flavonoids, alkaloids, glycosides and saponins (Table 1).

All four extracts of *E. chlorantha* stem bark at a dose of 3000 mg/kg *p.o.*, did not produce any mortality in the rats during the pilot acute toxicity study.

There was a significant ($P < 0.05$) increase in the level of serum total protein (TP) in the test groups when compared with the negative control/ untreated group (distilled water). Rats pre-treated with HX, CH, EE and ME extracts of *E. chlorantha* stem bark showed an insignificant increase ($P > 0.05$) in the serum albumin contents when compared with the untreated group. Groups pretreated with HX, CH, EE and ME extracts of *E. chlorantha* stem bark showed a significant increase in the serum globulin contents when compared with the negative control/ untreated group.

The test groups had a significant decrease ($P < 0.05$) in activities of AST, ALT and ALP when compared with the negative control/ untreated group. The extracts (HX, CH, EE and ME) also decreased significantly the activities of ALT and ALP relative to silymarin (positive control group) (Table 2).

The results reported in table 2 also showed that groups treated with HX, CH, EE and ME extracts of *E. chlorantha* stem bark had significantly decreased ($P < 0.05$) levels of serum bilirubin when compared with the negative control/ untreated group.

Liver sections from acetaminophen treated rats showed vacuolar degeneration and different stages of necrotic alterations in the hepatocytes surrounding the central veins. Focal mononuclear leucocytes inflammatory cells infiltration was observed in between the degenerated and necrotic hepatocytes, as well as in the portal area. There was marked congestion of portal vessels and central vein (Figure 1). Liver sections from rats pre-treated with silymarin showed diffused proliferation of Kupffer cells between the hepatocytes associated with dilatation in the portal vein and inflammatory cells infiltration in the portal area (Figure 2).

Liver of rats pre-treated with hexane extract of *E. chlorantha* prior to acetaminophen administration showed diffused proliferation of the Kupffer cells between the hepatocytes, associated with dilatation in the central vein, and focal inflammatory cells infiltration in the hepatic parenchyma (Figure 3). The liver of rats given chloroform extract of *E. chlorantha* before administration of acetaminophen showed dilatation in the central and portal veins with newly formed bile ductules, oedema and inflammatory cells infiltration in the portal area. Focal haemorrhage was noticed in the hepatic parenchyma (Figure 4). The liver of rats dosed with ethyl acetate and methanol extract of *E. chlorantha* stem bark showed no visible histopathological lesion (Figure 5 and 6, respectively).

Table 1. Phytochemical constituents of hexane, chloroform, ethyl acetate and methanol extracts of *Enantia chlorantha* stem bark.

Phytochemical	Hexane	Chloroform	Ethyl acetate	Methanol
Phenolics	-	+	++	++
Flavonoids	+	+	+	+
Alkaloids	++	++	++	++
Glycosides	±	+	±	+
Saponins	++	++	++	++
Tannins	-	-	-	-
Phlebotanins	-	-	-	-
Steroids	-	-	-	-

++ = Strongly positive, + = positive, ± = weakly positive, and - = not detected

Table 2. Serum biochemical values of rats administered with hexane, chloroform, ethyl acetate, methanol extracts of *E. Chlorantha* stem bark and the control groups

	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
TP (g/dL)	2.75 ± 0.15 [a]	1.28 ± 0.09 [b]	1.88 ± 0.23 [c]	2.65 ± 0.22 [a]	1.84 ± 0.02 [c]	1.46 ± 0.04 [b]	1.98 ± 0.19 [a]
ALB (g/dL)	0.85 ± 0.07 [a]	0.43 ± 0.09 [b]	0.72 ± 0.09 [a]	0.45 ± 0.05 [b]	0.80 ± 0.20 [a]	0.44 ± 0.08 [b]	0.88 ± 0.03 [a]
GLB (g/dL)	1.90 ± 0.18 [a]	0.85 ± 0.05 [b]	1.16 ± 0.09 [c]	2.20 ± 0.17 [a]	1.04 ± 0.02 [c]	1.02 ± 0.10 [c]	1.10 ± 0.08 [c]
AST (U/L)	35.46 ± 2.81 [a]	56.00 ± 1.33 [b]	38.50 ±2.26 [a]	34.50 ±0.63 [a]	43.04 ± 0.60 [c]	35.20 ±4.50 [a]	41.50 ±1.71 [c]
ALT (U/L)	41.39 ± 2.61 [a]	75.25 ±1.80 [b]	59.75 ± 2.40 [c]	42.25 ± 1.03 [a]	55.00± 1.41 [c]	46.00 ± 7.48 [a]	55.25 ± 3.35 [c]
ALP (U/L)	35.61 ± 3.11 [a]	72.25 ± 0.85 [b]	46.25 ± 6.76 [b]	34.00 ± 2.94 [a]	30.00±2.97 [a]	38.40 ± 4.35 [a]	46.75 ± 9.16 [b]
BIL(mg/dl)	6.56 ± 0.34 [a]	9.92 ± 0.17 [b]	8.57 ± 0.06 [b]	6.52 ± 0.36 [a]	9.05 ± 0.15 [b]	6.96 ± 0.94 [a]	7.95 ± 0.20 [c]

Values are expressed as mean ± SEM (n= 5 mice/ group).
TP- total protein ALB- albumin GLB- globulin
AST- aspartate aminotransferase ALT- alanine aminotransferase ALP- alkaline phosphatase
BIL- bilirubin
Means with different superscripts within rows are significantly different at $P < 0.05$

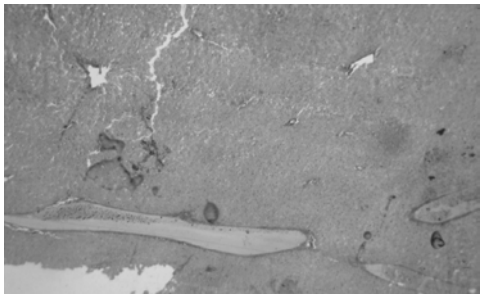

Figure 1. Shows vacuolar degeneration, necrotic hepatocytes, marked congestion of portal vessels and central vein. H & E stain (x100)

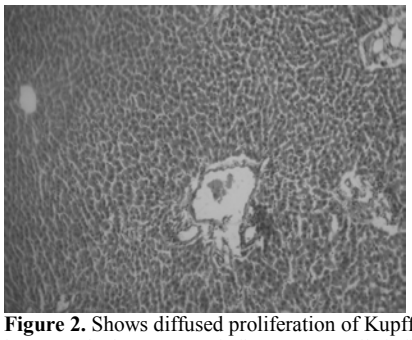

Figure 2. Shows diffused proliferation of Kupffer cells between the hepatocytes inflammatory cells infiltration.H & E stain (x100)

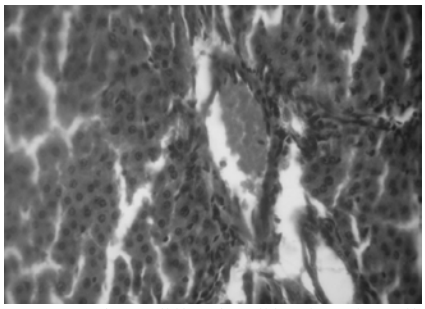

Figure 3. Shows diffused proliferation of Kupffer cells between the hepatocytes dilatation in the central vein and inflammatory cells infiltration in the hepatic parenchyma. H & E stain (x100).

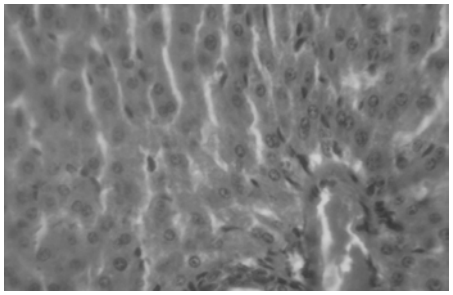

Figure 4. Dilatation in the central and portal veins with newly formed bile ductules, oedema and inflammatory cells infiltration in the portal area. Focal haemorrhage was noticed in the hepatic parenchyma (H & E stain(x100).

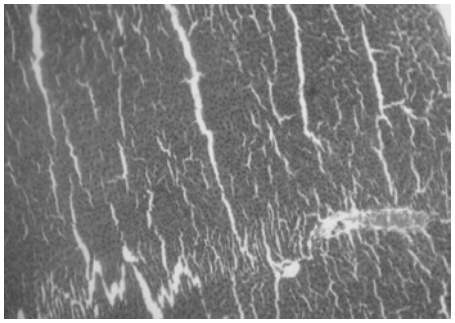

Figure 5. Shows no visible histopathological lesion. (H& E stain) x 100.

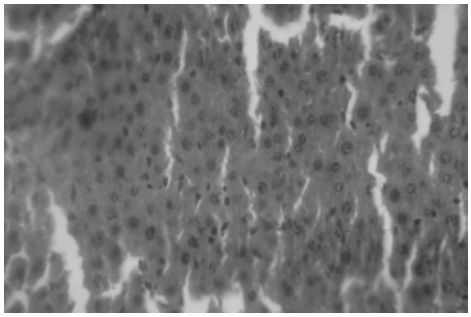

Figure 6. Shows no visible histopathological lesion .H& E stain (100x).

4. Discussion

The stem bark extracts of *E. chlorantha* was found to contain phenolics, flavonoids, alkaloids, glycosides and saponins. These are secondary metabolites which have been reported to cure a lot of diseases (Dongmo *et al,* 2007*;* Suman *et al,* 2011). The fact that methanol exhibited the strongest reactions; thus being able to extract more phytochemicals, could mean that there are more non-polar phytochemicals in the stem bark.

Acetaminophen is a common antipyretic agent, which is safe in therapeutic doses but can produce fatal hepatic necrosis in man, rats and mice with toxic doses (Dash *et al,* 2007). Protection against acetaminophen-induced toxicity has been used as a test for potential hepatoprotective activity by several investigators (Sabir & Rocha, 2008; Parmar *et al,* 2010).

Proteins are important organic constituents of the animal cells playing a vital role in the process of interactions between intra and extra cellular media (Waqar *et al,* 2004). In the present study, the untreated/ negative control group administered with acetaminophen showed a decrease in the level of serum total protein (TP) while pre-treatment with solvent extracts of *E. chlorantha* (500mg/kg) showed an increase in the level of serum protein. Being a part of cell membrane and as an enzyme, protein helps to balance sub cellular fractions. Protein and amino acids are also very important nutrients and they play a major role in the synthesis of microsomal detoxifying enzymes which help detoxify toxicants that enter into the animal's body (Abubakar *et al,* 2010). The reduction in the protein levels in the untreated/ negative control group might thus be as a result of their metabolism to liberate energy during acetaminophen toxicity. The liver is also an important site for the synthesis of many serum proteins (Ahsan *et al,* 2009). The reduction in serum total protein observed in the acetaminophen group may also be associated with decrease in the number of hepatocytes which consequently results in decreased hepatic capacity to synthesize protein. Pre-treatment with HX, CH, EE and ME extracts of *E. chlorantha* stem bark significantly increased TP indicating the hepatoprotective activity of the extracts most probably through hepatic cell regeneration (Olorunnisola *et al,* 2011). These results are in line with the report by Manokaran *et al*, (2008) that oral administration of *Aerva lanata* to acetaminophen treated rats showed increased serum protein level when compared to acetaminophen alone treated rats. Similarly oral administration of hydro-ethanolic extract (70%) of *Calotropis procera* flowers to acetaminophen treated rats showed significantly increased serum protein level (Setty *et al,* 2007). The highest increase in serum total protein content was noticed in the rats treated with the HX extract (this group had more than a two-fold increase) of *E. chlorantha* stem bark and this increase was statistically significant when compared with the acetaminophen treated rats. This also explains the corresponding reduction and increases in albumin in the untreated group and the test groups as about 60% of total serum protein is albumin (Musa *et al,* 2005).

It has been reported in several studies that liver enzymes are liberated into the blood whenever liver cells are damaged and enzyme activity in the plasma is increased (Chang, 2009). Thus ALT, AST and ALP activity and serum bilirubin level are largely used as most common biochemical markers to evaluate liver injury (Ajayi *et al,* 2009). Elevation of these liver enzymes is also associated with cell necrosis of many tissues especially the liver (Adedapo *et al*, 2004). The current study also confirmed these effects of acetaminophen overdose toxicity, as indicated by marked increases in serum hepatic enzymes in the control/ untreated group. This is in consonance with the findings of Vadivu *et al,* (2008) who stated that acetaminophen causes liver damage in rats and significantly ($P < 0.05$) increased the AST and ALT levels in serum when compared with silymarin which has a remarkable protection of serum AST and ALT levels towards acetaminophen induced hepatotoxicity. The significant ($P<0.05$) decrease in activities of these enzymes by the extracts may indicate that the plant extracts did not have necrotic effect on the liver. This may be due to the fact that the extracts offer protection and maintain the functional integrity of hepatic cells. The protective effect may be the result of stabilization of plasma membrane thereby preserving the structural integrity of cell as well as the repair of hepatic tissue damage caused by acetaminophen (Murugaian *et al,* 2008). The increased ALP concentration following acetaminophen administration is in line with existing literature that ALP synthesis is increased by cells lining bile canaliculi usually in response to cholestasis and increased biliary pressure (Gaw *et al.,* 1999). Increased level was obtained after acetaminophen administration and it was brought to near normal level by *E. chlorantha* treatment. This further signifies the curative nature of the extract against acetaminophen toxicity.

Serum bilirubin is considered an index for the assessment of hepatic function and any abnormal increase indicates hepatobiliary disease and severe disturbance of hepatocellular architecture (Martin and Friedman, 1992). Acetaminophen administration resulted in increased serum bilirubin level, (Table 2) thereby suggesting severe hepatic injury and confirming the hepatotoxic nature of acetaminophen. Treatment with *E. chlorantha* stem bark extracts significantly decreased the elevated level of total bilirubin in serum towards normalcy indicating its hepatoprotective efficacy. The hexane extracts demonstrated the highest potency in this regard.

Liver of rats administered with acetaminophen showed severe necrosis, with disappearance of nuclei. This could be due to the formation of highly reactive radicals because of oxidative threat caused by acetaminophen (Shardul, 2010). Histopathological changes of the group pre-treated with the extracts showed significant improvement in architecture. Pretreatment with the ethyl acetate and methanol extracts restored the hepatic architecture and protected the liver tissue from fatty and degenerative changes, by preventing the toxic chemical reaction. Although, necrotic changes were still evident in the liver of rats

pre-treated with the hexane and chloroform extracts, the severity of the damage was less intense significantly. The various phytoconstituents of the stem bark extracts of *E. chlorantha* might be helpful in the changes in the membrane, in the mitochondria or at the ionic level like calcium (Rang *et al*., 2003*)*.The extracts of *E. chlorantha* stem bark may have a role in the process of regeneration and prevention of fibrosis. However, our study has shown the centrizonal necrosis by acetaminophen and prevention of such changes and restoration to normalcy in the centrizonal area by extracts of *E. chlorantha* stem bark.

The possible mechanism responsible for the protection of the acetaminophen induced liver damage by the extract of *E. chlorantha* maybe a r esult of the extract acting as a free radical scavenger by intercepting the radicals involved in acetaminophen metabolism by microsomal enzymes or the phytochemicals constituents of the plant because a number of scientific reports indicate the role of certain flavonoids and steroids in hepatoprotection against hepatotoxins. The presence of these compounds in *E. chlorantha* may be responsible for the protective effect on acetaminophen induced liver damage in rats.

5. Conclusion

Based on t he above results, it could be concluded that hexane, chloroform, ethyl acetate and methanol extracts of *Enantia chlorantha* stem bark exert significant hepatoprotection against acetaminophen-induced toxicity.

The hexane extract of *E. chlorantha* showed better hepatoprotective activity in acetaminophen induced liver damage compared to chloroform, ethyl acetate and methanolic extract(s) as indicated by maximum prevention of increased serum biochemical parameters.

The stem bark extract of *E. chlorantha* extract may be hepatoprotective.

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Odonata of Wadi Al Mujib Catchment with Notes on the Impact of Wadi Al Mujib Dam, Jordan (Insecta: Odonata)

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Abstract

A total of 14 species pertaining to five families (Platycnemididae, Coenagrionidae, Gomphidae, Aeshnidae and Libellulidae) were identified along eight sites in the Wadi Al Mujib catchment. Collected species varied along the eight sampling sites, ranging from a single species from Al Mujib dam to a maximum of seven species in the waterfalls to the bridge site. In the present study, eight species are considered as new records to the Odonata of the Wadi Al Mujib catchment *(Ischnura elegans, I. evansi, I. fountaineae, I. senegalensis, Anax parthenope, Orthetrum sabina, Sympetrum fonscolombii* and *Zygonyx torridus*). Such changes over the past 35 years reflect the dynamics of dragonflies' spatial movement within their distribution range. Results shown in this study strongly indicates the negative impact of the Wadi Al Mujib dam on the dragonfly fauna, as a single species was recovered from the dam proper. This is mainly due to the sharp cliffs and water level fluctuation and the limited breeding areas. Few flat areas with scarce vegetation were identified around the dam. Water level fluctuation does not allow steady vegetation growth around the edges of the dam, yet, the abrupt water depth is not suitable for development of the larval stages of dragonflies. Sharp edges are not suitable for breeding and perching of these insects.

Key words. Dragonflies, Wadi Al Mujib, Jordan, Biodiversity.

1. Introduction

The Wadi Al-Mujib basin drains a large part of central Jordan. Its principal wadis form a deep canyon complex that developed through the Moab plateau, the eastern uplifted shoulder of the Dead Sea rift. The drainage development of the Wadi Al Mujib basin since the Late Oligocene regression of the Tethys has been submitted to the combined effects of different environmental changes: rift activity, neotectonic history and climate changes (De Jaeger and De Dapper, 2002). The catchment is fed by several tributaries extending from the east near the main spring in Um Al Rasas, wadi systems around Karak then join Wadi Al Hidan near Al Mujib Nature Reserve.

Two major biogeographical zones of Jordan are represented in the reserve, namely the Irano-Turanian and the Saharo-Arabian zones. These characteristics and variations enhance the formation of five vegetation types, which, as a result, enrich the diversity of both faunal and floral elements in the reserve. These major vegetation types are the Mediterranean non-forest, saline, water, tropical and steppe, with a total size area of 2.95, 10.38, 30.28, 39.39 and 97.42 km² respectively. The reserve is characterized by three major wadis with continuous water flow (Wadi Mujib, Wadi Hidan and Wadi Zarqa Ma'in).

Dragonflies and damselflies are among the most ancient insects that inhabited the earth. These remarkable species are considered as one of the most sensitive species of freshwater ecosystems together with other groups such as Ephemeroptera and Trichoptera. Their existence is dependent on the water quality and its availability as well as river structure. Both larvae and adults are predators feeding on aquatic and flying insects. They are often used as indicators for environmental health and conservation management (Corbet, 1999). Dragonflies are used in both basic and applied research due to the relative ease them (Kalkman *et al*., 2009).

The Odonata of Jordan received attention during the late seventies and early eighties by Dumont (1973, 1975). As a part of his dissertation, W. Schneider studied the dragonfly fauna of the Levant, including Jordan (Schneider 1981a,b, 1982a,b, 1985, 1986). Ever since, few studies were undertaken to explore this insect group.

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Amr *et al*. (1997) studied the dragonflies of the Azraq oasis and later Katbeh-Bader *et al.* (2002) examined the Odonata collections deposited in the Insect Museum at the Jordan University and at the Jordan University of Science and Technology. They reported a total of 47 species of dragonflies from Jordan, of which 29 were formerly known in the country. A review article with emphasis on conservation of dragonflies and their habitats in Jordan was published by Katbeh-Bader *et al.* (2004). Recently, Kunz *et al.* (2006) published a comprehensive study on the distribution of *Zygonyx torridus* in the Palaearctic, including new localities from Jordan.

The most important contribution on the dragonflies of Wadi Al Mujib catchment was undertaken by Schneider (1986). In his dissertation, he listed a total of nine species met along the Al Mujib catchment from 1981 to 1983. Three species were recorded from the upper reaches of Wadi Al Mujib, five from the current dam location and four along the wadi until it reaches the Dead Sea.

Dumont (1991) listed seven species from Wadi Al Mujib without referring to specific localities. Some of these species were already reported by Schneider (1986).

The objectives of this study are: to know the Odonata of Wadi Al Mujib catchment, identify key species that can be used as indicators and to define the effects of the Al Mujib dam on dragonflies populations.

2. Materials and Methods

2.1. Sampling Sites

Eight sampling sites were selected within the Wadi Al Mujib catchment in order to represent various habitats (Figure 1 and 2). The study was conducted during July 2011.

These sites were selected based on the overall habitat type and their accessibility. Some areas are very difficult to access due to the sharp ridges around the main flow of the water course. Additionally, the upper reaches of Wadi Al Mujib beyond Um Al Rasas are drained out due to water pumping and the main water course disappears before it reaches the main dam.

2.2. Description of the Sites Studied

Site 1: Um Al Rasas (E 35°53'46" N 31°23'59")

This is the main spring that feeds the Al Mujib river. This spring is surrounded by relatively thick vegetation of *Typha domingensis* and *Nerium oleander* and by a flat rocky area. Plastic water pipes to pump water for irrigation are scattered along the spring and the main stream. The area is not polluted by animal excrement or plastic.

Site 2: Al Mujib dam lake (35°49'26", N 31°25'48")

The lake created by the Wadi Al Mujib dam is located within the heart of the Wadi Al Mujib catchment. The elevation is about 208 m asl. The dam collects water from springs and main floods during winter. Maximum depth is 60 m and the total capacity reaches 32 million m3. The periphery of the lake consists of sharp rocky cliffs that were formed during the construction phase (Figure 2B). Very limited open areas are available around the water. Vegetation is very scarce due to the continuous flooding and water retraction. Only small patches of *Juncus* sp. are present in some areas.

Small scaled agriculture occurs along the margins of the lake and on the relief around. Water is pumped from the dam by means of pumping engines. Insecticides are used by farmer to spry their vegetables in an unorganized fashion.

Site 3: Under the dam (E 35°48'58", N 31°26'47")

This site is characterized by the presence of side pools with a dense vegetation of *Typha* sp. and Phragmites near the main stream. The latter is narrow and is bordered by rocky edges. Scattered *Tamarix* trees and *Nerium oleander* are in close vicinity to the main water course. Common frog (*Rana ridibunda*) and the Tree frog (*Hyla savignyi*) are not uncommon. Several species of freshwater fishes were identified.

