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FORWARD

Dear Collegues,

I used to start my message by the achievements we try always to do and by the idea that was burn to put between your hands our journal – IJST. Today, I write you about how our journal continues for ten years without stop, despite the challenges we faced, and despite all constraints that our beloved Arab countries have while they are looking for more development achievements. What I want to say, is that the only weapon, as well as the tool to proceed to the gate of development is science and how we can use and adopt all the ways that make our cultures, our thoughts and our talents and research efforts are converted into practices to improve life for us and for the coming generations and let the other parts of the world listen to us very appreciately.

Let me present my deepest thanking and great recognitions for all people and institutes who faithfully gave IJST their concerns, their cares, and their patiences to keep it as one of the leading journals in Arab and international worlds.

Thanks a lot for Prof. Jamal Abbas and Dr. Abdullah Al- Shebani from University of Kufa, Dr. Atheer Al- Douri , Prof. Hazim Al- Daraji from University of Baghdad, Prof. Waleed Al- Murrani for his endless support from Plymouth University, Prof. Abdulbari Abbas Al- Faris from University of Basrah, and finally to the one who stands always behind this great effort and performs her best with no disperence, non stopping, and with full of faith, loyalty and creative footprints at IJST, the Editorial Board Secretary of IJST. With you all, IJST is now here, and will continue as long as we breath, as we believe on our goal, and as we have the power from God to be with you.

IJST was a fruitful effort issued by the International Centre for Sciences and Technology – ICAST, which tries to take part in both globalization and revolution in information and communication technologies, because S&T development becoming not only the key elements of economic growth and industrial competitiveness, but also essential for improving the social development, the quality of life and global environment. ICAST took then a decision to establish a scientific alliance with TSTC (Tharwa for scientific Training & Consultations) and this alliance comes to support the efforts towards publishing IJST.

Today, we announce a new issue of our journal, that is the third issue from the tenth volume of IJST, Septemebr , 2015.

Finally, I hope that all significant figures of sciences whom joined the editorial board, the researchers, and the readers of our journal will keep IJST between their eyes and contribute in continuing its journey, with their remarks, valuable recommendations and their researching outcomes.

Thanks a lot for all who support IJST.

Editor-in-Chief

IJST

Abdul Jabbar Al- Shammari

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TABLE OF CONTENTS

** Articles in this issue are listed below according to field specialties order, starting by English section and followed by Arabic section.*

(I) ENGLISH SECTION

Continue>>>> English section

VETERINARY MEDICINE

ARABIC STUDIES AND RESEARCHES SECTION – قسم الدراسات والبحوث العربية (II)

ENGLISH SECTION

Investigating the effects of UV radiation and tissue culture techniques on anti-amylase inhibitor activity extracted from white kidney bean (*Phaseolus vulgaris* L.)

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ABSTRACT

Phaseolus vulgaris seeds were irradiated with UV-A, UV-B and UV-C radiation, and then grown in incubator for 8 days at 25±°C. Hypocotyls of newly germinated embryos were cultured on an MS medium containing 0.5mg/l BA and 1.5mg/l 2,4-D. Callus pieces from both UV treated and non-treated explants were lyophilized then α-amylase inhibitors was extracted with ammonium sulfate buffer under a mechanical stirring and centrifugation. It was dialyzed against water and freeze-dried. Total hydrolytic activity assay was used to determine the reduction in amylase activity when the extracted amylase inhibitor was added to reaction mixture. Results showed that UV-B significantly affected the mean % callus induction and callus fresh weight, also the activity of α-amylase inhibitors extracted from callus pieces of UV treated kidney bean was higher than those obtained from that of non-treated explants and the intact plant.

Keywords: *Phaseolus vulgaris,* germinated embryos, UV-A, UV-B and UV-C radiation.