Site 4: Al Sdeer (E 35°46'56", N 31°27'05"):

This site is about 5 km west to the dam. It is located within a deep gorge with relatively fast running water. Sides are rocky with a vegetation cover of *Typha* and *Phragmites* and small patches of *Juncus*, scattered *Tamarix* sp. and *Nerium oleander* (Figure 2C). Water depth varies from shallow to small ponds within the main water course with depth reaching up to 1 meter.

Site 5: Al Malaqee (E 35°36'11", N 31°26'48")

This site represents the junction between Wadi Al Mujib with Wadi Al Hidan tributary. Its name in Arabic,"Al Malaqee", means the meeting point. The upper junction of Wadi Al Mujib is densely vegetated and cannot be walked through. The area is characterized by a dense vegetation of *Typha* on both sides of the water course, while *Tamarix* and *Nerium oleander* grow more on the dry edges (Figure 2D). Water temperature is relatively higher than in other sections of the water catchment, due to mixing of hot springs feeding into Wadi Al Hidan. This area is quite rich in freshwater fishes that can be seen swimming against the water current. Few frogs, if any, were seen.

Site 6: Between Al Malaqee and Al Mujib Gorge (E 35°35'15", N 31°27'20")

This site extends from the beginning of the gorge leading to the waterfalls to Al Malaqee site. It extends for over four km from the beginning of the gorge until it reaches Al Malaqee. Water depth varies from shallow to up to 40 cm with variable dense vegetation of *Typha*, *Phragmites* and *Tamarix* (Figure 2E). In some instance, the water course is wide, reaching up to 3 meters.

Site 7: From the waterfalls to the bridge (E 35°34'59", N 31°27'40")

This area lies immediately after the gorge and extends to the Mujib Bridge. The water is shallow over a gravel bed. A few scattered vegetation is found, with mainly *Tamarix* and few *Juncus* communities. Edges are narrow with a gravel cover (Figure 2F).

Site 8: Mouth of Wadi Al Mujib to the Dead Sea (E 35°34'38", N 31°27'58")

This area is located near the Dead Sea. No open water flow exists as the river flows underground. The vegetation consists of *Tamarix*.

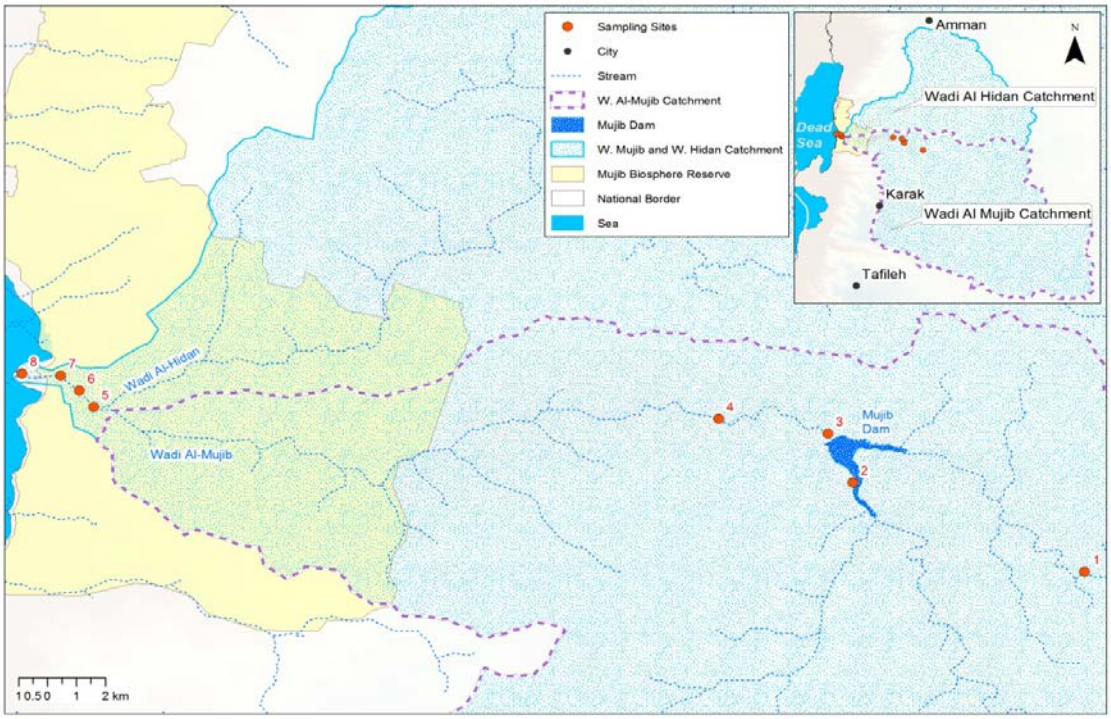

Figure 1. Study sites: **1**. Um Al Rasas. **2**. Wadi Al Mujib dam. **3.** Under Wadi Al Mujib dam. **4**. Al Sdeer. **5.** Al Malaqee. **6.** Between Al Malaqee and Al Mujib gorge. **7**. From the waterfalls to the bridge. **8**. Mouth of Wadi Al Mujib to the Dead Sea.

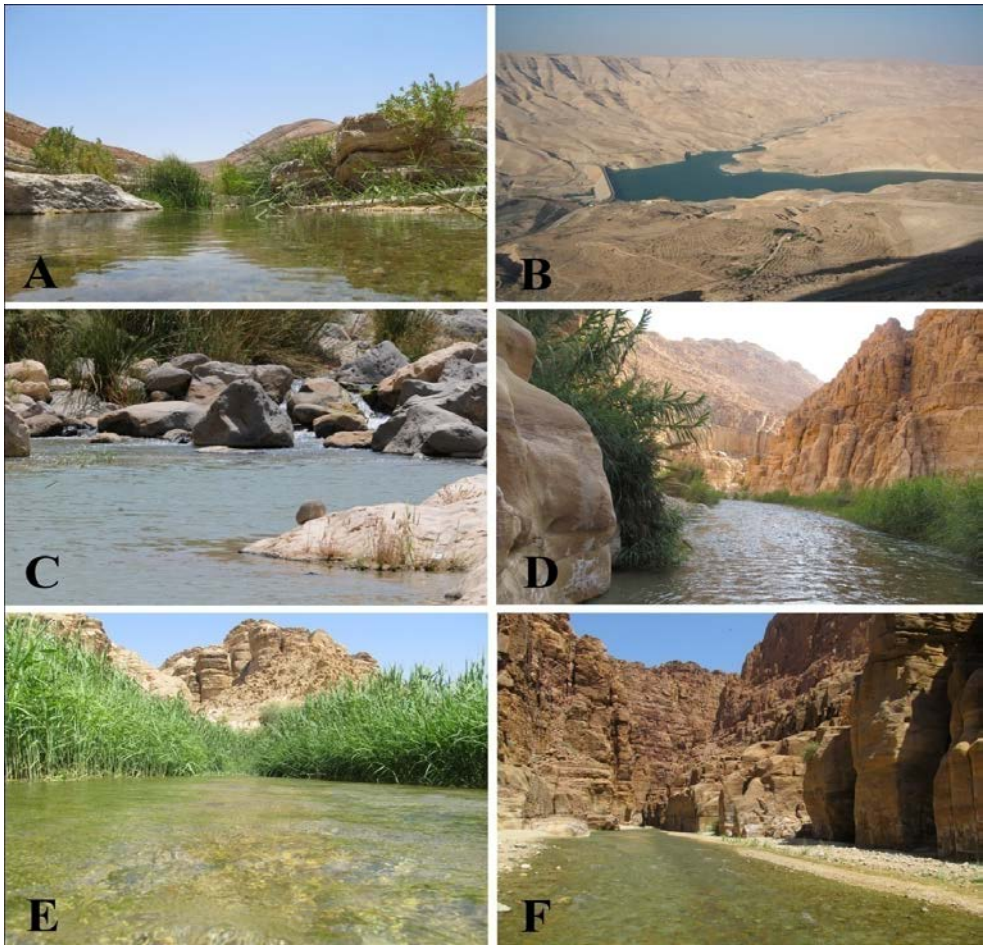

Figure 2. Habitats along Wadi Al Mujib Catchment. **A**. Um Al Rasas. **B**. Wadi Al Mujib dam. **C**. Al Sdeer. **D**. Between Al Malaqee and Al Mujib gorge. **E**. Al Malaqee. **F**. From the waterfalls to the bridge.

2.3. Sampling and Collecting Dragonflies

The vegetation (shrubs, low vegetation, trees, etc.) along Wadi Al Mujib was checked for the presence of dragonflies. In some instance, the vegetation was shaken to check for the presence of resting and perching adults. A handled aerial net with a wide opening was used to capture adult dragonflies.

In each site, a total of at least five hours was spent to check for the presence of Odonata. Transects along water edges were carefully examined for the presence of damselflies at rest on the low vegetation. Dragonflies usually occur on shrubs, trees and rocks in shaded areas. The team consists of two persons for the first three sites, and then additional persons joined the team for the rest of the sites. Both edges of the water courses were examined with a maximum distance from the water course not exceeding five meters.

Captured Odonata were kept in a plastic container and killed in a killing jar. Identification was followed according to Schneider (1986) and Dumont (1991). Both male and female genitalia were examined under the microscope to confirm their identification. The collected material was deposited in the University of Jordan Insects Museum (UJIM), was identified by Ahmad Katbeh-Bader and was later revised by Christian Monnerat (Switzerland).

3. Results

3.1. Species Diversity in the Sites Studied

A total of 14 species representing five families (Platycnemididae, Coenagrionidae, Gomphidae, Aeshnidae and Libellulidae) were identified during this work. The number of species collected varied from a locality to another and ranged from a single one at the Al Mujib dam to seven at Al Malaqee (Table 1). Among the families, the Libellulidae included the highest number of species with seven species.

Site 1: Um Al Rasas

Materials collected and examined: *Platycnemis dealbata,* 3 ♂♂ and 3♀♀, 20.7.2011; *Orthetrum chrysostigma*, 7♂♂ and 1 ♀, 20.7.2011; *Orthetrum brunneum*, 3 ♂♂ and 1♀, 20.7.2011; *Trithemis arteriosa*, 2 ♂♂ and 2 ♀♀, 20.7.2011. L. Al Azzam and R. Al Omari leg. throughout.

Remarks: Four species were collected and observed along the main spring of Um Al Rasas. *Platycnemis dealbata* was very common and was found among the lower part of thick *Typha belts*. Libellulidae species were also common with varying relative abundance.

Site 2: Wadi Al Mujib dam lake

Materials collected and examined: *Sympetrum fonscolombii*, 1 ♂, 10.7.2011, Z. Amr and O. Abed leg.

Remarks: During extensive survey for over eight hours, two specimens of *S. fonscolombii* were observed and only one specimen was collected. Both specimens were found perching on dry plants.

Site 3: Under the dam

Materials collected and examined: *Ischnura elegans*, 3 ♀♀, 11.7.2011; *Platycnemis dealbata*, 3 ♂♂ and 3 ♀♀, 11.7.2011; *Crocothemis erythraea*, 1 ♂ and 1 ♀, 11.7.2011; *Orthetrum chrysostigma*, 1 ♂, 11.7.2011. Z. Amr and O. Abed leg. throughout. Observed: *Anax parthenope*, 11.7.2011.

Remarks: Five species were collected and observed in this site. *Anax parthenope*, was observed among the thick vegetation near an open pool. Both *Ischnura elegans* and *Platycnemis dealbata* were common within the shady vegetation near the pools and also among the vegetation in the vicinity of the main water course. Perching males of *Crocothemis erythraea* and *Orthetrum chrysostigma* were less common.

Site 4: Al Sedeer

Materials collected and examined: *Platycnemis dealbata,* 3 ♀♀ and 1 ♂, 12.7.2011; *Paragomphus genei*, 1 ♀, 12.7.2011; *Trithemis arteriosa*, 2 ♂♂, 12.7.2011; *Orthetrum chrysostigma*, 1 ♀, 12.7.2011.Z. Amr and O. Abed leg. throughout.

Remarks: Four species of dragonflies were collected from this site. Most notably was the presence of *Paragomphus genei*. This species prefers open water such as open streams, rivers or pools.

Site 5: Al Malaquee

Materials collected and examined: *Platycnemis dealbata*, 1 ♂ and 1 ♀, 20.7.2011; *Orthetrum chrysostigma*, 3 ♂, 20.7.2011; *Trithemis arteriosa*, 3 ♂, 20.7.2011. L. Al Azzam and R. Al Omari leg. throughout.

Remarks: Three species were collected from this site. They were common, particularly *P. dealbata*. Males of *O. chrysostigma* were the most common Anisoptera observed. They were perching on s tones, rocks and among vegetation.

Site 6: Between Al Malaquee and the gorge

Materials collected and examined: *Platycnemis dealbata*, 1 ♂ and 1 ♀, 20.7.2011; *Paragomphus genei*, 1♂, 20.7.2011; *Crocothemis erythraea*, 1 ♀, 20.7.2011; *Orthetrum chrysostigma*, 3 ♂♂ and 1 ♀, 20.7.2011; *Trithemis arteriosa*, 5 ♀♀, 20.7.2011; *Zygonyx torridus*, 1 ♀, 20.7.2011. L. Al Azzam and R. Al Omari leg. throughout.

Remarks: This site exhibited high species diversity (6 species). This is mainly due to the wide sides of the water course in addition to the relatively thick vegetation. *Orthetrum chrysostigma*, *C. erythraea* and *T. arteriosa* were the most common species. Males of *O. chrysostigma* and *C. erythraea* were very distinctive, perching on vegetation and rocky areas.

Site 7: From the Waterfalls to the bridge

Materials collected and examined: *Ischnura senegalensis,* 1 ♂, 21.7.2011; *I. fountaineae*, 1♂, 21.7.2011; *I. evansi*, 1 ♀, 21.7.2011; *Platycnemis dealbata*, 1 ♀, 21.7.2011; *Paragomphus genei*, 1 ♀, 21.7.2011; *Orthetrum sabina*, 1 ♂, 21.7.2011; *Trithemis arteriosa*, 2 ♂♂, 21.7.2011. L. Al Azzam and R. Al Omari leg. throughout.

Remarks: This site exhibited the highest species diversity. Seven species were either collected or observed in this site.

Site 8: Mouth of Wadi Al Mujib to the Dead Sea

Materials collected and examined: *Ischnura fountaineae,* 1 ♂, 21.7.2011; *Trithemis arteriosa*, 2 ♂♂, 21.7.2011. L. Al Azzam & R. Al Omari leg. throughout.

Remarks: Two species were collected from this site (Table 1). Number of individuals was low compared to other sites.

Table 1. Odonata collected and observed along Wadi Al Mujib Catchment

		Collection sites							
Species	**Common name**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Suborder Zygoptera									
Family Platycnemididae									
Platycnemis dealbata	Ivory Feather leg	●		●	●	●	●	●	
Family Coenagrionidae									
Ischnura elegans	Blue-tailed Damselfly			●				●	
Ischnura fountaineae									●
Ischnura evansi	Evans' Blue tail Damselfly								●
Ischnura senegalensis									●
Suborder Anisoptera									
Family Gomphidae									
Paragomphus genei	Green Hooktail				●		●	●	
Family Aeshnidae									
Anax parthenope	Lesser Emperor			●					
Family Libellulidae									
Crocothemis erythraea	Common Scarlet-darter			●			●		
Orthetrum brunneum	Brown Skimmer	●							
Orthetrum chrysostigma	Epaulet Skimmer	●		●	●	●	●		
Orthetrum sabina	Green Skimmer							●	
Sympetrum fonscolombii	Red-veined Darter		●						
Trithemis annulata	Violet Dropwing								
Trithemis arteriosa	Red-veined Dropwing	●			●	●	●	●	●
Zygonyx torridus	Ringed Cascader						●		
Total		**4**	**1**	**5**	**4**	**3**	**6**	**5**	**4**

Sites: **1**. Um Al Rasas. **2**. Wadi Al Mujib Dam. **3**. Under Wadi Al Mujib Dam. **4**. Al Sdeer. **5** Al Malaqee. **6**. Between Al Malaqee and Al Mujib gorge. **7**. After the water falls to the bridge. **8**. Entrance of Wadi Al Mujib to the Dead Sea.

4. Discussion

4.1. Species Diversity of Odonata in the Al Mujib Catchment: past and present

The present Odonata fauna of the Wadi Al Mujib catchment includes 14 species belonging to five families. This represents about 30% of the total known species occurring in Jordan. Previously, Schneider (1986) reported eight species; four were not found during the present study (*Crocothemis sanguinolenta, Diplacodes lefebvrii, Orthetrum abbotti* and *Trithemis annulata*). Also, five species reported by Dumont (1991) were not found again (*Calopteryx syriaca, Crocothemis sanguinolenta, Epallage fatime, Onychogomphus flexuosus, Orthetrum abbotti* and *Trithemis annulata*) (Table 2). Schneider (1986) doubted the occurrence of *Calopteryx syriaca* from old reports.

Table 2. Comparison between the Odonata fauna in the present study with previous studies.

Species	Schneider (1986)	Dumont (1991)	Present study
Calopteryx syriaca	?	●	
Epallage fatime		●	
Ischnura elegans			●
Ischnura evansi			●
Ischnura fountaineae			●
Ischnura senegalensis			●
Paragomphus genei	●		●
Onychogomphus flexuosus		●	
Platycnemis dealbata		●	●
Anax parthenope			●
Crocothemis erythraea	●		●
Crocothemis sanguinolenta	●	●	
Diplacodes lefebvrii	●		
Orthetrum abbotti	●	●	
Orthetrum brunneum	●		●
Orthetrum chrysostigma	●		●
Orthetrum sabina			●
Sympetrum fonscolombii			●
Trithemis annulata	●	●	●
Trithemis arteriosa	●		●
Zygonyx torridus			●
Total	**9**	**7**	**15**

Six species were shared between the present and past studies, namely *P. genei, P. dealbata, C. erythraea, O. brunneum, O. chrysostigma*, and *T. arteriosa*.

Eight species are considered as new for the Wadi Al Mujib catchment (*I. elegans, I. evansi, A. parthenope, O. sabina, S. fonscolombii* and *Z. torridus*). Such changes over the past 35 years reflect the dynamics of dragonflies' spatial movement within their distribution range. This pattern of faunal changes was documented by Moore (2001), which reported radical changes in dragonfly communities in pond in the United Kingdom over the period 1962-1988. Such changes were attributed to changes in the aquatic flora, vegetation cover, presence of predators etc (Wellborn et al., 1996).

Within the Dead Sea basin, Furth (1983) reported 13 species of dragonflies. Among these, *A. parthenope, C. erythraea, O. brunneum, O. chrysostigma, S. fonscolombii, T. arteriosa, T. annulata* and *Z. torridus* were also recorded from the western side of the Dead Sea, that means they could easily colonize other localities in this area, to which Wadi Al Mujib pertains.

4.2. Conservation Status of Dragonflies in Jordan

Jordan is a small country with very limited water resources. Over the past sixty years, the human population increased enormously, that led to increased pressure on water resources. Dam constructions, altering natural surface waters increased in number to meet water demand for both agriculture and domestic use. Such activities and development took its toll on freshwater habitats and its biological communities.

Schneider (2004) revised the status of the Odonata of the Levant (Table 3). In his treatment, *C. syriaca* was considered as endangered and its status is now even worse. *Orthetrum abbotti* is considered as an Afrotropical

relict in Jordan. Such species requires further evaluation in Jordan.

Table 3. Conservation status of some levantine Odonata in Jordan, according to Schneider (2004).

Species	IUCN 2003	Suggested status 2004	Remarks
Calopteryx syriaca	EN	EN	Endemic
P. sublacteum mortoni		VU	Endemic
Pseudagrion syriacum			Endemic
Gomphus davidi			Endemic
Onychogomphus macrodon	VU	VU, DD	Endemic
Crocothemis sanguinolenta		VU (?), DD	Relict
Libellula pontica		DD	Endemic
Orthetrum abbotti		DD	Relict

The diversity of Odonata in the Levant is considered high and including a number of endemic and threatened species. At present, Jordan is witnessing increasing demand for water. In combination with the unpredictable effects of climate change, this suggests strongly that the conservation status of many species will deteriorate in the near future (Riservato *et al*., 2009), at such a point that when a new examination of their conservation status will be done, species like *Calopteryx hyalina* and *Onychogomphus macrodon* will certainly join the strongest categories of threat (CR a nd EN or CR, respectively).

Within the Mediterranean region, habitat loss and degradation caused by human activities is the major threat for both threatened and non-threatened dragonfly species. It is estimated that these threats are currently affecting 110 dragonfly species, including 30 of the 31 threatened species (Riservato *et al.,* 2009).

4.3. Impact of the Wadi Al Mujib Dam on Dragonflies Diversity

Results shown in this study strongly indicate the negative impact of the Wadi Al Mujib dam on t he dragonfly fauna. Only a single species (*S. fonscolombii*) was recovered from the dam proper. This is mainly due to the sharp cliffs and water level fluctuation. Few flat areas with scarce vegetation were found around the lake. Water level fluctuation does not allow a s teady vegetation growth around the banks. Moreover, the abrupt water depth is not suitable for the development of the larvae of the dragonflies. Sharp edges are not suitable for breeding and perching for these insects. So, *S. fonscolombii* may represent vagrant or migrant individuals from nearby areas or from other countries, and certainly not a breeding population given the depth of the dam and the absence of suitable breeding areas. Previously, five species (*C. erythraea, Diplacodes lefebvrii, O. brunneum, O. chrysostigma* and *P. genei*) were recorded at the present location of the Al Mujib dam (Schneider, 1986).

In addition, several species of alien freshwater fishes were found breeding in the dam (*Clarias gariepinus, Cyprinus carpio, Oreochromis aureus* and *Tilapia zillii*). They may also have a negative impact on the larval stages of dragonflies.

Globally, many dragonfly species have shown a dramatic decline in their distribution and abundance patterns since the second half of the 20th century (Westfall and May, 1996; Sahlen *et al.,* 2004; Inoue, 2004). This decline is mainly due to habitat destruction, eutrophication, acidification, pollution and water management (channelization, dams construction, modifications of the structure of rivers). Not all anthropogenic disturbances are harmful to dragonflies. In some instances, small dams play an important role in increasing the overall density of many lotic species (Samways, 1999).

Wilcove *et al.* (1998) gave a comprehensive review on endangered species due to habitat loss and occurrence of alien species. Thirty three species of Odonata were considered threatened due to these causes. The sensitivity of dragonflies to habitat structure and their amphibious habits make them well suited for use in evaluating long and short environmental changes (Kalkman *et al.,* 2008).

On the other hand, shallow farm dams with a relatively dense helophyte vegetation offer suitable habitats for Odonata communities. In southern Africa, farm dams, which are a n ecessary and characteristic feature of the agricultural landscape, attract dragonflies, each functioning as a small nature reserve (Samways, 1999).

4.4. Key species that can be used as indicators

Within the Wadi Al Mujib catchment, at least four species can be regarded as key species (Table 4). Although *Onychogomphus lefebvrii* and *Onychogomphus flexuosus* were not recorded during this study, the former species was reported by Dumont (1991). These species are very sensitive to changes in vegetation cover as well as to water quality. These species were selected since they were the most common species in the stretches exhibiting relatively high species diversity. Also, these species are easy to identify on the spot without the need for a close-up examination. These key species can provide information on changes in vegetation cover over seasons or due to man induced changes in a particular site. Also, they bring some information about water velocity and quality.

Table 4. Some selected species that can be considered as indicator species.

Species	Habitat Requirement	Remarks
O. lefebvri	Thick vegetation of *Typha* and slow running water	Found on medium sized vegetation
P. genei	Thick vegetation with trees and large shrubs in shaded areas with fast running water	Observed on shrubs, rocks and trees.
O. flexuosus	Thick vegetation with trees and large shrubs in shaded areas with fast running water	Observed on shrubs, rocks and trees.
Z. torridus	Thick vegetation with trees and large shrubs in shaded areas with fast running water	Observed on shrubs, rocks and trees.

Perhaps *Orthetrum abbotti* and *Crocothemis sanguinolenta* should be also considered as a major key species to the study area, since they are known only from the Dead Sea rift within the Levant. During the present study, we failed to record them. Additional effort is required during different seasons to confirm their occurrence in the Wadi Al Mujib catchment. Both were found in the past in this catchment (Dumont, 1977) and Monnerat and Hoess (2011) mapped them in several locations of the Dead Sea rift.

Several species of the dragonflies of Jordan disappeared or are on the verge of extinction (i.e. *C. syriaca*). Indeed, Schneider (1982b) pointed out earlier on such changes and their impact on the regional dragonfly fauna. For example, *E. fatime* was common all over the Jordan Valley and currently is very scarce. Similarly, *C. syriaca* populations are diminishing in previously known breeding sites (Katbeh *et al.,* 2004). This species is very sensitive to pollution and habitat structure degradation and could be eradicated easily from its natural habitats. Schneider (1982b) pointed out that the endemic *Pseudagrion syriacum* is threatened due to the overwhelming changes in freshwater ecosystems in the Jordan valley.

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Comparative Studies on Anti-hyperglycemic Effects of Ethyl Acetate and Methanol Extract of *Albizzia lucida* Benth Bark in Alloxan Induced Diabetic Rats

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Abstract

The decoction of the bark of *Albizzia lucida* Benth was used in ayurvedic system for management of diabetes mellitus. To confirm the traditional claim, the comparative anti-hyperglycemic efficiency of ethyl acetate extract of bark of *Albizzia lucida* Benth. (EEAL) and methanol extract of bark of *Albizzia lucida* Benth. (MEAL) was done in alloxan induced diabetic rats. Acute and sub-acute effect of oral administration of EEAL and MEAL (200 & 400 mg/kg, b.wt) on blood glucose was observed in different time interval for 10 days. By the end of the study, the collected blood sample and pancreas was used for estimation of serum lipid profile and histological studies. EEAL and MEAL showed that the serum blood glucose and serum lipids (total cholesterol, triglyceride, VLDL-cholesterol, LDL-cholesterol) was reduced significantly ($p < 0.01$) in dose dependent manner when compare to control group. At the same time MEAL was showed potent effect than EEAL. EEAL and MEAL does not affect the body weight and alter the histopathology of pancreas similar to that of normal. It was concluded that EEAL extract of bark of *Albizzia lucida* Benth and MEAL extract of bark of *Albizzia lucida* Benth. possess anti-hyperglycemic activity in alloxan-induced diabetic model rats.

Keywords: *Albizzia lucida* Benth., Ayurveda, Diabetes, Alloxan, Serum Lipid Profile, Histopathology, Anti-hyperglycemic.

1. Introduction

Diabetes is chronic metabolic disorder characterized by hyperglycemia due to absence or insufficient of circulating insulin levels (Holmann and Turner, 1991). Though insulin and various anti-diabetic drugs are available but insulin could not be used by oral and continuous use of synthetic agents leads severe side effects (Valiathan, 1998). Herbal medicines are prescribed universally even though their biological active constituents are unknown due to their potency with minimum side effects and relatively low cost (Verspohl , 2002; Villasen˜or and Lamadrid, 2006).

In folk medicine, the decoction of bark of *Albizzia lucida* Benth. (Family: Mimosaceae) is considered useful in diabetes, pregnancy and stomachache. It is also used as a medicine for water buffalo when given with salt (Bulusu Sitaram and Chunekar, 2006). Therefore, the present research was undertaken to verify the anti-hyperglycemic potential of ethyl acetate extract of bark of *Albizzia lucida* Benth. (EEAL) and methanol extract of bark of *Albizzia lucida* Benth. (MEAL) in alloxan induced diabetic rats.

2. Materials and Methods

2.1. Plant Material and Preparation of Extract

The bark of *Albizzia lucida* Benth. was collected from Tirumala hills, Chittoor district of Andhra Pradesh, India in March 2011. The plant was authenticated Dr. K. Madhava Chetty, Department of Botany, Sri Venkateswara University, Tirupati. The bark of *Albizzia lucida* Benth. was dried in shade and pulverized in the grinder-mixer to obtain a coarse powder, then passed through the 40 mesh sieve. A weighed quantity (100gm) of powder was subjected to continuous hot extraction with ethyl acetate and methanol in soxhlet apparatus for 48 hours. Then the extracts were evaporated at reduced pressure using rotary evaporator until all the solvent has been removed to give an extract sample. The percentage yield of ethyl acetate and methanol extract of *Albizzia*

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lucida Benth. was found to be 7.40 & 9.85%w/w respectively.

2.2. Preliminary Phytochemical Investigation of EEAL and MEAL

The preliminary phytochemical investigation was performed by using several standard phytochemical tests for the qualitative estimation of presence of various phytochemicals in ethyl acetate (EEAL) and methanol extract (MEAL) of bark of *Albizzia lucida* Benth. (Harbone,1973).

2.3. Animals

Male albino Wistar rats (200-250gm) were obtained from the animal house in Sree Vidyanikethan College of Pharmacy, Tirupati, Andhra Pradesh. The animals were maintained in a well-ventilated room with 12:12 hour light/dark cycle and water was given ad libitum. The experiments were performed after approval (Approval no: SVCP/IAEC/I-009/2011-12) of the protocol by the Institutional Animal Ethics Committee (IAEC) and were carried out in accordance with the current guidelines on the care of laboratory animals.

2.4. Acute Toxicity Study

The acute toxicity of EEAL and MEAL was determined as per the OECD guideline no. 423 (Acute toxic class method). Albino wistar rats (n=6) either sex selected and kept fasting for overnight providing only water. EEAL and MEAL was administered orally at the dose level of 2000mg/kg by oral needle and observed for 14days. Hence, 1/10th (200 mg/kg) and 1/5th (400 mg/kg) of the doses were selected for further study (OECD, 2002).

2.5. Anti-hyperglycemic Efficiency of Albizzia lucida Benth. Extracts

2.5.1. Induction of diabetes

Male wistar albino normoglycemic rats were injected intraperitoneally with alloxan monohydrate dissolved in normal saline at the dose of 120 mg/kg b.wt (Lachin and Reza , 2012; Chaudhary *et al*., 2012). After 3 days, the fasting blood glucose was checked and above 250 mg/dl of blood glucose reached rats was considered as diabetic rats. These diabetic rats were segregated into seven groups of six rats each group. Group I (Normal control): Vehicle 1% w/v CMC; 5ml/kg, b.w. p.o); Group II (Alloxan induced Diabetic control) received only vehicle (1%CMC; 5ml/kg, b.w. p.o); Group III & IV - Alloxan induced diabetic rats received the EEAL 200 & 400 mg/kg/day p.o suspended in 1% w/v CMC; Group V & VI – Alloxan induced diabetic rats received the MEAL 200 & 400 mg/kg/day p.o suspended in 1% w/v CMC; Group VII (Standard) Alloxan induced diabetic rats received Glibenclamide (2.5 mg/kg p.o) suspended in 1% w/v CMC, respectively.

2.5.2. Acute experimental study

Blood glucose level of all group rats were checked after 0, 1, 3, 6 & 9h of oral administration of single dose of test drugs.

2.5.3. Sub-acute experimental study

The same treatments continued with same dose once daily for 10 days. The blood samples collected from tail vein and measured the glucose level using commercially available glucose strips (Accu-Chek) using one-touch glucometer (Johnson-Johnson, India) on initial day, 3, 7, 10^{th} day respectively. After terminate the study, the animals were sacrificed by cervical dislocation. The blood was collected without anticoagulant and serum was separated by centrifugation at 6000 rpm for 5min. the pancreas organ of rats also separated carefully for histological study.

2.5.4 Estimation of serum lipid profile

Serum total cholesterol (TC), triglycerides (TG), LDL-C, VLDL, HDL-C were measured for all animals (n=6) from each group by commercially available diagnostic kits (Span Diagnostics, India). The serum low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) levels were calculated by Friedewald formula (Friedewald *et al*., 1972): VLDL = TG / 5; LDL = TC - (HDL + VLDL).

2.6 Histological Assessment of Pancreas

The separated pancreas was kept in 10% formaldehyde solution for histopathological studies and standard procedure followed for fixation. This study was done at pathology department of Sri Venkateswara University, Tirupati, Andhra Pradesh.

2.7 Statistical Analysis

The present research observations were signified as Mean ± Standard Error Mean. Statistical significance of dissimilarities amid the groups was evaluated by one way and multiple way analysis of variance (ANOVA) followed by Turkey test. *P* values less than 0.05 were deliberated as significance.

3. Results

3.1. Preliminary Phytochemical Investigation of EEAL & MEAL

Ethyl acetate and methanol extracts of bark of *Albizzia lucida* Benth. were showed proteins, steroids, alkaloids, flavonoids, terpenoids, tannins and phenolic compounds and saponins.

3.2. After single dose of EEAL & MEAL on serum blood glucose level in diabetic rats

EEAL and MEAL extracts were significantly ($P<0.01$) decreased the serum blood glucose level. In acute experimental study, single dose response of EEAL and MEAL in alloxan induced diabetic rats at 0, 1, 3, 6 & 9h. Table 1 and Figure 1 showed that the methanolic extract of bark of *Albizzia lucida* Benth. (MEAL 200 and 400mg/kg, p.o) effectively ($P<0.01$) reduced blood glucose level of diabetic rats (47.59% and 53.34%, respectively) than ethyl acetate extract of bark of *Albizzia lucida* Benth. (EEAL 200 and 400mg/kg, p.o) (26.34% and 37.49%, respectively). These reduced values were very close to that of glibenclamide (2.5mg/kg p.o) (61.74%). After oral administration of MEAL, acute reduction ($P<0.01$) of glucose level in time dependent manner when compared to EEAL.

3.3. After Multidose of EEAL & MEAL on serum blood glucose level in diabetic rats

In sub-acute experimental study, the blood glucose was determined on initial day, 3, 7, 10th day after oral administration of EEAL and MEAL (200 & 400mg/kg, p.o) (Table 2and Figure 2). Both doses of MEAL (200 and 400mg/kg, p.o) showed significant (P<0.01) chronic reduction of serum blood glucose level (54.59% and 57.73%, respectively) when compared to diabetic rats. MEAL effect was near to that of (P<0.01) of glibenclamide (2.5mg/kg p.o) (67.06%). Anti-hyperglycemic effect of MEAL was greater effect (P<0.01) than EEAL 200 and 400mg/kg, p.o (40.89% & 48.96%, respectively).

3.4. After Multidose of EEAL & MEAL on serum lipid profile and body weight in diabetic rats

The serum blood glucose was raised (P<0.01) accompanied by an increase in serum lipid profile such as total cholesterol (TC), triglycerides (TG), LDL-C, VLDL & HDL-C in alloxan-induced diabetic rats (Table 3 and Figure 3). MEAL (200 and 400mg) afforded greater reduction of TC, TG, LDL-C & VLDL, whereas HDL-C level was significantly (P<0.01) increased. Anti-hyperlipidemic effect of MEAL was great efficient than EEAL. Standard group rats showed significant (P<0.01) anti-hyperlipidemic effect with glibenclamide (2.5mg/kg p.o).

Table 4 and Figure 4 showed that the alloxan induced diabetic rats showed loss of body weight when compared to normal control and EEAL and MEAL (200 and 400mg/kg, p.o) treated rats. These results apparently recorded that treatment with EEAL and MEAL extracts showed better control in the loss of body weight.

3.5. Histopathological studies on islets of pancreas

Pancreatic beta cells of normal rats showed adorned islets with well defined border and normal allocation of islets of langerhans. The histopathological studies of alloxan induced diabetic rat pancreas illustrates that a atrophy, suppress the number of beta cells of islets of langerhans was noted when compared to control group. Necrosis and damage of islets was caused by alloxan. The oral administration of EEAL and MEAL to groups III, IV, V & VI of experimental rats showed in the recovery of beta cells necrosis and the restoration of the islets similar to that of control and standard groups (glibenclamide 2.5mg/kg p.o) treated animals (Figure 5).

Table 1. After single dose of EEAL & MEAL on serum blood glucose in alloxan induced diabetic rats

Groups	Serum Blood Glucose (mg/dl) (% of glucose reduction)				
	0 h	1 h	3 h	6 h	9 h
I - Normal control (1% w/v CMC)	86.67±1.856^{**a}	87.00±2.221^{**a}	84.00±1.00^{**a}	87.17±1.424^{**a}	84.83±1.276^{**a}
II - Alloxan induced Diabetic control	258.00±2.113	263.50±3.490	268.17±3.807	282.67±2.418	288.00±2.517
III - Alloxan + EEAL (200 mg/kg, p.o)	264.50±2.513^{b}	256.17±1.600^{b} (3.15)	243.50±1.147^{*b} (7.94)	223.33±1.453^{**b} (15.56)	194.83±1.400^{**b} (26.34)
IV - Alloxan + EEAL (400 mg/kg, p.o)	265.83±2.600^{b}	252.67±1.978^{b} (4.95)	234.00±2.129^{**b} (11.97)	193.17±1.400^{**b} (27.33)	166.17±1.424^{**b} (37.49)
V - Alloxan + MEAL (200 mg/kg, p.o)	267.17±4.102^{b}	254.17±1.400^{*b} (4.87)	237.33±0.667^{**b} (11.17)	197.83±2.227^{**b} (25.95)	140.00±0.931^{**b} (47.59)
VI - Alloxan + MEAL (400 mg/kg, p.o)	261.83±2.857^{b}	238.67±1.282^{**b} (8.85)	221.50±1.727^{*b} (15.40)	161.67±2.186^{**b} (38.25)	122.17±1.621^{**b} (53.34)
VII - Alloxan + Glibenclamide (2.5mg/kg, p.o)	266.17±3.380^{b}	233.83±2.372^{**b} (12.15)	208.00±1.033^{**b} (21.85)	135.67±1.229^{**b} (49.03)	101.83±2.167^{**b} (61.74)

All values expressed in mean ± SEM for six animals in each group (n=6);

All values compared with diabetic control groups. * p < 0.05 ; ** p < 0.01;

a – Normal control group I *Vs* diabetic control group II; b – Groups III, IV, V, VI & VII *Vs* diabetic control group II.

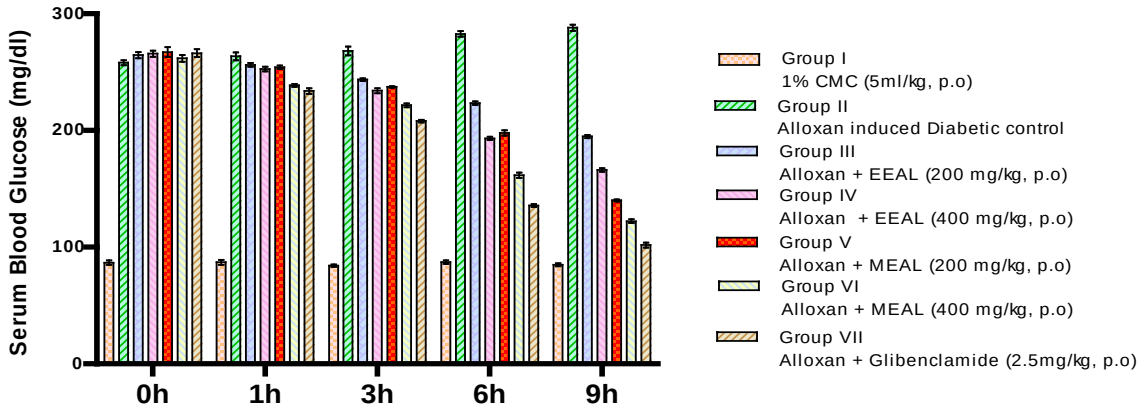


Figure 1. After single dose of EEAL & MEAL on serum blood glucose in alloxan induced diabetic rats

All values expressed in mean ± SEM for six animals in each group (n=6);

All values compared with diabetic control groups. * $p < 0.05$; ** $p < 0.01$;

a – Normal control group I *Vs* diabetic control group II; b – Groups III, IV, V, VI & VII *Vs* diabetic control group II.

Table 2. After Multidose of EEAL & MEAL on serum blood glucose in alloxan induced diabetic rats

Groups	Serum Blood Glucose (mg/dl) (% of glucose reduction)			
	0 day	3 day	7 day	10 day
I - Normal control (1% w/v CMC)	86.67±1.856^{**a}	87.50±2.802^{**a}	86.00±0.730^{**a}	83.83±0.946^{**a}
II - Alloxan induced Diabetic control	258.00±2.113	297.83±1.990	290.50±2.078	286.50±3.074
III - Alloxan + EEAL (200 mg/kg, p.o)	264.50±2.513^{b}	207.00±0.966^{**b} (21.74)	183.67±0.919^{**b} (30.56)	156.33±1.333^{**b} (40.89)
IV - Alloxan + EEAL (400 mg/kg, p.o)	265.83±2.600^{b}	178.33±1.333^{**b} (32.92)	149.00±1.528^{**b} (43.94)	135.67±0.715^{**b} (48.96)
V - Alloxan + MEAL (200 mg/kg, p.o)	267.17±4.102^{b}	176.33±1.726^{**b} (34.00)	145.17±0.946^{**b} (45.66)	121.33±1.145^{**b} (54.59)
VI - Alloxan + MEAL (400 mg/kg, p.o)	261.83±2.857^{b}	154.00±1.238^{**b} (41.18)	120.00±1.732^{**b} (54.17)	110.67±1.542^{**b} (57.73)
VII - Alloxan + Glibenclamide (2.5mg/kg, p.o)	266.17±3.380^{b}	96.67±0.919^{**b} (63.68)	93.67±1.174^{**b} (64.81)	87.67±2.459^{**b} (67.06)

All values expressed in mean ± SEM for six animals in each group (n=6);

All values compared with diabetic control groups. * $p < 0.05$; ** $p < 0.01$;

a – Normal control group I *Vs* diabetic control group II; b – Groups III, IV, V, VI & VII *Vs* diabetic control group II.

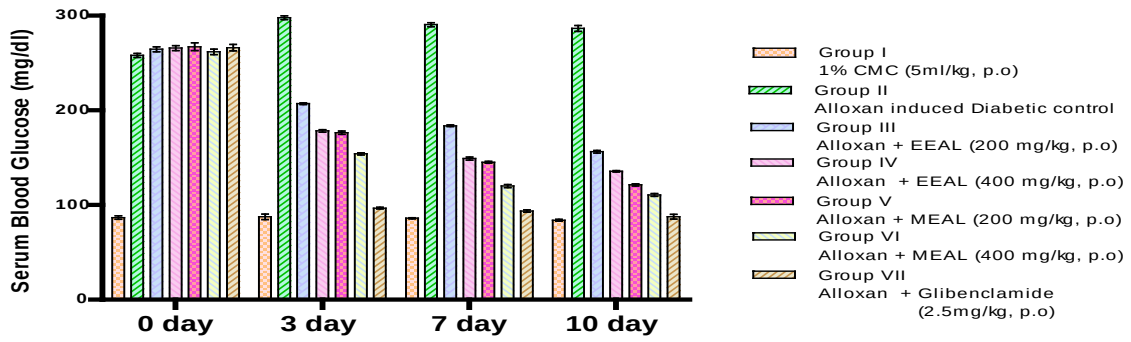


Figure 2. After Multidose of EEAL & MEAL on serum blood glucose in alloxan induced diabetic rats

All values expressed in mean ± SEM for six animals in each group (n=6);

All values compared with diabetic control groups. * $p < 0.05$; ** $p < 0.01$;

a – Normal control group I *Vs* diabetic control group II; b – Groups III, IV, V, VI & VII *Vs* diabetic control group II.

Table 3. After Multidose of EEAL & MEAL on serum lipid profile in alloxan induced diabetic rats

Groups	Serum Lipid Profile (mg/dl)				
	Total Cholesterol (mg/dl)	Triglycerides (mg/dl)	LDL- C (mg/dl)	VLDL- C (mg/dl)	HDL- C (mg/dl)
I - Normal control (1% w/v CMC)	119.67±1.282^{**a}	115.83±1.493^{**a}	40.33±1.833^{**a}	23.17±0.299^{**a}	56.17±0.833^{**a}
II - Alloxan induced Diabetic control	233.33±2.951	163.33±1.667	168.00±2.266	32.67±0.333	29.67±0.494
III - Alloxan + EEAL (200 mg/kg, p.o)	173.83±1.014^{**b}	150.00±1.238^{**b}	106.33±1.077^{**b}	30.00±0.248^{**b}	37.50±0.563^{**b}
IV - Alloxan + EEAL (400 mg/kg, p.o)	147.83±1.579^{**b}	146.83±0.703^{**b}	80.80±1.838^{**b}	28.37±0.141^{**b}	37.67±0.803^{**b}
V - Alloxan + MEAL (200 mg/kg, p.o)	139.83±0.543^{**b}	141.33±1.116^{**b}	69.90±0.834^{**b}	28.27±0.223^{**b}	41.67±0.422^{**b}
VI - Alloxan + MEAL (400 mg/kg, p.o)	123.83±1.014^{**b}	126.50±1.118^{**b}	52.20±1.108^{**b}	25.30±0.224^{**b}	46.17±0.401^{**b}
VII - Alloxan + Glibenclamide (2.5mg/kg, p.o)	116.67±1.282^{**b}	115.00±0.730^{**b}	42.83±1.237^{**b}	23.00±0.146^{**b}	50.83±0.909^{**b}

All values expressed in mean ± SEM for six animals in each group (n=6);

All values compared with diabetic control groups. * $p < 0.05$; ** $p < 0.01$;

a – Normal control group I Vs diabetic control group II; b – Groups III, IV, V, VI & VII Vs diabetic control group II.