الملخص باللغة العربية

في هذه التجربة، تم تعريض بذور نبات الفاصولياء للأشعة فوق البنفسجية نوع UV- A، UV -B والأشعة فوق البنفسجية نوع C– ، ونمت البذور في حاضنة لمدة 8 أيام في درجة حرارة 25 ± C ° ، وجرى زرع الأجنة النامية حديثا على وسط MS المحتوي على 0.5 ملغم/ لتر BA و1.5 ملغم/ لتر 2.4-D، ثم جففت قطع الكالس الناتجة من الأجزاء النباتية المعرضة وغير المعرضة للتشعيع بالتجميد، وتم استخلاص مثبط إنزيم ألفا أميليز باستخدام كبريتات الأمونيوم تحت التحريك الميكانيكية والطرد المركزي، وتم استخدام طريقة فحص النشاط التحللي المائي لتحديد الانخفاض في فعالية الأميليز بعد إضافة مثبط الأميليز إلى خليط التفاعل.

أظهرت النتائج أن نسبة كل من استحداث الكالس والوزن الطري للكالس قد تأثرت معنويا بالمعاملة UV -B ، كما أن فعالية مثبط إنزيم ألفا أميليز المستخلص من قطع الكالس الناتج من الاجزاء النباتية المعرضة للإشعاع كانت أعلى من تلك الموجودة في الأجزاء النباتية غير المعرضة للإشعاع والنبات الكامل.

INTRODUCTION

Phaseolus vulgaris, the green bean, kidney bean, or common bean, is an herbaceous annual plant in the Fabaceae (legume or bean family) and grown worldwide for its edible fruit, either the dry seed or the unripe fruit, both of which are referred to as beans. It is also occasionally used as a vegetable, and the straw can be used for fodder. Along with other species of the bean genus (Phaseolus), most of those members acquire nitrogen through an association with rhizobia, a species of nitrogen-fixing bacteria (1).

The common bean is a highly variable species with a long history of cultivation. All of the wild members of the species have a climbing habit, but the many cultivars are classified as bush beans or pole beans, depending on their style of growth. These include the kidney bean, the navy bean, the pinto bean, and the wax bean. The other major types of commercially grown bean are the runner bean (*Phaseoluscoccineus*) and the broad bean (*Viciafaba*). Production of beans is well distributed worldwide, with countries in Asia, Africa, Europe, Oceania, South and North America among the top bean growers. Brazil and India are the largest producers of dry beans, while China produces, by far, the largest quantity of green beans. Worldwide, 23 million tons of dry common beans and 17.1 million tons of green beans were grown in 2010 (2).

Effect of tissue culture techniques

The use of *in vitro* culture methods for the selection of variant types in ornamentals has been documented for many years especially for flower color, plant morphology and also physiological characters. Induced variability does not seem to be different from that known to occur spontaneously. However, mutagen treatment could increase mutant frequency severely (3). Although some variants such as changes in flower color may emerge from spontaneous mutations at relatively high rates, mutation frequency of many useful traits is very low, in *vitro* methods could lead to the occurrence of variation through the new phenotype produced. Variation refers to the differences of genetic variation of cells whereby the characteristics of mother plant is delivered to the new plant (4).Plant biotechnology together with conventional breeding methods offer scope in bean improvement and it could be increased and the seed quality (5). Also a reliable and efficient *in vitro* culturesystem that may lead to efficient differentiation, shootdevelopment and whole plant regeneration becomes an essentialprerequisite for improvement of common bean throughgenetic transformation/ mutagenesis protocols. In addition to geneticimprovement, the*in vitro* culture forms an important tool for therecovery and conservation of germplasm (6).

A study conducted by (7), examined the callus cultures induction of white seed inducedmutant obtained from *Phaseolus vulgaris*L., and reported that callus cultures were initiated from the axillary leaves, axillary shoots,node, internode, and root segments, the initiation and growth ofcallus were evaluated on MS medium with 3% sucrose, 0.4% agar,1.5 mg/lBAP, and three levels of IAA. The highest callus relativegrowth was obtained on medium with 0.5 mg/l IAA and 1.5 mg/lBAP.