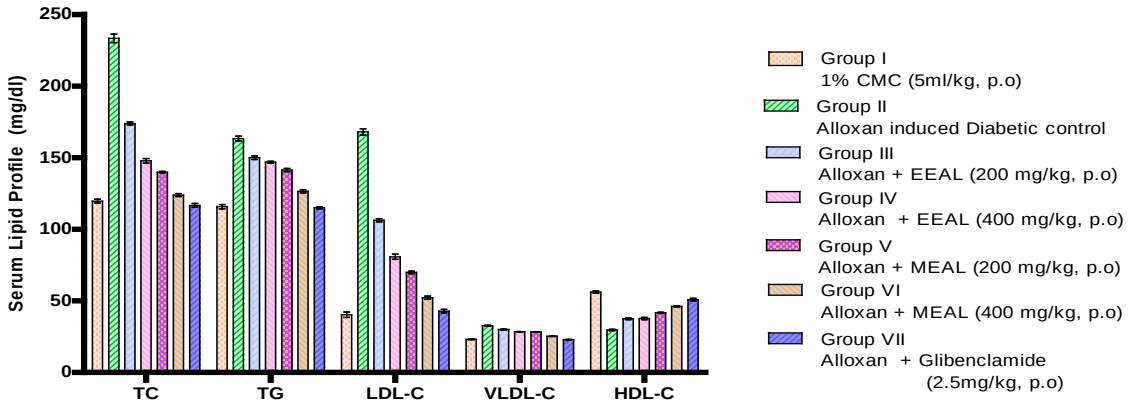


Figure 3. After Multidose of EEAL & MEAL on serum lipid profile in alloxan induced diabetic rats

All values expressed in mean ± SEM for six animals in each group (n=6);

All values compared with diabetic control groups. * $p < 0.05$; ** $p < 0.01$;

a – Normal control group I *Vs* diabetic control group II; b – Groups III, IV, V, VI & VII *Vs* diabetic control group II.

Table 4. Efficiency of EEAL & MEAL on body weight in alloxan induced diabetic rats

Groups	Body weight (g)	
	Initial	Final
I - Normal control (1% w/v CMC)	164.83±1.621[a]	194.17±0.703[**a]
II - Alloxan induced Diabetic control	163.50±1.586	158.67±1.202
III - Alloxan + EEAL (200 mg/kg, p.o)	164.33±1.229[b]	171.00±1.033[**b]
IV - Alloxan + EEAL (400 mg/kg, p.o)	163.83±0.601[b]	177.17±1.493[**b]
V - Alloxan + MEAL (200 mg/kg, p.o)	164.00±1.528[b]	173.33±1.308[**b]
VI - Alloxan + MEAL (400 mg/kg, p.o)	164.33±1.174[b]	185.17±1.138[**b]
VII - Alloxan + Glibenclamide (2.5mg/kg, p.o)	167.50±1.607[b]	187.33±0.843[**b]

All values expressed in mean ± SEM for six animals in each group (n=6);
All values compared with diabetic control groups. * $p < 0.05$; ** $p < 0.01$.
a – Normal control group I *Vs* diabetic control group II.
b – Groups III, IV, V, VI & VII *Vs* diabetic control group II.

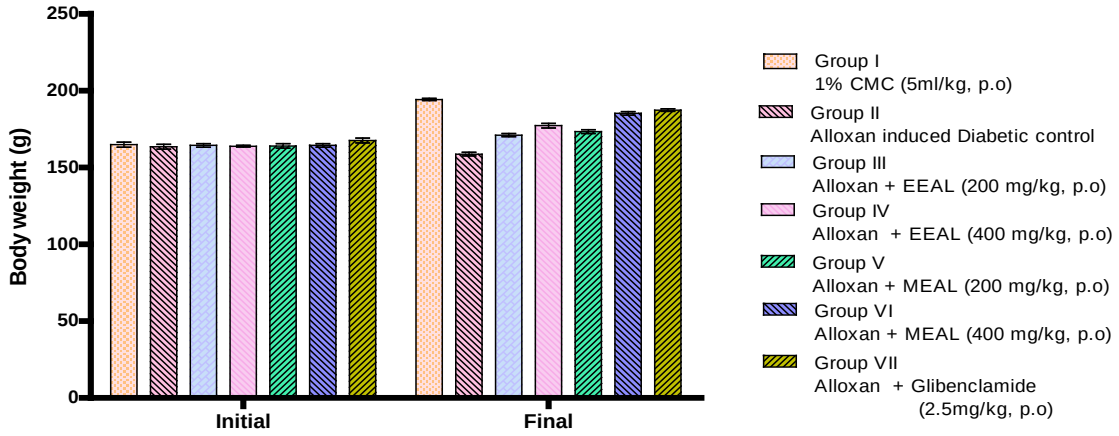
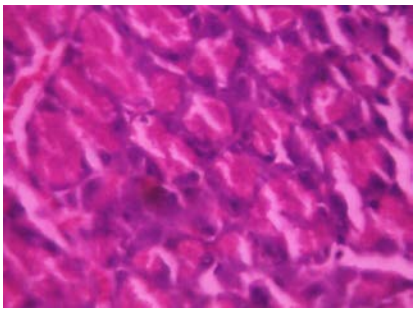
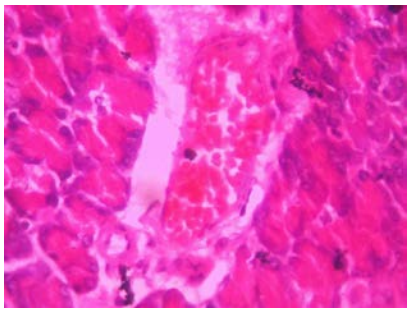


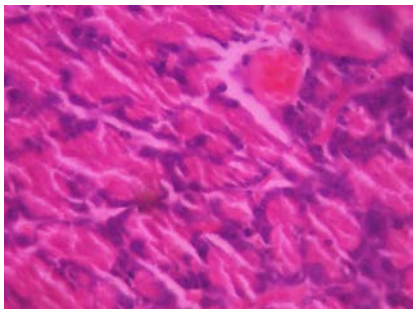
Figure 4. Efficiency of EEAL & MEAL on body weight in alloxan induced diabetic rats
All values expressed in mean ± SEM for six animals in each group (n=6);
All values compared with diabetic control groups. * $p < 0.05$; ** $p < 0.01$.
a – Normal control group I *Vs* diabetic control group II.
b – Groups III, IV, V, VI & VII *Vs* diabetic control group II.



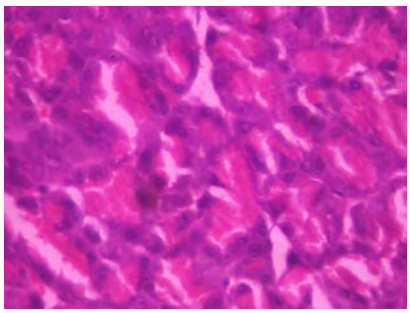
I - Normal control (1% w/v CMC)



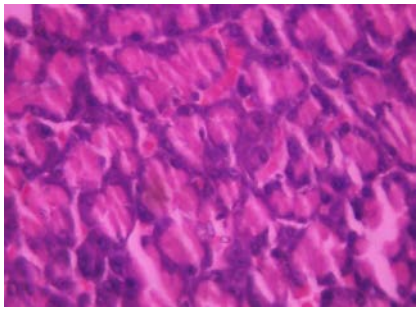
II - Diabetic control



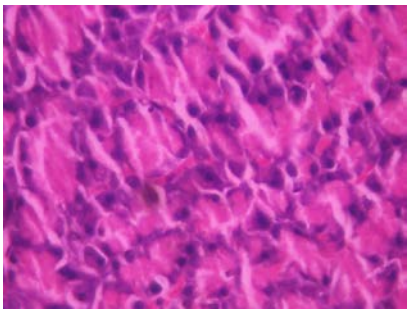
III - Alloxan + EEAL (200 mg/kg, p.o)



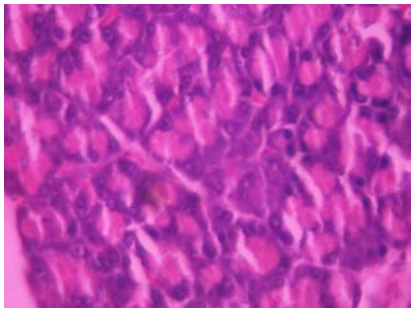
IV- Alloxan + EEAL (400 mg/kg, p.o)



V- Alloxan + MEAL (200 mg/kg, p.o)



VI - Alloxan + MEAL (400 mg/kg, p.o)



VII - Alloxan + Glibenclamide (2.5mg/kg, p.o)

Figure 5. Histopathological studies on pancreas

4. Discussion

Ethyl acetate extract of bark of *Albizzia lucida* Benth. (EEAL) and Methanol Extract of bark of *Albizzia lucida* Benth. (MEAL) was screened for discover the scientific evidence of decoction of this bark of *Albizzia lucida* Benth. used for treatment of diabetes in Ayurveda (Sitaram and Chunekar , 2006). Nevertheless, presence of triterpenoid saponins which possess anti-hyperglycemic, anti-hyperlipidemic and anti-angiogenic properties has been reported in previous literature (Tan *et al*., 2008; Joseph and Jin, 2013).

The intraperitoneal injection of alloxan monohydrate (β-Cytotoxin) induces chemical diabetes in albino rats causes hyperglycemia, hyperlipidemia due to impaired insulin release and insulin resistance. The acute and sub-acute administration, the effect of EEAL and MEAL was reduced blood glucose significantly. Glibenclamide (sulphonylureas) produce hypoglycemia by enhancing the release of insulin and these drugs are very potent in alloxanized diabetic rats. Hence our results displayed that the acute and sub-acute anti-hyperglycemic effects of EEAL and MEAL might be potentiating the insulin secretion, it was similar to that of Glibenclamide (Meliani *et al.,* 2011).

In alloxanized diabetes also correlated with hyperlipidemia and hypertriglyceridemia. Observation of hyperlipidemia, the total cholesterol (TC), LDL-C, VLDL was increased & fall of HDL-C due to underutilization of glucose leads to excess mobilization of fat from adipose tissue. At the same time, oral administration of EEAL and MEAL was suppresses the mobilization effect by increased the glucose utilization. Moreover, EEAL and MEAL showed significantly decreases the triglycerides (TG) by activating or releasing the lipoprotein lipase resulting in regulates the metabolism of lipids (Nammi *et al.*, 2003).

Additionally, EEAL and MEAL treated animals body weight was not affected and it is comparable to that of normal control animals. In histological studies on pancreas, there is no necrosis and damage of islets of langerhans in oral administration of EEAL and MEAL treated groups. It was accepted the potency and safety of EEAL and MEAL.

In conclusion, our observations of the present research clearly demonstrated that Ethyl acetate extract of bark of Albizzia lucida Benth. (EEAL) and Methanol Extract of bark of Albizzia lucida Benth. (MEAL) exerts markable anti-hyperglycemic and anti-hyperlipidemic efficiency due to the presence of biologically active constituents with possible multiple mechanism involving both pancreatic and extra pancreatic effects. But still more research is needed for find out its specific mechanism of action and long term effects in diabetes mellitus.

5. Conflict of Interest

The authors declare that there are no conflicts of interest.

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Bacteriological and Mycological Assessment for Water Quality of Duhok Reservoir, Iraq

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Abstract

Duhok dam reservoir is situated in Duhok city, Iraq. It is an artificial reservoir which supplies water for crops land and orchard around the canal throughout its path. The objective of this investigation is to assess physical, chemical and microbiological aspects of aquatic ecosystem in the lake. The water quality variables (water temperature, pH, EC, total dissolved nitrogen and phosphate, SO_2 , BOD_5 and microoganisms) were being measured seasonally during 2011. The results reported that high conductivity and sulphate concentrations were recorded during different seasons. Heterotrophic plate count and faecal colifom exceed Iraqi and WHO standards for drinking purposes. Statistically, no differences were found between studied sites for all variables. Microbiological isolates, counts, total occurrence and diversity index were more in sediment than in water samples. The occurrence of mycobiota was surveyed by three isolation methods 19 fungal species assigned to 14 genera were isolated. The most frequent species in order were: *Aspergillus* spp*., Penicillium* spp. and *Eurotium* spp. The occurrence of keratinophilic fungi was detected in the sediment by hair baiting method The most frequent genera isolated in this study were *Chrysosporium* spp. , *Trichophyton* spp. and *Microsporum* spp.

Keyword: Water Quality, Duhok Reservoir, Bacterial Count, Fungal isolation, Biodiversity.

1. Introduction

Water quality performs an important role in the health of human beings, animals, and plants. Surface water quality is an essential component of the natural environment and a matter of serious concern today (Liu *et al.,* 2011). Rivers and reservoirs play a major role in drinking water, agricultural use, fishery, and electricity production, so protection of water quality is a very important issue and it should be kept at acceptable levels (Venkatesharaju *et al*., 2010). The variation of water quality is the essential combination of both anthropogenic (such as urban, industrial, agricultural activities and the human exploitation of water sources) and natural contributions (such as precipitation rate, weathering processes and soil erosion) (Pejman *et al*., 2009). Deterioration of lake and river water quality is common in many aquatic systems and potential causes are usually various including point and non- point sources of pollution (Pisinaras *et al.,* 2007).

In a w ell- balanced aquatic ecosystem, the quality of water plays a cr itical role between, the organisms and environment which is also extremely important for the health of the ecosystem (Akbulut *et al.,* 2010). In water quality assessment the microbial community has special significance, especially in terms of protecting public health. Coliform bacteria, normally present in intestinal tract of humans and worm- blooded animals, can secondary be found on plants, in the soil and in waters. Although the occurrence of primarily non- pathogenic refers to the presence of disease- causing organisms, they reach natural waters mainly during rainfall, through runoff from agricultural and urban lands, as well as through drainage (Radojevic *et al.,* 2012). Total coliform (TC) is used as a parameter giving basic information on microbiological quality of surface waters (WHO, 2008). For more than a century the presence of coliform bacteria in drinking and recreational waters has been taken as an indication of fecal contamination, and thus of a health hazard. Total coliform and thermotolerant (fecal) coliform (FC) indicator tests are common public health tests of the safety of water and wastewater which might be contaminated with sewage or fecal material (APHA, 1998).

Historically, water has played a significant role in the transmission of human disease. Typhoid fever, cholera, infectious hepatitis, bacillary and amoebic dysenteries and many varieties of gastrointestinal diseases can all be transmitted by water (Rompre *et al.*, 2002).

The qualitative and quantitative composition of fungi in water sediments depend on the origin and composition of waste water sediments, stabilization degree of their organic

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matter, hydration degree and structure. It was postulated that keratinophilic fungi may be utilized as microbiological indices for the transformations of organic matter of waste water sediments as well as of the degree of their deactivation from the sanitary standpoint (Ulfig and Korcz, 1991).

Duhok dam is a high earth fill dam with central clay core and gravel shell. The main aim of the dam was irrigation of the agricultural areas inside Duhok city and areas around it till Summel city through a tunnel, now the reservoir area of the dam is used for supplying Duhok city with water beside it became a touristic area (Mustafa and Noori, 2013). It is 60 m (197 ft) tall and can withhold 52,000,000 m3 (42,157 acre.ft) of water. The dam has a bell-mouth spillway with a maximum discharge of 81 m3/s (2,860 cu ft/s) (Wikipedia, 2008).

The main objectives of this study were to assess the microbial water quality by detecting the presence of coliforms and fungi, as well as to determine the spatial and temporal pattern in the community structure of biota richness and relations to some physico- chemical parameters, in order to know the water quality of Duhok dam and their suitability to using it for different purposes.

2. Materials and Methods

2.1. Study Area

Duhok dam reservoir was established in 1988 on Duhok rubar (River) forming an impoundment of surface area around 256 hectares, coming third in Iraqi Kurdistan region, northern part of Iraq (Figure 1) after Dokan and Derbandikan reservoirs. Duhok impoundment is located about 2 Km to the north of Duhok city center of latitude 36° 50' 49" and longitude 43° 00' 33". It is an artificial reservoir with maximum depth exceeding 60 meter having an area of 6.8 Km^2 (1.7 Km width, 4 Km length) (Al-Nakhabandi, 2002).

The reservoir is situated on a hilly plane surrounded from the south by a mountain Chai Spii, whereas from the north by a chain of Zahio mountain. Most parts of the area are formed from the slopes and steep mountains crossed by numerous valleys all of which generally direct the water flow of rain, and snow melts through different creeks and canals to the main Duhok rubar, because of the steep slopes toward the center of the reservoir. This reservoir (rubar Duhok canal) supplies water for crops land and orchard around the canal throughout its path (Al-Ganabi, 1985). The establishment of the reservoir extended the irrigation nowadays to about 4600 hectares on the west of Duhok town, extending to approach the main international road Zakho. The source of the water to the dam is mainly rain, snowmelt and the main tributaries Sundor and Gurmava that on their joining made up Duhok rubar.

The impoundment has almost a fan- like shape with an elongated part to the north west, where the forest vegetation and orchards have been permanently under the water, the west and east shores of the impoundment are arable lands, therefore, the use of the fertilizers and pesticides are common, whereas in Spring and Summer seasons views of cattle and sheep for grazing were common in the area. The outflow of the impoundment is through the spillway discharge.

Figure 1. Maps of: Duhok city, Iraq, Duhok Dam Lake and studied sites.

2.2. Sample Collection

Water samples were collected at surface (0- 20cm) from three sites, while sediment samples were collected from two sites at depth 1m near banks of the reservoir (sites 1 and 3) in four different seasons during 2011. All samples were kept in a 2L sterilized plastic bottles, and stored in insulated cooler containing ice and delivered on the same day to laboratory and all samples were kept at 4 °C until processing and analysis.

2.3. Analytical Methods

2.3.1. Physico- chemical analysis

Water quality parameters includes: water temperature was measured by using a thermometer (accurate to nearest 0.1 °C), pH using pH- meter (Philips, 4014, UK), electrical conductivity using (EC meter, Philips, 4025, UK). The BOD_5 by the Winkler azid method, Sulfate by titrimetric method (APHA, 1998). Total nitrogen wet mineralized by using potassium persulphate as described by (MacKareth *et al.*, 1978), persulphate digestion method was used for total dissolved phosphate as described by (Lind, 1979).

2.3.2. . Microbiological analysis

For the bacteriological analysis of water samples, Coliform test was performed by the most probable number (MPN) technique (Benson, 1998) and heterotrophic plate count (aerobic) by Pour Plate method (Sugita *et al.*, 1993). Standard MPN technique was applied using glucose azide broth for isolation of fecal streptococci (APHA,1998)

Detection of *Salmonella* spp was done by the enrichment of water samples on Selenite F broth, followed by isolation of the typical organism on selective medium, Xylose Lysine Deoxycholate Agar (XLD). Detection of *Vibrio cholerae* was done by enriching the samples in 1% alkaline peptone water for 6 to 8 hours followed by isolation on Thiosulphate Citrate Bile salt sucrose (TCBS) agar medium (Collee *et al.*, 1996). For *Pseudomonas aeruginosa* both MacConkey agar , Nutrient agar were used as presumptive cultures and Mannitol salt agar and Blood agar were used to isolate *Staphylococcus aureus* (Benson, 1998). All colonies with different characteristics on their selective media were identified on the basis of their colonial, morphological and biochemical properties following Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994).

The fungi were isolated from the water samples seasonally by using two methods: The direct plate and the dilution plate, two types of growth media were used for isolation of fungi Potato dextrose agar (PDA) and Sabouraud's dextrose agar (SDA) supplemented with chloramphenicol (50 mg/l) and cycloheximide (500 mg/l). While for the isolation of fungi from soil sediment two methods were used: A dilution of 10^{-3} was chosen for the estimation of the fungal total count and the hair bait technique of Vanbreuseghem (1952) was used to isolate keratinophilic fungi. For this purpose, sterile Petri dishes were half filled with the soil samples and moistened with water and baited with burying sterile human hair in the soil. These dishes were incubated at room temperature (20 ±1 °C) and examined for fungal growth over a period of four weeks. After observing the growth under a stereoscopic binocular microscope it was cultured on SDA supplemented with chloramphenicol (50 mg/l) and cycloheximide (500 mg/l) (Deshmukh and Verekar 2006).

2.4. Statistical Analysis

Statistical analysis was conducted for the data using software program (SPSS version 20). One way ANOVA (Analysis of variance). Post hoc test (Duncan) was applied to determine significant differences in spatial and in temporal variation. All data are expressed as mean ± SE. A *P* value of 0.05 was considered as the limit for statistical significance.

3. Results and Discussion

Seasonal variation of physico-chemical and bacteriological characteristics are given in Table 1. The maximum temperature was 28 ºC recorded during summer and minimum during winter 9 ºC. The fluctuation in water temperature usually depends on the season, geographic location and sampling time.

Table 1. Seasonal variation of physico- chemical and biological characteristics of water for Duhok dam. (data represented as mean± SE).

Variables	Winter	Spring	Summer	Autumn
Water temperature (ºC)	9.0± 0.0^{a}	28± 0.0^{b}	27.1± 0.16^{c}	22± 0.0^{d}
pH	7.7± 0.057^{a}	8.3± 0.081^{b}	8.0± 0.043^{c}	7.9± 0.052^{c}
EC (µs.cm^{-1})	753± 0.88^{a}	817± 4.72^{b}	1237± 6.22^{c}	1038± 0.66^{d}
Total Nitrogen (µg.l^{-1})	18.6± 6.21^{a}	117.9± 37.7^{b}	98.6± 30.0^{b}	5.8± 2.26^{a}
Total phosphate (µg.l^{-1})	1.5± 0.31^{a}	7.98± 4.52^{a}	7.4± 3.80^{a}	0.73± 0.18^{a}
Sulphate (mg.l^{-1})	548.8± 9.93^{a}	518± 39.3^{a}	486± 39.5^{a}	579± 13.8^{a}
BOD_5(mg.l^{-1})	1.26± 0.14^{a}	1.16± 0.16^{a}	2.2± 0.15^{b}	0.93± 0.07^{a}
Heterotrophic plate count (CFU.ml^{-1})	291± 18^{a}	348000± 326^{a}	51600± 351^{a}	1296± 266^{a}
Fecal coliform MPN.100m^{-1})	1.0± 0.1^{a}	8.3± 1.3^{a}	10.6± 1.5^{a}	0.0± 0.0^{a}
Fungi (CFU.ml^{-1})	1.0± 0.05^{a}	7.6± 0.28^{b}	1.6± 0.07^{a}	2.6± 0.08^{ab}

Note: Values in each row with different letters are significantly different at $P \leq 0.05$ according to Duncan test. Values in rows with same letters are not significantly different.

Highest microorganisms counting recorded during spring and summer seasons. Statistically significant differences ($P \leq 0.05$) were observed between seasons regarding to microorganisms counts. The minimum microorganisms count in winter might be due to cold climate condition, which is not been supportive for bacterial and fungal duplication in a greater extent (Venkateesharaju *et al.*, 2010).

The pH value was more than 7 and the maximum value was recorded during spring season (pH max. 8.3). High photosynthesis rate during spring adversely affect the pH value. These results came in agreement with the results of Al- Nakshabandi (2002) who also worked on Duhok reservoir. Conductivity values varied from 753 to 1237 µs. cm^{-1} for winter and summer seasons respectively. The high salt concentration of water shows that significant dissolution and/ or precipitation reactions are taking place in the lake depending upon the solubility constants of different minerals present in the lake (Millero, 2001). The minimum value observed in rainy season is due to dilution with the rain water and maximum owing to evaporation and high water temperature (Aqel, 2012). The maximum limit of conductivity value exceeds the desirable limits for drinking water (WHO, 1984). While, water quality classified as high salinity type C3 for irrigation purposes (Ayers and Wescot, 1994). Al- Nakshabandi (2002)

recorded higher electrical conductivity value for the same impoundment.

Total dissolved nitrogen and total dissolved phosphorus determination is important in assessing the potential biological productivity of surface waters, increasing concentration of phosphorus and nitrogen compounds in lakes and reservoirs leads to eutrophication (Welch and Tindell, 1992). Maximum concentration observed during Spring 117.9 and 7.98 $\mu g.l^{-1}$ for total nitrogen and total phosphorus respectively with minimum concentration recorded during autumn season.

The sulphate concentration of natural water is an important factor in determining their suitability to using it for different purposes (Sawyer and McCarty, 1978). High sulphate content recorded during studied period and never fall below 486 m $g.l^{-1}$. It exceeds maximum permissible range of Iraqi standard (1986) and WHO (2011) drinking water quality standard. Statistically significant difference ($P \leq 0.05$) was observed with conductivity value. Similar results were obtained by Al- Nakshbandi (2002) and Duhoki (1997) at the same reservoir.

BOD_5 represents the amount of oxygen that microbes need to stabilize biologically oxidizable matter. Higher BOD_5 value was recorded during Summer season (2.2 $mg.l^{-1}$). This may be due to high water temperature and bacterial counts. Statistically significant differences ($P \leq 0.05$) was found between different seasons.

High heterotrophic plate counts during all seasons were recorded with maximum number 348 x 10^3 and 51.6 x 10^3 CFU during Spring and Summer seasons respectively. The maximum permissible level of Iraqi drinking water standard must not exceed 50 $CFU.ml^{-1}$. It may reveals the human activity (Tourist and waste disposal) as bacterial population was estimated in higher concentration from water samples collected from the bank of the lake (Shafiq *et al*., 2011).

Faecal coliforms in the present investigation exhibits more counts during summer followed by spring seasons. During both seasons it exceeds Iraqi standards (1986) for drinking purposes. The presence of FC suggests that water may have been contaminated with faeces either of human or animal origin (Omezuruike *et al*., 2008).

Total fungal count was high during spring season (7.6 $CFU.ml^{-1}$), this may be related to human activities and waste disposal around lake bank.

Statistically, there are no significant differences ($P \leq 0.05$) between all studied sites for physico- chemical and biological water characteristics of Duhok reservoir (Table 2).

Table 2. Physico-chemical and biological characteristics of water for Duhok dam (data represented as mean± SE).

Locations	Seasons	Heterotrophic plate count ($CFU.gm^{-1}$)	Fecal coliform (MPN. $100m^{-1}$)	Fungi x 10^3 ($CFU.gm^{-1}$)
	Winter	$3.1x10^2$	0	21
Site 1	Spring	7 x 10^3	4	12
	Summer	5 x 10^5	4	4
	Autumn	5 x 10^2	0	10
	Winter	2.3 x 10^2	21	9
Site 3	Spring	5.2 x 10^5	4	13
	Summer	8 x 10^4	21	9
	Autumn	9 x 10^2	0	8

As noticed in Table 3, the results showed higher microbial count for all groups in sediment samples compared with water samples. The highest heterotrophic plate count and faecal coliform count were recorded in sediments of site 3. This site characterized by the presence of some vegetative plants near the bank. The presence of coliforms group in collected samples generally suggests that a certain selection of water and sediment samples may have been contaminated with faeces either of human and animal origin and other more dangerous microorganisms could be present (Omezuruike *et al*., 2008). Bano (2006) reported that presence of bushes, shrubs or plants makes likely possible that smaller mammals may have been coming around these water bodies to drink water, thereby passing out faeces into the water. In addition to, tourist activities nears this site that exposed to more pollutant sources.

Table 3. Seasonal variation of microbial count in sediment of Duhok dam.

Variables	Site1	Site2	Site3
Water temperature (ºC)	21.5± 4.3 [a]	21.5± 4.3 [a]	21.6± 4.4 [a]
pH	8.02± 0.11 [a]	7.96± 0.17 [a]	7.99± 0.09 [a]
EC ($\mu s.cm^{-1}$)	967± 111 [a]	960± 109 [a]	957± 109 [a]
Total Nitrogen ($mg.l^{-1}$)	80± 43.5 [a]	73.5± 32 [a]	27.1± 9 [a]
Total phosphate ($mg.l^{-1}$)	2.55± 0.9 [a]	8.63± 4.2 [a]	2± 0.5 [a]
Sulphate ($mg.l^{-1}$)	491± 36 [a]	561± 22.8 [a]	546.8± 12.6 [a]
BOD_5 ($mg.l^{-1}$)	1. 5± 0.35 [a]	1.35± 0.25 [a]	1.32± 0.26 [a]
Heterotrophic plate count (($CFU.ml^{-1}$)	18.2± 1.03 [a]	2.21± 0.81 [a]	280± 24.1 [a]
Fecal coliform MPN .$100m^{-1}$)	3.5± 2 [a]	1.75± 1.03 [a]	9.75± 4.95 [a]
Fungi ($CFU.ml^{-1}$)	3.75± 0.24 [a]	2± 0.2 [a]	4± 0.52 [a]

As shown in Table 4, *E. coli* was found in water sample almost during Winter and Summer seasons. In sediments, it was more abundant and recorded in all seasons except Autumn season. *E. coli* is the most widely adopted indicator of faecal pollution and they can also be isolated and identified simply (Kumar *et al*., 2010). *E.coli* has frequently been reported to be the causative agent of traveler's diarrhoea, urinary tract infection, hemorrhagic colitis, and haemolytic uraemic syndrome (Al-Otaibi, 2009). *Streptococcus faecalis* with *E. coli* are good indicators of gastrointestinal diseases.

The presence of such bacteria indicates the possible presence of faecal material (Leclerc *et al*., 1996). *Pseudomonas aeruginosa* has been isolated in both water and sediment samples. *Pseudomonas* can, in rare circumstances, cause community acquired pneumonias as well as ventilator associated pneumonias, being one of the most common agents isolated in several studies (Radojevic *et al.*, 2012; Fine *et al*., 1996). Karfistan and Arik-Colagolu (2005) enter *Pseudomonas* bacteria as indicators of microbiological water quality during their study on Manyas lake. *Staphylococcus aureus* regarded as important indicators of the whole aquatic ecosystem health, including fish, and birds via the food web (Kumar *et al*., 2010). Most of these isolated bacteria species from Duhok water dam have been isolated in different water bodies in other studies (Bano, 1996; Omezuruike et al., 2008; Uzoigwe and Agwa, 2012). No detection of *Vibrio cholerae* was found in water and sediment samples of Duhok water dam.

Table 5. A total of 5 fungal genera (9 species) were identified in water samples and 9 genera (12 species) were identified in sediment sample. The most dominant species includes *Aspergillus niger* (28%), *A. flavus* (16%), *A. ochracecus* and *Pencillium* spp. (10%) in water samples, while in sediment samples the most common species were *A. niger* (22%), *Rhodotorulla* spp. (19%), *A. flavus* (16%) and *Rhizopus* spp. (10%).

Higher diversity index (H`= 2.136) was accounted in sediment rather than water samples (H`= 1.956).

The fungal communities were identified at the all four seasons in water samples. The highest number of taxa, 6 species were collected in Spring season followed by 4 species in Autumn and 3 species in Summer and Winter.

In sediment samples, highest number of taxa, 8 species were observed during Autumn season, 5 species collected during Spring and Winter seasons and 4 s pecies wereidentified during Summer. Seasonal changes in the water temperature have been shown distinct effects on occurrence and percentage of fungal communities and compositions. The reason of low fungal isolation genera in Duhok water dam may be due to absent of plants growing along its banks, with exception of few macrophytes near site 3. Luo *et al*., (2004) commented that riparian vegetation had been regarded as an important factor influencing freshwater fungal communities through availability of detritus for these organisms. Higher species isolation, occurrence and diversity index (H`) and counting in sediments than in water, may be related to availability of organic detritus on sediments that supply a good sources of food and habitat for these decomposers (El-Dohlob and Ali, 1981). It seems quite clear that the availability of organic matter, pH and water temperature play important role in the existence and propagation of aquatic fungi in lakes (Mahmoud and Abou- Zeid, 2002).

Table 4. Isolation of some bacteria in water and sediment of Duhok dam.

Samples	Bacterial isolation	Seasons												Total occurrence
		Winter			Spring			Summer			Autumn			
		Site 1	Site 2	Site3	Site 1	Site 2	Site3	Site 1	Site 2	Site3	Site 1	Site 2	Site3	
Water	*E. coli*			+				+	+	+				4
	Salmonella spp.						+	+	+	+				4
	Pseudomonas aeruginosa	+		+	+									3
	Streptococcus faecalis				+		+							2
	Staphylococcus aureus (MRSA)			+										1
	Vibrio cholera													
Total		1		3	2		2	2	2	2				14
Sediment	*E. coli*	+		+	+		+	+		+				6
	Salmonella spp.						+	+		+				3
	Pseudomonas aeruginosa	+		+										2
	Streptococcus faecalis									+				1
	Staphylococcus aureus (MRSA)	+		+						+				3
	Vibrio cholera													
Total		3		3	1		2	2		4				15

Table 5. The occurrence of fungi at different seasons in water and sediment of Duhok dam

Samples	Fungal isolation	Seasons												Total occurrence
		Winter			Spring			Summer			Autumn			
		Site 1	Site 2	Site3	Site 1	Site 2	Site3	Site 1	Site 2	Site3	Site 1	Site 2	Site3	
Water (CFU.ml^{-1})	*Aspergillus candidus*	1						1						2
	Aspergillus flavus				5		4							9
	Aspergillus niger				2	1	3		1	1	1		2	11
	Aspergillus ochraceous				4									4
	Emericella spp.	1										2		3
	Penicillium spp.		1			1						2		4
	Rhodotorulla glutins						2							2
	Cladosporium spp.						1							1
	Eurotium sp.									2			1	3
Total		2	1		11	2	10	1	1	3	1	4	3	39
Sediment x 10^3 (CFU.ml^{-1})	*Aspergillus flavus*	2		2	1		4	2		2			1	14
	Aspergillus fumigates				2									2
	Aspergillus niger			3	5		5			3			3	19
	Aspergillus ochraceous				4									4
	Cladosporium sp.										5			5
	Emericella sp.										2			2
	Mucor sp.										1			1
	Neosartoria sp.	2						2						4
	Rhizopus sp.			4						4	1			9
	Rhodotorulla sp.	17												17
	Alternaria sp.										1			1
	Penicillium citrinum						4						4	8
Total		21		9	12		13	4		9	10		8	86

Microsporum gypseum, *Chrysosporium* spp., *Trichophyton spp*. and *Absidia spp*. were isolated by hair-baiting technique (Table 6). Among the isolated species, *M. gypseum* and *Trichophyton spp*. were common agent of dermatophytosis (tinea) in human and animals that can cause many problems for human health (Hedayati and Mirzakhani, 2009. In addition, *Chrysosporium spp*. and *Absidia spp*. keratinophilic and saprophytic fungi, were isolated by this technique. These results are partially agree with that found by (Hedayati and Mirzakhani, 2009).

Table 6. Keratinophilic fungi isolated in sediment samples from different sites of water Sari city, by the hair-baiting technique.

Fungal genera	Spring	Summer	Autumn	Winter
*Absidi*a spp.			+	
Chrysosporium spp.	+	+	+	+
Microsporum spp.	+			
Trichophyton spp.			+	

4. Conclusion

High water temperature was 28 ° C recorded during Summer. All tested water samples were alkaline in their nature. Water quality characterized by high salt concentration according to electrical conductivity values, exceeded guideline standards for drinking and irrigation purposes. Sulphate content exceeded Iraqi standard for drinking water quality and may be attributed to some sulphur springs discharged into Duhok water dam. Fecal coliform group was detected in all studied sites. Higher microbial count, isolation and diversity index were found in sediment rather than water samples. This may be related to organic detritus which used as microhabitat and food sources for microbial growth. Isolation of some pathogenic microorganisms (bacteria and fungi) in water and sediment samples, may be due to non point sources of pollutant discharged into Duhok water dam, can cause problems for human health as used for drinking and swimming activities. Fourteen fungal genera were isolated during this investigation.

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Effects of *Theileria lestoquardi* Infection on Haematological and Biochemical Parameters in Experimentally Infected Desert Ewes

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Abstract

In an experimental infection of sheep with *Theileria lestoquardi* some biochemical and haematological parameters were studied in ten Sudanese desert ewes together with 10 clinically healthy ewes. The obtained results showed a significant (P<0.05) decrease in haemoglobin, packed cell volume and white blood cells counts compared to the control group. The decrease in Hb concentrations and PCV% was observed 7-10 weeks after tick application whereas; the decrease in WBCs occurred at week 5 and week 6 after tick application. There was also a significant decrease (P<0.05) in serum total proteins, and serum globulins values of the infected group at week 3- week7 after tick application. A significant increase (P<0.05) in serum urea and serum creatinine values in the infected group was noticed during the first 7-8 weeks in the infected group compared to the control one's.

Key words: *Theileria lestoquardi,* Sheep, Haematological, Serobiochemical, Sudan

1. Introduction

Tick- borne diseases of small ruminant are of highly economic importance in many countries. Malignant theileriosis of sheep and goats caused by *Theileria lestoquardi* is considered among the most important small ruminant diseases and constituted an obstacle to the industry in countries like the Sudan (Bakheit *et. al*., 2006). When the infested sheep develop theileriosis, *Theileria* schizonts are demonstrated in liver, spleen, lung, kidney lymph node and peripheral blood (Hong *et. al*., 2003).

Theileria lestoquardi, was shown to be widely distributed in main sheep grazing areas in Sudan where 16.3% of sheep surveyed showed reactive antibodies in IFAT (Salih *et. al.*, 2003). Biochemical investigations were very important to understand the host parasite relationship and to study the metabolism of the parasite, which may be helpful in the diagnosis and therapeutic processes (Yadav *et. al.*, 1986).

2. Materials and Methods

2.1. Experimental Animals

This study was carried out at the Department of Radioisotopes, Central Veterinary Research Laboratories, Khartoum, Sudan. It was conducted on Sudanese desert ewes. The ewes were clinically healthy, free of ticks, parasitic infections including theileriosis and *Brucellosis*.

Before the commencement of the experiments, the animals were kept in the premises of the Veterinary Research Administration for five weeks to adapt themselves to the new environment and to be examined for any clinical detectable diseases such as brucellosis, trichomoniasis, theileriosis and any reproductive disorders or abnormalities. All animals were dosed with broad spectrum antihelmenthic and anti coccidial. The animals were fed on green forage and supplemented with a commercial pelleted diet. Ewes were randomly divided into two groups (A and C) of ten each. Each group was housed separately at the Department of radioisotopes barns of 20 square meters each. They were provided with metal feeding troughs and plastic containers for water. Ewes of group A were experimentally infected with *Theileria lestoquardi*, whereas; ewes in group C remained as the uninfected control.

2.2. Infection with Theileria lestoquardi

A ram which was naturally infected with Theileria lestoquardi was brought from Atbara, North Sudan by the aid of the regional veterinary research laboratory in Atbara. This ram was particularly brought as it was proved to carry 10% parasitaemia the parasitaemia was further confirmed in the department of ticks at Soba. This sheep was used as the source of infection.

Flat nymphs of *Hyalomma anatolicum* were applied on this ram to pick up the infection using ear bags according to

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the method described by Bailey (1960). Engorged nymphs collected and kept in the laboratory to the second stage (flat adult). Infected flat adults emerging from the nymphs were allowed to feed again on the experimental ewes.

Establishment of infection was monitored daily by measuring rectal temperature. Thin blood smears from the ear vein were prepared daily, fixed with absolute methanol and then stained with fresh Giemsa stain.

2.3. Collection of Samples

Weekly Blood samples were collected from the jugular vein into plain and heparinized vacutainer tubes from each ewe throughout the study. Sera were separated and kept at -20 ^{0}C until analyzed. All the haematological parameters were estimated by the methods described by Schalm (1965).

Total protein was determined using Biurret reagent as described by King and Wooton (1965), Albumin was determined according to Bartholmew and Delany (1966) while, Urea level was determined according to the method outlined by Evans (1968) using Randox commercial kits (Diamond Road ,Crumlin,Co.Antrim,United Kingdom, BT29 4QY).

2.4. Statistical Analysis

Using (SPSS) version 10.0 data were analyzed using student T-test analysis, differences were considered significant at $P<0.05$ level.

3. Results

3.1. Haematological Parameters

3.1.1. Haemoglodin concentration

The weekly mean Hb concentration, PCV and total white blood counts were monitored for 10 weeks in both groups of sheep post tick application. The starting concentrations of Hb at the first week was >8.0 g/dL then gradual decrease was noticed in the infected group which showed lower Hb values compared to the control group (Table 1), however, the difference was significant ($P<0.05$) in weeks 7-10 reaching lowest concentration at week 10 (< 7.0 g/dL). While in control group the mean Hb concentration remained as high as (7.6- 8.9g/dL) throughout the study period.

3.1.2. PCV

The mean values of PCV in the infected group were found to be lower than that of the control group ranging between (22-28%) and (26-29%), respectively (Table 1). However, the differences were statistically significant ($P<0.05$) at weeks 8,9 and10 representing the lowest value (<22%).

3.1.3. White Blood Cells (WBCs)

The mean WBCs counts were (8.1±2.12and 8.3±2.24) in the control and infected group, respectively. As shown in Table (1) there was a gradual decrease in WBCs counts in the infected group and at week 5 and week 6 the counts dropped to significant levels ($P<0.05$). However, at week 7 to week10 the counts increased to the starting level (8-11) in both groups.

Table 1. Haematological values (mean± SD) in *Theileria lestoquardi*-infected Sudanese desert ewes

Weeks post tick application	Hb	PCV	WBCs
1	8.27±1.20[a] 8.10± 1.35[a]	25.11±4.7[a] 24.11±5.62[a]	8.12±2.12[a] 8.36±2.24[a]
2	7.65±1.12[a] 6.91±1.49[a]	28.72±8.3[a] 25.56±6.44[a]	7.96±3.23[a] 7.52±3.25[a]
3	8.40±0.95[a] 7.44±1.04[a]	27.34±5.16[a] 23.00±5.17[a]	7.02±1.82[a] 6.69±1.66[a]
4	8.00±0.61[a] 7.88±0.79 [a]	29.47±4.71[a] 27.88±4.58[a]	6.20±2.56[a] 6.10±1.93[a]
5	7.97±0.78[a] 7.91±1.04[a]	28.79±4.9 [a] 28.11±5.25 [a]	6.20±2.84[a] 4.96±1.41[b]
6	8.38±0 .64[a] 8.26±0.64[a]	25.87±4.73[a] 23.00±4.44[b]	7.20±2.25[a] 6.31±4.65[b]
7	7.67±1.13[a] 6.78±1.10[b]	26.00±5.04[a] 25.43±4.65[a]	6.92±2.11[a] 6.78±2.23[a]
8	8.35±1.82[a] 4.20±0.01[b]	27.93±5.16[a] 21.89±6.39[b]	9.13±2.16[a] 9.33±2.29[a]
9	8.55±0.33[a] 6.92 ±1.7[b]	26.82±5.56[a] 22.15±6.41[b]	11.0±3.24[a] 8.92±1.09[a]
10	8.90±1.13[a] 6.98±1.27[b]	27.64±5.39[a] 22.30±4.00[b]	8.45±1.75[a] 8.92±1.09[a]

Values with different small superscripts within the same column were significantly different at $P<0.05$

3.2. Biochemical Parameters

3.2.1. Serum Total Proteins

Decreases in the weekly serum total protein concentrations was observed for up to 10 weeks post tick application in the infected animals compared to the controls (Table 2). However, the difference was significant ($P < 0.05$) at weeks 3 –week7, whereas, in week 8- 10, the mean values of serum total proteins showed insignificant difference ($P>0.05$) between the two groups.

3.2.2. Serum albumin

Although the mean values of serum albumin during the sampling period of 10weeks, were slightly lower in the infected group compared to the control group, yet the differences were insignificant ($P>0.05$).

3.2.3. Serum globulins

A gradual decrease in the mean concentration of serum globulin was noticed in the infected group from (> 4.0g/dl) at week 3 to (<3.0g/dl) at week 7 post infection (Table 2),however, at week 8 the values began to increase to the control level and by week 10 there were no significant difference in globulin concentration between both group.

3.2.4. Serum creatinine

The mean serum creatinine concentrations (mg/dl) were significantly higher in the infected group of sheep compared to the control one's (Table 2). These differences were observed during the first 7 weeks reaching the highest value (22.05±14.4 mg/dl) at week 5. Then it started to drop again to the baseline level. Later, at week 10 the

creatinine levels reached its lowest values in both groups (4- 4.3 mg/dl).

3.2.5. Serum urea

The weekly serum urea concentrations varied between (30 - 34mg/dl) in the infected group which is significantly higher ($P<0.05$) than in the control group (28-31mg/dl) during the first eight weeks, whereas, during weeks 9-10, there were no significant differences in urea concentration of both groups where they showed relatively constant value (32.4-32.9mg/dl) as presented in Table 2.

Table 2. Serobiochemical values (mean± SD) in *Theileria lestoquardi*-infected Sudanese desert ewes

Weeks post tick application	Total Proteins (g/dl)	Albumins (g/dl)	Globulins (g/dl)	Creatinine (mg/dl)	Urea (mg/dl)
1	7.31± 0.32 [a]	3.32 ±0.33[a]	3.98± 0.54 [a]	5.32± 5.86 [a]	34.76± 5.58 [a]
	7.34± 2.05[a]	3.31± 0.82 [a]	4.03± 1.37 [a]	7.59± 5.03 [b]	33.86± 3.19 [b]
2	7.45 ± 0.49 [a]	3.38± 0.32 [a]	4.07± 0.76 [a]	5.46± 0.87 [a]	32.84± 3.26 [a]
	7.73± 2.62[a]	3.41± 0.57 [a]	4.32± 2.01 [a]	10.14± 3.85 [b]	34.24± 2.45 [b]
3	7.35 ± 0.78 [a]	3.00± 0.27 [a]	4.34± 0.88 [a]	4.26± 3.74 [a]	31.38± 2.89 [a]
	5.56± 1.11[b]	2.84± 0.97 [a]	2.72± 1.03 [b]	8.99± 5.99 [b]	32.89± 7.19 [b]
4	7.27 ± 0.37 [a]	3.12± 0.32 [a]	4.15± 0.51 [a]	6.83± 3.59 [a]	28.51± 4.10 [a]
	6.11± 0.15[b]	2.99 ±0.61[a]	2.87± 0.78 [b]	11.34± 2.55 [b]	30.02± 3.19 [b]
5	7.19 ± 0.51 [a]	3.26± 0.58 [a]	3.93± 0.81[a]	7.00± 3.07 [a]	30.71± 3.62 [a]
	5.73±. 69 [b]	3.14 ±0.27[a]	2.83± 0.68[b]	22.05± 14.7 [b]	33.71± 6.63 [b]
6	7.24 ± 0.40 a	3.24± 0.24 a	3.97± 0.51a	6.16± 5.51 a	31.52± 4.92 a
	5.98± 1.36b	3.09± 0.43 a	2.84± 1.12b	10.27± 4.17 b	32.88± 6.24 b
7	7.37 ± 0.44 a	3.21± 0.17 a	4.16± 0.54 a	4.29± 2.20 a	31.39± 2.39 a
	5.83± 1.25b	3.07 ±0.22a	2.98± 1.21 b	9.21± 3.59 b	34.78± 3.90 b
8	7.19 ± 0.38 a	3.31± 0.33 a	3.89± 0.60 a	10.26± 1.21 a	31.70± 4.06 a
	7.15± 1.96a	3.12 ±0.35a	3.79± 1.59 a	12.74± 4.12a	33.36± 3.32 b
8	7.17 ± 0.36 a	3.13± 0.29 a	4.04± 0.49 a	7.38± 3.79 a	32.40± 3.24 a
	7.63± 1.69a	3.12± 0.52 a	4.25± 0.91 a	7.12± 4.38a	32.95± 11.34a
10	7.19 ± 0.59 a	3.15 ±0.31a	4.15± 0.52 a	4.30± 2.81 a	32.31± 4.25 a
	7.54± 0.82a	3.11 ±0.42a	4.16± 0.29 a	4.13± 3.25 a	32.59± 5.68 a

Values with different small superscripts within the same column were significantly different at $P<0.05$

4. Discussion

Attempts to explain the haematological changes on pathophysiological basis, revealed that in infection with *T. lestoquardi,* there was a marked decrease in haemoglobin, packed cell volume and white cell counts. This decrease fluctuated in weeks following tick application, after which, the values returned back to normal. Our findings were similar to the findings of other worker (Ahmed, 2004; Mehta *et al*., 1988; Rayules and Hafeez, 1995; Sandhu *et al*., 1998; Singh *et a l.,* 2001). Nazifi *et al*. (2010) also reported that, as the parasitaemia increased, a significant decrease was observed in RBCs, PCV and Hb. In contrast, with an increase in the parasitaemia rate, a s ignificant increase in the mean corpuscular volume, haptoglobin (Hp), serum amyloid A (SAA), ceruloplasmin and fibrinogen was evident. The decline of the above mentioned values might probably be attributed to the destruction of erythrocytes by macrophages in the lymph nodes, spleen and other organs of the reticuloendothelial system as previously suggested. Singh *et al.* (2001) and Omer *et. al.* (2002) reported that these changes in blood parameters pictured by the decrease in haemoglobin, packed cell volume and white blood cell counts may finally lead to the occurrence of severe anemia.

With respect to the biochemical changes as a result of *T. lestoquardi* infection, the study demonstrated that there was an apparent decrease in the concentrations of serum total protein, serum albumin and serum globulins. This decrease fluctuated in weeks following the tick application, after which, these values returned back to normal. This finding is in line with that of Ahmed (2004), Singh *et al*. (2001), Ramazan and Uguruslu (2007), and Yadav and Sharma (1986). However, Sandhu *et al.* (1998) reported an insignificant decrease in these parameters. Stockham *et al.* (2000) attributed the decrease in the concentrations of the serum protein and the serum albumin to the extra vascular accumulation of proteinaceous fluids resulting from affected lymph nodes. On the other hand, Singh *et. al.* (2001) and Omer *et al.* (2003) attributed the decrease in serum proteins to hypoalbuminaemia and hypoglobulinaemia arising from liver failure.

Our findings indicated that ewes infected with *Theileria lestoquardi* had higher concentrations of serum creatinine and serum urea. This increase fluctuated in week3-7 following tick application, after which, they returned to normal levels. These findings were in agreement with the findings of Ramazan and Uguruslu (2007), Ahmed (2004), and Yerham *et al*. (1998) who attributed the increase in creatinine to damage observed in the liver and kidney in babesiosis in sheep. The increase in urea level was similar to that reported by Singh *et al*. (2001) and Sandhu *et al*. (1998). However, our observation contradicts that of Omer *et al.* (2003) who showed significant decrease in urea and creatinine in cattle naturally infected with *Theileria annulata.*

5. Conclusions and Recommendations

In conclusion it could be stated that infection of desert sheep with *Theileria lestoquardi* had significantly and adversely affected the haematological and biochemical parameters under this study.

Further investigations were needed to trace the causes of such fluctuations in serum and blood parameters.

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Computational Prediction of Binding of Methyl Carbamate, Sarin, Deltamethrin and Endosulfan Pesticides on Human Oxyhaemoglobin

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Abstract

Pesticides are used to control insects and pests. However, their use has become so frequent that they may create problems for non target animal species. Methyl carbamate, sarin, deltamethrin and endosulfan pesticides are used to control insects and pests. In the current study computational prediction of binding of these pesticides on human oxyhaemoglobin using Molegro Virtual Docker (MVD) and evaluating the comparative Mole Dock Score, Root-mean square deviation (RMSD), affinity, interacting residues of human oxyhaemoglobin, number of Hydrogen bond interaction, docking score, Protein steric interaction energy (Protein EvdW) and interacting interaction of residues. In the present study the energy bound conformation with lower value of selected ligands shows hydrogen bonding and electrostatic interactions. The binding affinity of selected pesticides is found to be in decreasing order i.e., sarin > methyl carbamate > deltamethrin > endosulfan. All the pesticides bind with the serine 133. Frequent alterations in the expression of serine (amino acid) due to pesticide interaction with oxyhemoglobin may lead to produce carcinogenic cells in human beings.

Key words: Pesticide, Human oxyhaemoglobin, MVD, RMSD, Protein steric interaction energy, Serine.

1. Introduction

Due to increase in population scarcity of food is likely to create immense pressure to full fill the demand of food production. Nearly 29% of food produced is destroyed by insects, pests, birds, rats, etc. With the increasing pressure for food production, there is a great increase in the use of insecticides and pesticides to reduce the loss due to insects and pests (Tomlin, 2006). Though their use reduces the loss from insects and pests, on the other hand, they have adverse effects on crops nutrient value and leads to hazardous results on non target animals as well as human beings (Dich *et al*., 1997). Methyl carbamate, sarin, deltamethrin and endosulfan are widely used pesticides in homes, gardens and agriculture (Bradberry *et al.,* 2005, Ayaz *et al.*, 2013). They are used in commercial crops, recreational uses and control variety of pests and inhibit cholinesterase enzyme activity, thus they have similar symptomatology during exposure of acute and chronic toxicity (Lifshitz *et al.*, 1997, Burr & Ray, 2004). They can produce a variety of acute health problems and also carries several ecological risks (Lifshitz *et al.*, 1994, Goswamy *et al.*, 1994, Saxena and Saxena, 1997).Haemogrammic studies in albino rat after pesticide intoxication were revealed by Shakoori *et al.* (1992), Saxena and Tomer (2003), Shah *et al.* (2007) and Saxena and Saxena (2010). They reported that total erythrocyte count, hemoglobin concentration, packed cell volume, mean corpuscular volume were decreased after pesticide toxicity. It becomes necessary to carry out hematological examination to evaluate the normal and abnormal physiological states of the body. Our cells require regular supply of fuel and oxygen. Blood has capability to fulfill these requirements for proper functioning of cells resulting in maintaining good health. Low hemoglobin is the main cause of anemia. Further, low hemoglobin indicates lower levels of oxygen in the blood, which often causes shortness of breath (Shakoori *et al.*, 1988; Saxena *et al.,* 2009). Low hemoglobin level may also exasperate extant heart problems (Villarini *et al.*, 1998). In this work, we report computational prediction of binding of methyl carbamate, sarin, deltamethrin and endosulfan pesticides on human oxyhaemoglobin and evaluate the comparative Mole Dock Score, RMSD, binding affinity, interacting residues of receptor human oxyhaemoglobin, number of H-bond interaction, docking score, protein EvdW and interacting Interaction of residues using MVD.

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2. Materials and Methods

Three dimensional X ray crystallized structure of human oxyhaemoglobin (PDB: 1HHO, 2.1 Å resolution) was downloaded from the protein data bank (Shaanan, 1983). The downloaded protein have the two chain hemoglobin A (oxy) (alpha chain) with 141 r esidues and hemoglobin A(oxy) (beta chain) with 146 r esidues .The protein was taken as receptor protein and most suitable site was predicted by using q s itefinder ligand binding site prediction (http://www.modelling.leeds.ac.uk). Because of priority of site, oxyhaemoglobin has been selected for docking with ligand methyl carbamate (CID_11722), sarin (CID_7871), deltamethrin (CID_40585), and endosulfan (CID_6434141) pesticides, recently use for plant protection and control pests in homes & gardens. The selected ligands (pesticides) were downloaded from Pub Chem Compound (http://www.ncbi.nlm.nih.gov). Docking study was done with Molegro Virtual Docker (MVD). It is automated docking software with fast processing. The binding site cavity detection was performed by q site finder ligand binding site prediction tool (Figure 1). The docking simulation was performed by using docking software, namely MVD for the selected pesticides (ligands) and oxyhaemoglobin (protein).It shows mole dock score, RMSD, affinity (the estimated binding affinity in kj /mol), docking score, protein EvdW and interacting interaction (the interaction energy among the pose and the cofactor), number of H-bond and interaction between interacting residues of receptor human oxyhaemoglobin, which indicates towards the formation of stable complex among ligand and receptor molecule (Thomsen and Christensen, 2006). MVD visualizer is used for interaction site prediction.

3. Results and Discussion

The comparative results obtained (using MVD) from docking simulation are given inTable-1. The interaction analysis for binding of human oxyhaemoglobin with methyl carbamate, sarin, deltamethrin and endosulfan have been done to find out the residues that are involved in binding site residues and number of hydrogen bonds are involved in interaction among selected pesticides, Table - 2.The energy bound c onformation with lower value of selected ligands shows hydrogen bonding and electrostatic interactions are given in Figures 2a, b, 3a, b, 4a, b and 5a, b, for methyl carbamate, sarin, deltamethrin and endosulfan respectively.

Docking energy for most of pesticides were found favorable for hemoglobin A (oxy) (alpha chain) rather than hemoglobin A (oxy) (beta chain) which shows that these compounds can get stuck into hemoglobin A (oxy) (alpha chain) due to positive interaction (Singh, 2012). The methyl carbamate shows very high affinity to bind with human oxyhaemoglobin and it interacts with Phe 98, Ser133, Val132, Ser102, Leu 120 residues of human oxyhaemoglobin. All these residues involved in binding belong to the cavity-1. The methyl carbamate forms 5 hydrogen bonds with Phe 98, Ser133, Val132, Ser102,Leu 120 (Table 2 and Figure 2 (a, b)). The hydogen bonding is very significant for interaction of biomolecules. Sarin forms low binding energy complex as compare to methyl carbamate which shows binding affinity with Ser 133, Thr 134, Phe 98, Lys 99, Ala 130, Thr137and forms three hydrogen bond with Ser 133, Thr 134 (Table 2 and Figure 3 (a,b)). The deltamethrin forms low binding energy complex as compare to sarin, it shows binding affinity with Ser133, Ser102, Tyr 35, Ala 130, Asp 126, Phe 98, Lys 99, Leu 105 residues of human oxyhaemoglobin. Whereas, deltamethrin formed three hydrogen bond with Ser133, Ser102, Tyr 35 r esidues (Table 2 and Figure 4 (a,b)).The Endosulfan forms low binding energy complex as compare to methyl carbamate shows binding affinity with Ser133, Asp 126, Leu 129, Ser102, Phe 98, Lys 99, Ala 130, Tyr35 and forms one hydrogen bond with Ser133 residue of human oxyhaemoglobin [Table 2 and Figure 5 (a, b)). The binding affinity of selected pesticides methyl carbamate, sarin, deltamethrin and endosulfan at the active site of human oxyhaemoglobin using MVD is in decreasing order i.e. sarin > methyl carbamate > deltamethrin > endosulfan. The binding of pesticide with oxyhaemoglobin also supported by *in vitro* studies. Mourad, 2005 reported a fall in hemoglobin contents after insecticide intoxication in farm workers. Further, Shakoori *et al.* (1992), Khan and Ali (1993), Saxena *et al.* (2009), and Saxena and Saxena (2010) also observed a significant reduction in heamoglobin concentration after pesticide toxicity.

Even today our understanding of the relationship between pesticides and human health is limited due to numerous methodological problems faced in laboratory studies. Computational methods can turn out very useful for comparing vitro results for new hypotheses. Docking study shows that amino acid residue i.e., Ser133 is common in binding for all the pesticides. Serine is a polar amino acid and important for catalytic function of many enzymes (Serine, 2013).Further, the OH group of serine phosphorylates and form kinase enzyme. Serine kinases also play an important role in cellular homeostasis due to phosphorylate transcription factors, regulate cell cycle, and arrange properly cytoplasmic and nuclear effectors (Blume-Jensen and Hunter, 2001; Weichenthal *et al.*, 2012). Capra *et al.* (2006) revealed that some serine kinase might play a tumor suppressor role and have a cau sal role in certain malignancies. The misregulation of kinases enzyme stimulate tumor growth, metastasis and poor clinical outcome (Warner *et al.,* 2003; Freeman and Whartenby, 2004; Eckerdt *et al.,* 2005). It has been also reported that frequent alterations in the expression of serine kinases causes cancer in human being (Edelman *et al*., 1987).

Table 1. Comparative Docking Simulation Result of Selected Pesticides Methyl Carbamate, Sarin, Deltamethrin and Endosulfan with Human Oxyhaemoglobin Protein from X-ray Crystallized Data of Protein Data Bank (PDB:1HHO) using MVD.

S. No.	Ligands	MoleDock Score	RMSD	Affinity	Protein EvdW	Intracting	Docking Score
1.	Methyl Carbamate	-45.0674	0.118821	-26.473	-34.8891	-47.1982	-48.0108
2.	Sarin	-51.4733	1.51773	-24.1619	-49.6729	-53.5958	-52.1534
3.	Deltamethrin	-124.791	1.76639	-36.9168	-129.268	-134.458	-123.268
4.	Endosulfan	-80.0703	3.52641	-42.6401	-75.845	-76.882	-80.3116

Table 2. Human Oxyhaemoglobin from X-ray Crystallized data of Protein Data Bank (PDB: 1HHO), protein residues interact with selected pesticides Methyl Carbamate, Sarin, Deltamethrin and Endosulfan using MVD (highlighted residues are involved in H-bonding interaction with ligands).

S. No.	ligands	Interacting residues of receptor Human oxyhaemoglobin	No. of H-bond interaction
1.	Methyl Carbamate	Ser133, Ser102,Leu 120 Phe 98, Val132	05
2.	Sarin	Ser 133, Thr 134, Phe 98, Lys 99, Ala 130, Thr137,	02
3.	Deltamethrin	Ser133, Ser102, Tyr 35, Ala 130, Asp 126, Phe 98, Lys 99, Leu 105,	03
4.	Endosulfan	Ser133, Asp 126, Leu 129, Ser102, Phe 98, Lys 99, Ala 130, Tyr35,	01

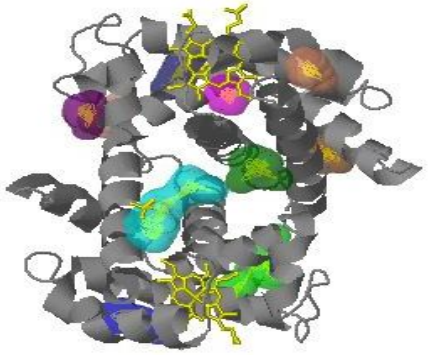

Figure1. Binding site for oxyhaemoglobin.

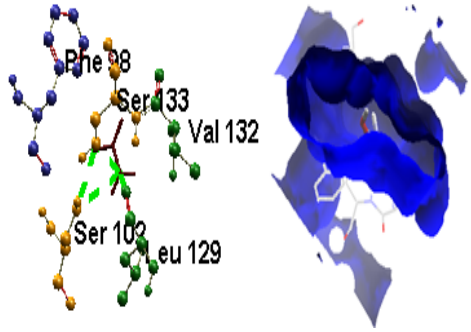

Figure2. (a) Docked conformation of hydrogen bonding view and (b) with Electrostatic interaction of Methyl Carbamate with interacting Human Oxyhaemoglobin protein at the active site cavity.

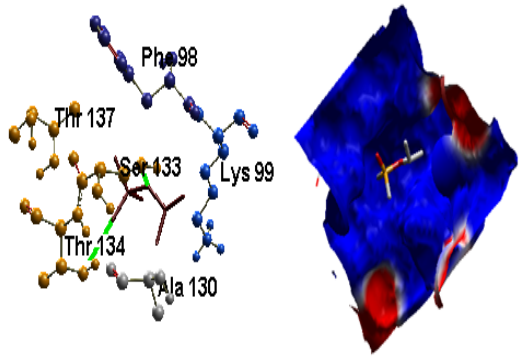

Figure3. (a) Docked conformation of hydrogen bonding view and (b) with Electrostatic interaction of Sarin with interacting Human Oxyhaemoglobin protein at the active site cavity.

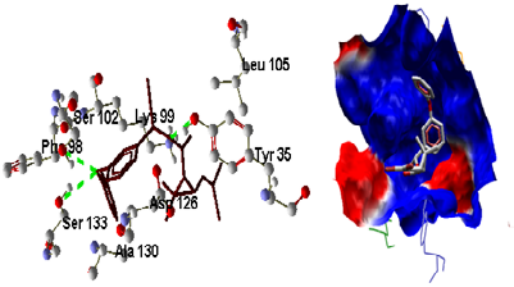

Figure4. (a) Docked conformation of hydrogen bonding view and (b) with Electrostatic interaction of Deltamethrin with interacting Human Oxyhaemoglobin protein at the active site cavity.

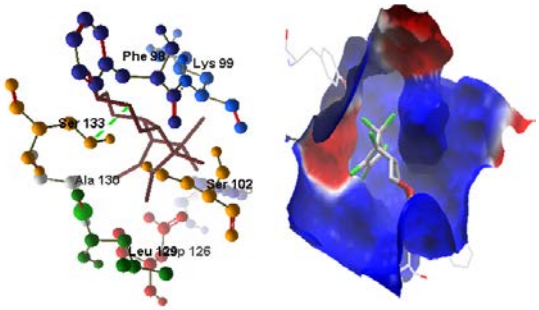

Figure5. (a) Docked conformation of hydrogen bonding view and (b) with Electrostatic interaction of Endosulfan with interacting Human Oxyhaemoglobin protein at the active site cavity.

4. Conclusions

We predict that pesticides bind human oxyhaemoglobin with varying affinities, and all tested pesticides bind to serine 133 which might lead to cancer. Thus, pesticides have both short term and long term

hazardous effects and, therefore, their use on crops & plants should be limited to a certain extent.

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New Records of Arthropod Ectoparasites of Bats from North-Eastern Algeria

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Abstract

Eight species of ectoparasites were recovered from nine bat species in north-eastern Algeria. Seven species belong to class Insecta and two species belong to class Arachnida. Insecta is represented by three orders namely, Diptera, Hemiptera and Siphonaptera. Two species of dipteran ectoparisites identified as *Phthiridium biarticulata* and *Brachytarsina falvipinnis*, belongs to Streblidae and Nycteribiidae, respectively. Order Hemiptera was represented by two species (*Cimex pipistrellus* and *Cimex lectularius*). One species of Siphonaptera was identified as *Ischnopsyllus octactenus*. Two species of arachnids were identified as *Rhipicephalus sanguineus* and *Spinturnix myoti*.

Key Words: Bats**,** Acarina, Flies, Hemiptera, Siphonaptera, Northeast Algeria**.**

1. Introduction

Our knowledge on the ectoparasites of bats of Algeria is very limited. Anciaux de Faveaux (1976) provided a list of insect parasites on Algerian bats based on previous records including Falcoz (1923), Séguy (1933) and Aellen (1955) for Nycteribiidae and Streblidae. Amr and Qumsiyeh (1993) reported five species of Nycteribiidae and Streblidae. Other reports included records of Spinturnicidae from Algeria such as Deunff (1977) and Uchikawa *et al.* (1994), and for Cimicidae (Usinger, 1966).

In the course of extensive collection of bats in north-eastern Algeria, nine bat species were collected. The present study documents the ectoparasites associated with bats collected from north eastern Algeria.

2. Materials and Methods

A total of 283 bats representing nine species were captured using mist-nets and hand-nets. Hand-nets were used to capture bats of cave roots, while mist-nets were used in open habitats. Bats were collected from eight localities within north-eastern Algeria during August, November and December 2012. Bats were identified according to Dietz and von Helversen (2010).

Each bat was individually examined and their ectoparasites were stored and labelled in a vial containing 75% alcohol. "N" and "NP" designate the number of bats examined and the number of recovered parasites respectively. Collected ectoparasites were identified according to Hopkins and Rothschild (1956), Rudnick (1960), Dusbabek (1962), Usinger (1966), Radovsky (1967), Theodor (1967), Beaucourn (1961), and Hůrka (1982).

Collected parasites were cleared and mounted using Hoyer's medium for mites and Canada balsam for ticks and the other insects on microscopic slides. Specimens were deposited in the Pasteur Institute in Algeria (L'Institut Pasteur d'Algérie).

3. Results and Discussion

Bat species

A total of 283 bats representing nine species (Family Vespertillionidae: *Eptesicus serotinus* (N=26), *Hypsugo savii* (N=17), *Myotis capaccinii* (N=15), *Myotis punicus* (N=32), *Pipistrellus kuhlii* (N=42), *Pipistrellus pipistrellus* (N=83); Family Rhinolophidae: *Rhinolophus*

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ferrumequinum (N=22), *Rhinolophus hipposideros* (N=18); Family Miniopteridae: *Miniopterus schreibersii* (N=28) were collected from eight localities within north-eastern Algeria (Table 1).

Table 1. Localities and bat species collected

Localities	Coordinates		Bat Species collected
	E	N	
Benmhidi	36.77	7.87	*M. capaccinii*
El Chatt	36.83	7.82	*M. capaccini*
El Hadjar	36.80	7.74	*P. pipistrellus* and *P. kuhlii*
Grotte	36.92	8.52	*Rh. hipposideros* and *M. schreibersii*
Kehf Lagareb	36.90	8.54	*Rh. ferrumequinum* and *M. punicus*
Kehf Nasser	36.92	8.55	*P. kuhlii* and *H. savii*
Sidi Amar	36.75	7.70	*P. pipistrellus*
Sidi Kassi	36.75	7.97	*E. serotinus*

Ectoparasites

Eight species of ectoparasites belonging to four orders were recovered from nine bat species from north-eastern Algeria. Three species of bat flies (Insecta: Diptera: Streblidae) *Brachytarsina falvipinnis* and (Insecta: Diptera: Nycteribiidae) *Phthiridium biarticulatum* and *Penicillidia dufouri* were recovered. Order Hemiptera was represented by two species (*Cimex pipistrellus* and *Cimex lectularius*), while order Siphonaptera was represented by a single species (*Ischnopsyllus octactenus*). One ixodid tick (*Rhipicephalus sanguineus*) and one species of mesostigmatid mites (*Spinturnix myoti*) were collected (Table 2).

Class Insecta
Order Diptera
Family Streblidae

Brachytarsina flavipennis Macquart 1851
Material examined: ex. *Rh. ferrumequinum*, (N=22, NP=10), Kehf Lagareb, 10-11.VIII.2012.
Remarks: From Algeria, it was recovered from *M. schreibersii, R. mehelyi* and *Rh. hipposideros* (Anciaux de Faveaux, 1976) and *Myotis blythi* (Amr and Qumsiyeh, 1993). This is the only streblid known within the Mediterranean area associated with horseshow bats such as *Rh. blasi, Rh. ferrumequinum, Rh. hipposideros* and *Rh. mehelyi* from Cyprus, Jordan and Syria (Amr and Qumsiyeh, 1993; Walter and Ebenau, 1997; Ševèík *et al*., 2013).

Family: Nycteribiidae
Phthiridium biarticulatum Hermann, 1804
Material examined: ex. *P. kuhlii,* (N=, 42, NP=16), Kehf Nasser, 8.XI.2012; ex. *Rh. ferrumequinum,* (N=22, NP=7), Kehf Lagareb, 8.XII.2012; ex. *Rh. hipposideros,* (N=18, NP=13), Kehf Lagareb, 8.XII.2012; ex. *E. serotinus*, (N=26, NP=10), Sidi Kassi, 9.XII.2012; ex. *M. punicus,* (N=32, NP=19), Kehf Lagareb, 8.XII.2012.

Table 2. Summary for ectoparasites recovered from nine species of bats from north-eastern Algeria

Bat species	Ectoparasites recovered
Family Vespertillionidae	
E. serotinus (Schreber, 1774)	*Phthiridium biarticulatum*
H. savii (Bonaparte, 1837)	*Rhipicephalus sanguineus*
M. capaccinii (Bonaparte, 1837)	*Cimex lectularius*
	Rhipicephalus sanguineus
M. punicus Felten, 1977	*Phthiridium biarticulatum*
	Penicillidia dufouri
	Spinturnix myoti
	Rhipicephalus sanguineus
P. kuhlii (Kuhl, 1817)	*Phthiridium biarticulatum*
	Cimex pipistrelli
	Ischnopsyllus octactenus
P. pipistrellus (Schreber, 1774)	*Cimex pipistrelli*
	Ischnopsyllus octactenus
Family Rhinolophidae	
Rh. ferrumequinum (Schreber, 1774)	*Brachytarsina flavipennis*
	Phthiridium biarticulatum
	Penicillidia dufouri
Rh. hipposideros (Bechstein, 1800)	*Phthiridium biarticulatum*
	Cimex lectularius
Family Miniopteridae	
M. schreibersii (Kuhl, 1817)	*Rhipicephalus sanguineus*

Remarks: It was previously recovered from *M. blythi, M. mehelyi* and *M. schreibersii* from Algeria (Amr and Qumsiyeh, 1993). This bat fly is distributed within the circum-Mediterranean and associated with cave dwelling bats such as *Rh. ferrumequinum* (Ševèík *et al*., 2013).

Penicillidia dufouri (Westwood, 1825)
Material examined: ex. *Rh. ferrumequinum,* (N=22, NP=24), Kehf Lagareb, 9.XI. 2012 and 8.XII.2012; ex. *M. punicus***,** (N=32, NP=2), Kehf Lagareb, 8.XI.2012 and 8.XII.2012.
*Remarks***:** It was previously recorded from four Algerian bat species: *Myotis blythi* and *Rh. hipposideros* (Theodor, 1967) and from *M. blythi*, *M. schreibersii* and *Rh. euryale* (Amr and Qumsiyeh, 1993). This is a bat ectoparasite with a wide range of distribution extended from Europe, North Africa and Asia, reaching to India (Krištofik and Danko, 2012). It is usually associated with bats of the genus *Myotis*, however, it was recovered from a wide range of species including *M. schreibersii* and *Rh. euryale* (Hůrka, 1980). In the present study, *Rh. ferrumequinum* is an additional new host for this nycteribiid fly. It was found among 17 *Rh. ferrumequinum* individuals.

Order Hemiptera
Family Cimicidae
Cimex lectularius (Linnaeus, 1758)
Material examined: ex. *M. capaccinii,* (N=15, NP=10), Benmhidi (El-Tref), 6-7.XI.2012; ex. *Rh. hipposideros,* (N=18, NP=14), Grottem 7-8.XI.2012.
Remarks: C. lectularius was collected from maternity roosts of *M. myotis* (Morkel, 1999).
Cimex pipistrelli Jenyns, 1839

*Material examined***:** ex. *P. kuhlii,* (N=42, NP=4), El Hadjar, 7.XI.2012; ex. *P. pipistrellus*, (N=83, NP=18), Sidi Amar, 6-7.XI.2012.
Remarks: The species was reported mainly among bats of the genus *Pipistrellus* within the Palaearctic region (Balvín, 2008). It was recovered from other species in Europe including *Myotis myotis* and *Nyctalus noctula* (Krištofik and Danko, 2012) and from maternity roosts of *M. myotis* (Morkel, 1999).

Order Siphonaptera
Family Ischnopsyllidae
Ischnopsyllus octactenus (Kolenati, 1856)
*Material examined***:** ex. *P. kuhlii,* (N=42, NP=4), El Hadjar, 7.XI.2012; ex. *P. pipistrellus,* (N=83, NP=16), El Hadjar, 7.XI.2012.
Remarks: This is a western Palaearctic species with distribution extending from Morocco, Spain and Great Britain to Middle Asia (Hůrka, 1997). This flea was reported from *P. kuhlii* in Tunisia (Beaucournu and Kock, 1996). It is mostly associated with bats of the genus *Pipistrellus* (Beaucournu and Launay, 1990).

Class Arachnida
Order Mesostigmata
Family Spinturnicidae
Spinturnix myoti (Kolenati, 1856)
*Material examined***:** ex. *M. punicus,* (N=32, NP=55), Kehf Lagareb, 8.XI.2012 and 8.XII.2012.
Remarks: This bat mite has a wide range of distribution through Europe, North Africa and Asia (Krištofik and Danko, 2012). It is mostly associated with bats of the genus *Myotis*, but reported from other genera as well (Stanyukovich, 1997).

Order Ixodida
Family Ixodidae
Rhipicephalus sanguineus (Latreille, 1806)
*Material examined***:** ex. *M. capaccinii*, Benmhidi, 6-7.XII.2012; ex. *M. punicus,* Kehf Lagareb, 8-19.XII.2012; ex. *M. schreibersii*, Grotte, 7-8.XII.2012; ex. *H. savii,* Kehf Nasser, 8.XI.2012 and 8.XII.2012.
Remarks: Other ixodid ticks were reported from a variety of bats such as *Ixodes simplex* and *Ixodes vespertilionis* (Krištofik and Danko, 2012). *Ixodes vespertilionis* are the most common known ectoparasites of bats in Europe (Beaucournu, 1961). Most of the recovered ticks were females, and about 20% were nymphs.

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Evaluation of Immunomodulatory Effects of Antiepileptic Drug Phenytoin

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Abstract

The immunomodulatory effects of phenytoin (PHN), used as an antiepileptic agent were investigated *in vivo* using female Balb/c mice. The main aim of this study was to investigate the immunotoxicity of PHN. Animals were divided into six groups, eight animals per group. Group I, served as a control group, received only the vehicle. Groups (II-VI) were received a daily intraperitoneal dose of PHN (2.5, 5, 10, 15 and 25 mg/kg, respectively) over a period of 21 days. PHN has shown a significant decrease in the animal body weight. The relative weights of animal's spleens were also decreased significantly at doses of (10, 15, and 25 mg/kg). PHN showed a significant decrease in the percentage of circulating neutrophils and lymphocytes and an increase in the percentage of circulating eosinophils. The result showed a marked suppression in antibody production capacity as a humoral immune response and a suppression in the Delayed type hypersensitivity response as a cell mediated immune response in PHN-treated mice compared to the control group. Detectable changes have also been noticed in the histology of the footpad tissue and spleen.

Key words: Phenytoin, Immunotoxicity, Balb/c mice, DTH, Humoral Immune Response, Hemagglutination Titer Assay.

1. Introduction

The Immune system is a collection of organs, cells and tissues that work together to protect our body from potentially harmful infectious agents and certain tumor cells (Ponce *et al*., 2009). Immunotoxicology is an important portion of the safety evaluation of drugs and chemicals (Descotes, 2006). Immunotoxicology studies focuses on the modulation of the immune system following exposure to environmental chemicals and drugs. The modulation may include immunosuppression (non- specific), immunostimulation, hypersensitivity, or autoimmunity (Veraldi, 2006; Descotes, 2005). There is increasing evidence that many toxic effects on the immune system components and their functions takes place as a result of drug treatments or chemical exposure (Rooney *et al.*, 2008). Therefore, it is very important to identify and evaluate the potential effects of chemical compounds that produce immunotoxicity during the processes of drug development (Spanhaak, 2006). Determining mechanism of immunotoxicity is an important issue in understanding the clinical relevance of the observed adverse effects. For instance, changes in blood cellular elements could suggest immunosuppression (Schulte, 2006).

Phenytoin (PHN) is an antiepileptic drug which can be useful in the treatment of epilepsy .This drug acts to suppress abnormal brain activity that has been seen in seizure by reducing electrical conductance among brain cells by stabilizing the inactive state of voltage-gated sodium channel, PHN known as (5,5-diphenylimid-azolidine-2,4-dione).Molecular formula: $C_{15}H_{12}N_2O_2$. It is white crystalline powder or granule with melting point 296 c^o, insoluble in water, while it is soluble in ethanol, acetone and ether (Bernaskova *et al*., 2010).

The usual human therapeutic dosage of PHN is (1 g) orally divided in 3 doses (400 mg, 300 mg, 300 mg) given at 2 hour intervals (Ratanakorn *et al*., 1997). Due to risk of serious side effects of PHN, it must be titrated gradually over several weeks to reach this target dose range (Keppel, 1998) PHN is associated with adverse side effects, depending on dosage , side effects occurred in 1/3 of the patients, especially those whose serum concentration was more than 20µg/ml (Beier *et al.*, 1978), increasing with higher serum concentrations and in combination therapy, including diplopia, nystagmus. Allergic skin rash, change in blood counts such as leukopenia , and impairment of hepatic function are seen and may return to normal after dose reduction (Walia, 2004). In some cases, serious allergic reaction, such as

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skin inflammation with large area flaking (exfoliative dermatitis) (Pelekanos *et al.,* 1991). Hematological side effects of antiepileptic drugs occur infrequently but remain a potential cause of severe toxicity. It is recommended that patients receiving PHN are necessary to have a complete blood count (CBC) every two weeks. According to the international monitoring guidelines, total white blood cells count (WBCs) and differential WBC count must be performed, with PHN treatment.Other hematological effects of PHN include eosinophilia also has been reported in 34% of PHN treated patients (Yang *et al.*, 2011; Bjornsson *et al.,* 2007; and Fararjeh *et al*., 2008). The aim of the present study was to investigate the toxic effect of PHN on immune system cells and tissues in mice by assessing humoral and cell mediated immunity. Routine hematological parameters, screening for changes in organ and body weight**,** hemagglutination titer (HA), and delayed type hypersensitivity (DTH) in PHN-treated animal groups were tested. Histological examination of the spleen and inflamed tissue was also evaluated for each animal (Putman *et al*., 2003).

2. Materials and Methods

2.1. Animals

Adult female balb/c mice (6-8 weeks old; 18-20 g weight) were obtained from The Hashimate University animal house, Zarqa', Jordan. Animals were housed in plastic cages containing saw-dust bedding and adapted for 1 week in the lab prior to usage. The animal's room was maintained at a temperature of (23 ± 2 °C) with relative humidity (50 ± 20 %) under a 12 hour light/dark cycle (lights on at 07:00). All animals were maintained at standard laboratory food and tap water ad libitum. Animals were cared for in accordance with the guide to the care and use of experimental animals. All procedures involving animals were reviewed and approved by the institutional review board (IRB) issued by The Hashemite University.

2.2. Chemicals

One hundred milligram of phenytoin sodium (Goedecke AG/Germany) was purchased from local drug store (Jordan). Phosphate buffered saline (PBS), RBC lysing buffer, Sheep- RBCs (SRBC) and RPMI-1640 medium were purchased from Sigma (USA).

2.3. Phenytion Administration

Phenytoin (One hundred mg) was dissolved in 8 ml of absolute ethanol (12.5 mg/ml). PHN was made up in a concentrated stock solution and diluted to an appropriate dilution with PBS for intraperitoneal (i.p.) injection each day of experiment (Tomson *et al*., 2007; Wyllie *et al*., 1991).

2.4. Dose and Exposure Schedules

Mice were randomly divided into six groups (I-VI), each of 8 animals. Group I (control group) received Phosphate buffer saline (PBS) and the same percentage of drug solvent (ethanol). Groups II–VI (treatment groups) received the corresponding doses of PHN. Animals were treated with PHN in PBS intraperitoneally for 21 consecutive days. Phenytion was administered at a volume of 10 ml/kg. The drug doses were 0.5, 1.0, 2.0, 3.0 and 5.0 X times of the human therapeutic dose, and were equal to 2.5, 5, 10, 15, and 25 mg/kg respectively. Mice were sacrificed by cervical dislocation 4 hours after the administration of the last drug dose.

2.5. Organ and Body Weight Ratio

The weights of animals were recorded at the beginning of the study and 4 hours after the last day of treatment (day twenty one). On the day of experiment, animals were sacrificed by cervical dislocation and body organs such as spleen, liver, kidneys and thymus were removed, and weighed. Connective and adipose tissue were removed from these organs before weighing. The relative weight of the organs of each mouse was calculated as organ weight (mg)/ body weight (g).

2.6. Determination of the Hematological Parameters

Blood was collected from the retro-orbital plexus of each mouse before being sacrificed using heparinized capillary tubes. 0.2 ml of blood were collected in a sterile (K_2-EDTA) anticoagulated tubes (Minicollect®, Impromini , China) so that the blood to anticoagulant ratio was 1:0.075 v/v. Routine hematological parameters were assessed including hemoglobin content, packed cell volume percent (PCV%), red blood cell (RBC) count, and WBC count. Blood films were prepared and stained with Gimsa stain for each treated animal for differential WBCs counts, slides were observed under light microscope (Nikon, China). Blood samples analyses were confirmed by Mindray BC 2800 he matology analyzer (Jiangsu, China) at Hamdan institution for medical equipment.

2.6.1. Serum Antibody Titer: Hemagglutination (HA) Titer Assay

Seven days before ending the treatments, mice were immunized by i.p injection of 10^8/50 µl Sheep-RBCs (SRBCs) in PBS (Lee, 2004). Phenytoin treatments were continued up to 21 days. At the end of experimentation (day 21), sera were prepared from peripheral blood samples, then 25 µl of twofold diluted sera in PBS were placed in the wells of a U-shape 96-microtiter plates. All samples were challenged with 25 µl of 5% v/v SRBCs suspension and mixed according to (Fararjeh *et al*., 2008). The plates were incubated at 37 °C for 1 h. Hemagglutination was then observed according to (Riahi *et al.,* 2010).

2.7. Delayed-type Hypersensitivity Response (DTH)

Delayed-type hypersensitivity response (DTH) was determined for all groups using a modified method of Bin-hafeez *et al.,* 2003. On the 14th day of the treatment, animals were immunized with 10^8/50 µl SRBCs. After seven days of immunization (day twenty of the experiment), all animals were again challenged with a booster dose of 10^8/50 µl SRBCs in the right hind footpad according to (Bin-hafeez *et al.,* 2003; and Fararjeh *et al*., 2008). The left hind footpad was injected with an equal volume of PBS to serve as trauma control for nonspecific swelling. Increased volume of footpad was measured 24 h after the last challenge with SRBCs using digital plethysmometer LE 7500 (Harvard, UK) and the differences between right and left hind footpad volumes were calculated (Dietert and Holsapple, 2007).

2.8. Histological Examination

The collected tissues of right footpad and spleen of each treatment group of mice were fixed in 10% formalin and sectioned using Shandon rotary microtome (Egenolf, 2011). Various sections (4-5 µm thickness) were prepared and stained with Hematoxylin–Eosin (H–E). Histological changes in these organs were examined under light microscope (Nikon, china) by an experienced pathologist and scored according to the degrees of changes in cellular infiltration and architectural distortion as described by (Kugelberg *et al*., 2005; and Fararjeh *et al.*, 2008).

Scoring for the presence of the mononuclear and polymorphonuclear leukocytes in the dermal layer was considered as follow; 0, neither mononuclear nor polymorphonuclear leukocytes were present; 1, (1-3 cells per field); 2, (5-7 cells per field); and 3, abundant occurrence of mononuclear and polymorphonuclear leukocytes (more than 7 cells per field) (Kugelberg *et al.,* 2005). The presence of the mononuclear and polymorphonuclear leukocytes in the muscular layer was also scored in a similar fashion. Histological changes in spleen were scored according to the white pulp, red pulp, and trabecular changes. Negative sign (-) indicates no changes observed; +, minimal changes; and ++, readily detectable changes.

2.9. Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM) of eight independent experiments. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison post-test using Graphpad Prism version 5 software package. In the figures, asterisks represent a statistically significant, increase or decrease, compared to control, where * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

3. Results

3.1. Effect of PHN on Organ and Body Weight

At the beginning of the study, all animals have almost comparable body weights since none of the groups showed any significant difference between groups as calculated by multiple comparison test. None of the used doses of PHN caused mortality in the treated animal groups. Animals treated with PHN showed a significant decrease in their body weight at the highest used doses (15 and 25 mg/kg) when compared to the control group . However, neither the subtherapeutic nor therapeutic doses of PHN (2.5, 5 and 10 mg/kg, respectively) showed any significant difference to the control group .On the other hand, PHN at the doses of (10, 15 and 25 mg/kg) caused a decrease in the relative (% body weight) of spleen .However, only (15 and 25 mg/kg) PHN doses showed a significant decrease in relative weight of thymus .No effects were observed in liver and kidney relative weights at any given doses when compared to the control group (Table 1) .

Table 1. Effects of PHN on Red blood cells (RBCs) parameters of different treatment groups of Balb /c mice females.

PHN (mg/kg)	RBCs parameters		
	RBCs count ($x10^6/mm^3$)	PCV (%)	Hemoglobin (g/dl)
Control	8.70±1.28	45.99±3.82	14.66±1.27
2.5	8.69±1.19	43.54±6.66	14.51±2.22
5	8.56±0.89	47.55±4.35	15.85±1.45
10	8.67±0.93	45.00±5.33	15.00±1.77
15	7.05±1.60	44.63±4.43	14.88±1.47
25	7.03±1.53*	45.64±5.99	15.21±1.99

Data are means± SEM of eight animals. (PHN; phenytoin, PCV; packed cell volume, RBC; Red blood cells). * $P < 0.05$ when compared to control.

3.2. Effect of PHN on Hematological Parameters

Hematological tests of the peripheral blood revealed that mice treated with PHN caused a significant decrease in erythrocytes count at the highest dose 25 mg/kg (Table 1). Also, PHN caused a significant decrease in leukocytes count at the doses (15, 25 mg/kg), while doses (2.5, 5, 10 mg/kg) of PHN did not show any significant change compared to the control group (Table 2). Moreover, PHN caused a s ignificant decrease in the percentage of circulating peripheral blood neutrophils and lymphocytes at 15, 25 mg/kg doses. However, there is no significant difference in the percentage of peripheral blood leukocytes among the lower three doses. Interestingly as shown in Table 2, PHN caused a significant dose dependent increase in the percentage of eosinophils. A significant increase was also found between 5, 10, 15 and 25 mg/kg doses. While none of the PHN doses used appears to have any effect on other hematological parameters (Table 1 and Table 2).

Table 2. Effects of PHN on White blood cells (WBCs) parameters of different treatment groups of female Balb/c mice

PHN (mg/kg)	WBCs parameters				
	WBCs count ($x10^3/mm^3$)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)
Control	7.21± 0.97	23.33± 2.54	65.81± 2.86	10.09± 1.69	0.75± 0.71
2.5	7.22± 1.99	22.48± 5.71	62.16± 9.71	9.34± 1.85	1.25± 0.46
5	5.89± 1.17	22.44± 2.70	61.9± 14.06	9.66± 0.79	2.5± 1.19*
10	5.94± 1.21	22.69± 3.17	56.13± 17.41	10.15± 1.48	2.38± 1.51*
15	5.03± 1.41**	19.21± 2.17*	43.3± 12.9**	10.18± 1.83	3.5± 0.9***
25	3.7± 0.9***	16.4± 2.2***	41.8± 8.8**	8.99± 1.66	4.1± 1.3***

Data are means± SEM of eight animals. (PHN; phenytoin, WBC; white blood cells).* $p< 0.05$ when compared to control, **$p< 0.01$ when compared to control,*** $p< 0.001$ when compared to control.

3.3. Effect of PHN on Serum Antibody titer: Hemagglutination (HA) titer

Hemagglutination titer at the doses of (15, and 25 mg/kg) showed significant inhibition ($p<0.001$) in the concentration of the anti-SRBCs antibodies expressed as

antibody titer when compared with the control group (Figure 1).

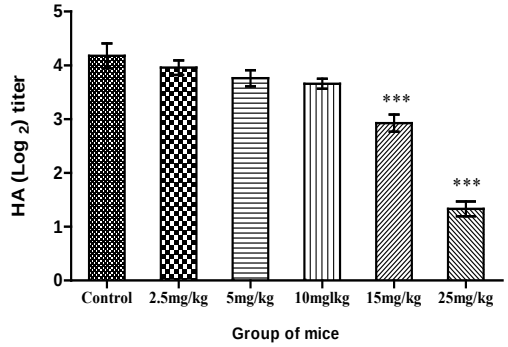

Figure 1. Effect of PHN on the anti-SRBCs antibodies production assessed by the hemagglutination $\log_2$ titer assay. Data are means ± SEM (n=8). ***p <0.001 when compared to control animals

3.4. Effect of PHN on Delayed- type Hypersensitivity Response (DTH)

The subtherapeutic dose 2.5mg/kg and the therapeutic dose 5 mg/kg PHN did not show any significant change on DTH response compared to the control group. However, a significant suppression of the DTH response was determined after a s ubsequent injection of antigen (SRBCs) at 10 mg/kg dose or more (Figure 2).

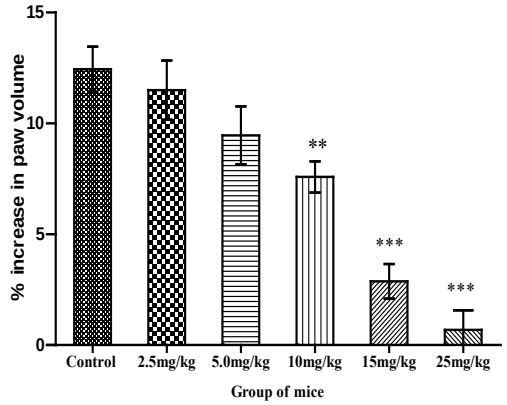

Figure 2. Effect of PHN on delayed-type hypersensitivity (DTH) measuredas the difference between SRBC- injected and PBS-injected hind footpad volumes. Mice received daily i.p injection of the indicated doses of PHN for 21 days. Data are means ±SEM of eight animals. **p < 0.01 and ***p <0.001 when compared to control animals.

3.5. Effect of PHN on Histology of Footpad Tissue

Microscopic examination of the footpad tissues showed an inflammatory infiltrate of leukocytes (polymorph-onuclear and mononuclear leukocytes) after being challenged with SRBCs, in both hypodermis and muscle layers.

Treatment with PHN caused a significant decrease in the inflammatory infiltrate at the doses of 15 and 25mg/kg . No effects were observed in other inflammatory parameters at all doses of PHN (Table 3 and Figure 3).

Table 3. Effects of PHN treatment on right footpad tissue in mice receiving (2.5, 5, 10, 15 and 25 mg/kg), as daily i.p. injection for 21days.

Inflamed tissue	Inflammation score (Presence of mononuclearand polymorphonuclear Leukocytes)	
Treatment mg/kg	Dermal layer	Muscular layer
Control	2.12±0.22	1.37±0.26
2.5	2.12±0.29	1.37± 0.18
5	1.62±0.26	1.25±0.16
10	1.62±0.26	1.0±0.32
15	1.12±0.22*	0.87±0.22
25	0.5±0.18***	0.5±0.18*

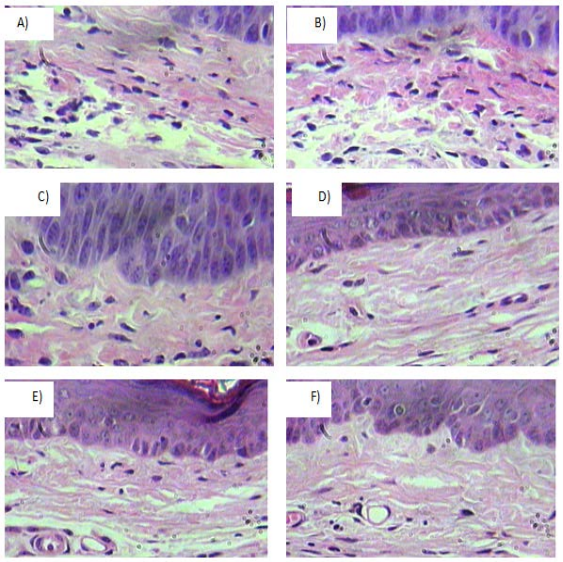

Figure 3. Inflamed footpad representative tissue sections of control and PHN-treated animals stained with hematoxylin eosin shown at high dry power field (40 x). (A); right footpad tissue of control group showing infiltration of polymorphonuclear and mononuclear leukocytes after SRBCs challenge. (B-F); PHN treated groups (2.5, 5, 10, 15 and 25 mg/kg, respectively) showing a decrease in the inflammatory infiltration of polymorphonuclear and mononuclear leukocytes.

3.6. Effect of PHN on Histology of Spleen

Microscopic examination of spleen showed a decrease in the density (atrophy) of the spleen white pulps at doses of 15 a nd 25 mg/kg (Figure 4). No other significant changes in other spleen histological parameters were seen at any used doses of PHN.

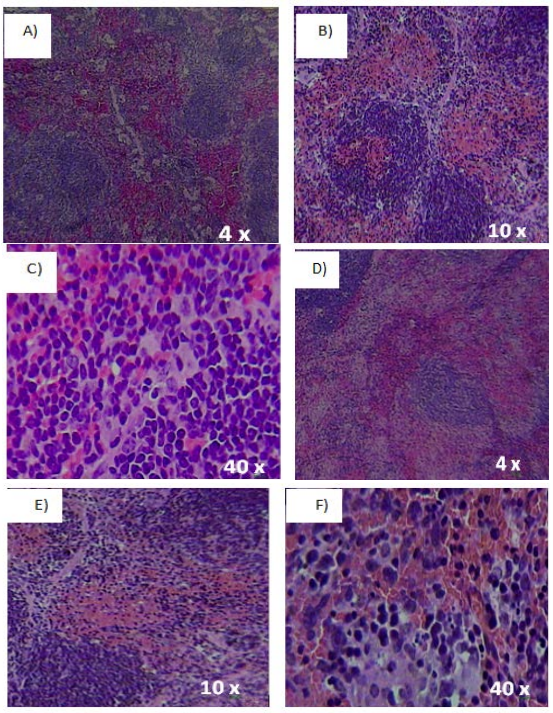

Figure 4. Spleen sections of control and PHN-treated animals stained with hematoxylin- eosin shown at different magnifications. (A-C) normal spleen (control); (D-F); 25 mg/kg PHN- treated animal showing white pulp atrophy.

4. Discussion

In the current study, the unhealthy effect of PHN has been confirmed by the significant decrease in body weights measured in PHN-treated mice at doses of 15 and 25 mg/kg. In the present study, mice treated with PHN have low food consumption. PHN alter one of the fundamental processes of the human body weight regulation, by altering appetite, metabolism, or absorption of calories. It has been noticed that mice treated with PHN exhibited mild desire for sleep which might indicate a reduction in food intake which results in body weight loss. Moreover, PHN (15, 25 mg/kg) treated mice showed a remarkable signs of behavioral abnormalities manifested by fainting and drowsiness (not calculated). These behavioral abnormalities were associated with reduction in body weights. This effect is consistent with the study of Ben-Menachem (2007) that has reported similar effect suggesting body weight loss.

Phenytoin caused a significant decrease in the relative weight of spleen at doses of (10, 15 and 25 mg/kg), this effect was confirmed by the observation of induced atrophy of the white pulp of spleen in a similar fashion. The spleen is a major lymphoid organ that plays a critical role in the primary humoral and secondary immune response. B lymphocytes partially mature in the bone marrow enter the circulation, and then populate peripheral lymphoid organs, including spleen and lymph nodes, were they complete their maturation process. Spleen white pulp plays also an important part in the initiation of immune responses by B cells to foreign antigens in the blood. This might be the reason behind the relative lymphocytopenia found in peripheral blood of treated mice.

In addition, spleen contains a large number of tissue resident leukocytes, therefore white pulp atrophy will reflect a reduction in the percentage of total leukocytes in the peripheral blood.

It has been shown that the PHN caused a significant decrease in the Red Blood Cells (RBCs) at the highest used dose (25mg/kg) which may be due to suppression of the bone marrow, that's why PHN may reduce the production of erythropoietin (EPO) hormone, thus the erythropoiesis process which is stimulated by EPO can be also affected by PHN. Likewise, PHN caused a significant decreased in the total leukocytes counts and in the percentage of neutrophils and lymphocytes. Leucopenia, neutropenia, and lymphocytopenia may reflect the toxicity or immunomodulatory effects of PHN in treated group of mice. These findings were consistent with the common occurrence of leukopenia in human model by a cohort study which has been conducted by (Blackburn, 1998).

A significant dose dependent increase in the percentage of eosinophils have been demonstrated in PHN treated mice at doses equal to or more than 5 mg/kg which is correspondent to the human therapeutic dose and this was in concordance with the study that has reported similar effect suggesting elevation of IgE with eosinophilia (Chen *et al.,* 2010). The decrease in humoral immunity response to PHN was manifested by suppression of antibodies production, tested as anti-SRBCs antibodies titer which shows a suppression of antibodies at treated doses of 15 a nd 25 mg/kg. White pulp atrophy, and spleen relative weight decrease observed in this study is a common finding following the administration of certain immunosuppressive drugs, and are accompanied by the decreased ability of the animal immune system to produce antibody. Suppression of DTH reaction by PHN could be not only due to the reduction in the availability of factors essential responsible for the maintenance of T cell proliferation, but also for the recruitment and activation of macrophages. Reduction in DTH may be due to any block in the antigen of SRBCs processing or presentation of these antigens by macrophages for T lymphocytes. The exact mechanism whereby PHN inhibits T cell activation is not known yet. However, it could take place via blocking of antigen specific receptors or acquisition of responsiveness to interleukins. Suppression of the delayed type hypersensitivity response has been demonstrated by PHN in this study at doses of 10, 15, and 25 mg/kg and these results were accompanied by a study by Dietert *et al.* (2010), showing that the inhibitory effects of PHN on cell mediated immune response in animal.

Moreover, DTH responses measured in the skin of the mice footpad have been used to assess cell mediated immunity in vivo. The loss of DTH reaction serves as an indicator of deteriorating cell-mediated immune function. A decreased in the infiltration of the mononuclear and polymorphonuclear leukocytes in the mouse footpad tissue, which has been demonstrated in histological examination at 15 and 25 mg/kg doses of PHN was accompanied by suppression in DTH response. These results might explain the inhibitory effect of PHN on the cell mediated immune responses.

In conclusion, the current study shows that Phenytoin had an inhibitory effect on the innate, adaptive and cell mediated as well as humoral immune response at human subtherapeutic, therapeutic and high doses. These results demonstrate an immunosuppression effect of PHN in mice. The effectiveness of PHN in the treatment of schizophrenia and other related diseases should be counterbalanced by its immunotoxicity. Further research studies should be considered to study the significance of using PHN in patients under long-term treatment. As well, studies on human models should be required to study the toxic effect of PHN on the immune system.

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Short Communication

Prevalence of Lactose Intolerance in Primary School Children in Qena Governorate, Egypt

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Abstract

Most people are born with the ability to digest lactose. Approximately 75% of the general population loses this ability, to some extent, going into adulthood (adult hypolactasia), while others retain such ability. The aim of the present study was to determine the prevalence of lactose intolerance, and its correlation with gastrointestinal symptoms, in primary school children aged 6-12 years in Qena Governorate, Egypt. A cross-sectional study was carried out on 300 school children with clinical suspicion of lactose intolerance. Biological and clinical data were obtained from children's parents or guardians. The history of diarrheal attacks especially following ingestion of milk or dairy products, as well as the incidence of diabetes in the children or family history of such diseases was also obtained. The children were instructed to maintain a low fiber diet without lactose for 48 hours prior to the day of examination. After 12 hours of fasting lactose tolerance test was carried out. The data obtained revealed that 74% of the participants in the study were intolerant to lactose. However, only 56.8% of lactose-intolerant children had positive clinical history of abdominal pain, abdominal distension or diarrheal attacks following ingestion of milk or dairy products. The prevalence of lactose intolerance in the studied cohort increased with age. Such genetically determined intolerance was 58% at 6-7 years of age and increased to 90% by the age of 11-12.

Keywords: Lactose Intolerance, Adult Hypolactasia, Qena Governorate, Cross-Sectional Study, Egypt

1. Introduction

Lactose is a d isaccharide that is abundant in mammalian milk. It is hydrolyzed into glucose and galactose by the enzyme lactase, which is located in the brush border (microvilli) of the small intestine. In most infants, intestinal lactase activity is maximal during the perinatal period. However, after 2-12 years of age two distinct groups emerge: those with low lactase activity (hypolactasia) or a "lactase non-persistence" group, and a "lactase persistence" group of individuals who retain their neonatal level of lactase activity into adulthood (Mattar *et al*., 2012; Genauer *et al*., 2010; NDDIC, 2009).

Lactose intolerance is generally a l ifelong inherited condition, but can be a temporary result of infection or some other insult to the jejuna mucosa. Lactose malabsorption occurs in three main types: primary, secondary and congenital. The most common form is primary adult hypolactasia. Secondary or acquired hypolactasia, can result from any gastrointestinal illness that damages the brush border or significantly increases transit time in the jejunum (Swagerty *et al.,* 2002). Lifelong complete absence of lactase [congenital hypolactasia] is rare. Recognition of these conditions is important, as its gastrointestinal symptoms are easily managed by simple dietary adjustments (Swagerty *et al*., 2002; Matter *et al*., 2012). Searching available literature did not reveal any previous studies regarding the prevalence of lactose intolerance in Upper Egypt. The Ethics Committee of Assiut University; approved the protocol of the present study and all experimental procedures were in accordance with the Helsinki Declaration of 1975, as revised in 1983. The present cross-sectional study was undertaken to determine the prevalence of lactose intolerance among primary school children in Qena governorate and the extent of cooperation of the community in such work. It was felt that determining the extent of this problem is important, as part of an overall study to categorize the different types of diarrheal diseases, which constitute serious morbid conditions in young school children in the less privileged communities.

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2. Subjects and Methods

A cohort of 300 children aged 6-12 years, including 153 males and 147 females in different primary schools in Qena governorate was recruited to participate in the study. Exclusion criteria included children with known chronic illnesses or family history of such diseases like diabetes, children with overnight fasting blood glucose level >126 mg/dl, those with any intestinal or allergic disorders or those with history of recent gastroenteritis to avoid causes of secondary hypolactasia.

The aim of the study was explained to the parents or guardians and written consents were obtained. Biological and clinical data were obtained through a written protocol including name, gender, age, weight, history of diarrheal attacks, especially following ingestion of milk or dairy products as well as chronic diseases or family history of such diseases, including diabetes mellitus.

Diagnosis of lactose intolerance depended mainly on family history and blood glucose level following ingestion of standard doses of lactose (Swagerty *et al*., 2002; Law *et al*., 2010). For performing the lactose tolerance test, the parents were instructed to maintain the children on a low fiber diet without lactose for 48 hours prior to examination. After 12 hours fasting, a baseline concentration of blood glucose was measured using portable glucometer (Medi Smart of Switzerland-Brilliant). Subjects then ingested an oral load of lactose of 2 g/kg body weight, with a maximum of 50 g, as 20% aqueous solution. Blood glucose level was measured again after 2 hours. The test was considered positive when intestinal symptoms occurred and the increase in blood glucose level was less than 20 mg/dl above the fasting level (Joneja, 2003; Law *et al*., 2010).

2.1. Statistical Analysis

The data were subjected to statistical analysis and tabulation using SPSS program Version 10. Means and standard deviations were used to describe numeric variables. The variables of categorical types were given as a number and percentages. Comparisons of continuous variables were performed using the Student-t test for independent samples.

Differences were considered significant when $p<0.05$. Comparisons for categorical variables were carried out using the $\chi 2$ test value calculated using the Epi Info 2000 Program. Results were presented to fulfil the objectives of the study.

3. Results

There was equal sex distribution in the studied cohort, where 51% were males and 49% were females. The measured body weights of the children were used to calculate the oral lactose load. Biological and clinical history data are presented in Table 1. The overall results showed that 222 children were lactose intolerant, representing 74% of the studied group. There were no statistically significant differences between the lactose tolerant and intolerant children with regard to age and gender. However, a positive correlation could be detected between lactose intolerance and history of gastrointestinal symptoms of abdominal pain and distension or diarrhea following ingestion of dairy products. The concentrations of fasting blood glucose and the two hours postprandial glucose level in the different age groups are presented in Table 2. It was found that the prevalence of lactose intolerance steadily increased with age from 58% in children aged 6-7 years up to 90% in the 11-12 year group (Figure 1).

The results also indicated that only 56.8% of lactose intolerant children had positive clinical history of gastrointestinal symptoms, following ingestion of milk or other dairy products, while such symptoms were negative in the remaining 43.2%. The prevalence of the positive symptoms in the children in different age groups is presented in Table 1.

Table 1. Body weight ranges and history of symptoms in the studied children.

Age Group (years)	Male/Female Ratio	Body Weight Range (Kg)	Positive History of G.I Symptoms
6-7	27/23	20-25	54%
7-8	23/27	20-35	30%
8-9	26/24	20-35	48%
9-10	26/24	20-50	56%
10-11	27/23	20-50	62%
11-12	24/26	31-over 50	56%
Overall Results	153/147	_____	74%

Table 2. Concentrations of fasting blood glucose and two-hours postprandial in Lactose intolerant and Lactose tolerant children.

Age Group (years)	Lactose Intolerant Children		Lactose Tolerant Children	
	Fasting Blood Glucose mg/dl	Two-hours postprandial mg/dl	Fasting Blood Glucose mg/dl	Two-hours postprandial mg/dl
6-7	93.3 ± 7.77	91.3 ± 8.33	92.9 ± 9.28	139.3 ± 18.73*
7-8	93.0 ± 7.96	90.7 ± 8.17	90.6 ± 9.27	137.1 ± 13.79*
8-9	93.6 ± 8.39	94.8 ± 7.55	88.7 ± 10.54	130.0 ± 10.76*
9-10	94.1 ± 7.50	93.1 ± 7.75	93.0 ± 9.21	133.9 ± 6.25*
10-11	92.6 ± 7.28	92.0 ± 7.66	83.75 ± 6.84	126.5 ± 8.88*
11-12	92.8 ± 8.14	94.6 ± 7.60	82.40 ± 4.92	123.8 ± 10.91*

Data presented as Mean ± Standard Deviation (Mean ± SD)
*Significantly different from the fasting blood glucose level

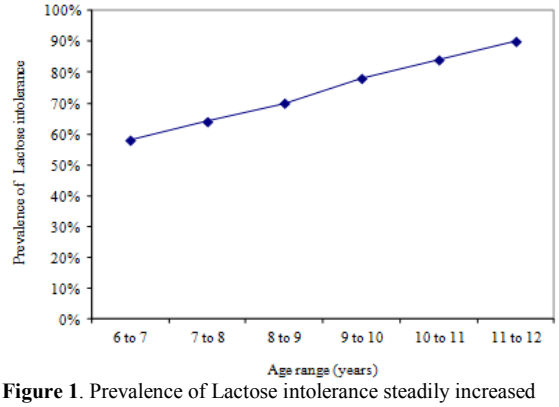

Figure 1. Prevalence of Lactose intolerance steadily increased with age

4. Discussion

Lactose intolerance, also called lactase deficiency or hypolactasia is the inability to digest lactose, a disaccharide found in milk and to a lesser extent in milk-derived dairy products. It is estimated that 75% of adults worldwide show some decrease in lactase activity during adulthood (Mattar *et al*., 2012, Morales *et al*., 2011, Swagerty *et al*., 2002). Such prevalence is very close to the overall result obtained in the present work, where 74% of the studied cohort was found to be lactose intolerant. The observation in the present study that the incidence of lactose intolerance increases with age is important since many published reports treated the studied population as a single unit and paid incomplete attention to age specific considerations (Tursi, 2004).

Lactose intolerance is a slowly progressive decline in available activity of the enzyme lactase. Such activity can be influenced by intestinal transit time and/or the additional foods concomitantly consumed with lactose (Morales *et al*., 2011; Hollox, 2005). In this respect, it should be recognized that lactose malabsorption is neither a homogeneous event nor an all-or-none phenomenon having its origins in a single etiology (Swallow, 2003). The appearance of GI symptoms depends on the amount of ingested milk and the degree of lactase deficiency. In case of partial lactase deficiency the GI symptoms appear only on ingestion of large amounts of milk or milk products (Heyman, 2006), this can be explained on the basis that the prevalence of GI symptoms depends on the amount of ingested milk and the degree of lactase deficiency, as they may have partial lactase deficiency so GI symptoms appear only on ingestion of large amounts of milk or dairy products statuses (Semenza *et al*., 2000; Troelsen, 2005; Heyman, 2006). Children should be investigated for other causes for the appearance of the GI symptoms, like for example milk allergy (Shaw & Davies, 1999; Labayen *et al*., 2001). The lack of correlation with gender probably indicates that if the clinical condition is genetically determined, it is not linked to the sex chromosomes. A genotyping study should be carried out to determine the variant that confers lactose tolerance in the studied population, which was beyond the scope of the present work.

5. Conclusion

The Prevalence of lactose intolerance in primary school children in Qena governorate is progressively increased with age. Not all lactose intolerant children have positive clinical history of abdominal pain and\ or abdominal distension and or diarrheal attacks following ingestion of milk or milk products as this related to many factors mainly, the amount of lactose ingested and the degree of activity of lactase enzyme. Symptoms usually disappear when you remove milk products or other lactose containing products from the diet.

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