Effects of UV radiations

The spectrum of ultraviolet (UV) reaching the Earth's surface has been divided into lower energy UV A (320-400 nm), higher energy UV B (280-320 nm), and UV C (254-280 nm) regions. The response of the plants to any given dose of radiation is species specific. Those parts of the ultraviolet daylight spectrum that particularly have attracted the most interests are UV-B (280-315 nm) and to less extent UV C bands (8). Ultraviolet radiation plays a key role in several biological functions, sometimes detrimental (e.g. DNA damage, immune suppression, cataracts) and others beneficial (e.g. assimilation of vitamin D, diminishing of risk of some internal cancers). However, there is no general health benefit in exposing crops and medicinal plants to extra UV B and UV C radiations (9). It is well known that plants sense UV radiation in different ways although the molecular nature and cellular localization of the primary 'receptor' of the radiation is still unknown (10).

Radiation is one of the physical factors that initiate mutations of plant cells when exposed to certain dosages. Morphological changes were observed when intact and *in vitro* plants were exposed to radiation (11). Radiation could initiate or inhibit the growth and differentiation of *in vitro* tissue cultured cells. The effects of radiation include changes in the plant cellular structure and metabolism e.g., dilation of thylakoid membranes, alteration in photosynthesis, modulation of the anti-oxidative system and accumulation of phenolic compounds (12).Investigation was carried out to find whether enhanced ultraviolet radiation influences the *Malvaparviflora* L., *Plantagomajor* L., *Rumexve sicarius* L. and *Sismbriumerysimoids* Desf. of some annual desert plants. The results indicated that the chlorophyll contents were affected by enhanced UV radiation. The chlorophyll a, b, and total contents were decreased compared with the control values and reduced with the enhanced UV radiation, but the carotenoid was increased compared with the control and also reduced with the enhanced UV radiation. So, the contents of chlorophylls varied considerably. The protein content was decreased significantly in both root and shoot systems compared with the control values but, it was increased with increasing wave lengths of UV-radiation of all tested plants. *R. vesicarius* showed the highest protein contents among the investigated plants (13).

Properties of α-amylase inhibitors from*Phaseolus vulgaris L.*

Common beans have 3 isoforms of alpha amylase inhibitor (alpha- 1, alpha-A12, alpha-AIL). The alpha-AI isoform has anti-amylase activity in humans. This enzyme is found in the embryonic axes and cotyledons in the seed and not in other organs of the plant. It is not active against plant alpha-amylases and is therefore classified as an anti-feedant or seed defense protein. The alpha amylase inhibitor prevents starch digestion by completely blocking access to the active site of the alpha-amylase enzyme (14). Factors that affect the activity of the alpha-AI isoform inhibitor are pH, temperature, incubation time and the presence of particular ions. The optimum pH for the inhibitor is 4.5 to 5.5 and the optimal temperature is 22 to 37°C. There is no activity at 0°C and the inhibitor is completely inactivated by boilingfor 10 minutes. The ideal incubation period has beenrecorded as 10 minutes, 40 minutes and 120 minutes bythree different researchers. The different incubationtimes are thought to be due to the use of different testconditions; namely a pH of 6.9 for the longer incubationperiods and a pH of 4.5 for the shortest (15).

Obesity, and resultant health hazards, which include diabetes, cardiovascular disease and metabolic syndrome, are worldwide medical problems. Control of diet and exercise are cornerstones of the management of excess weight. Foods with a low glycemic index may reduce the risk of diabetes and heart disease as well as their complications. As an alternative to a low glycemic index diet, there is a growing body of research into products that slow the absorption of carbohydrates through the inhibition of enzymes responsible for their digestion. These products include alpha-amylase and glucosidase inhibitors (16). The common white bean (*Phaseolus vulgaris*) produces an alpha-amylase inhibitor, which has been characterized and tested in numerous clinical studies. A specific and proprietary product named Phase 2 has demonstrated the ability to cause weight loss with doses of 500 to 3000 mg per day, in either a single dose or in divided doses. There have been no serious side effects reported following consumption of Phase 2. Gastro-intestinal side effects are rare and diminish upon extended use of the product. In summary, Phase 2 has the potential to induce weight loss and reduce spikes in blood sugar caused by carbohydrates through its alpha-amylase inhibiting activity (14).

MATERIALS AND METHODS

Seeds treatments with UV radiation:

White kidney bean (*Phaseolus vulgaris*) obtained from the local Iraqi markets. Seeds were treated with UV light in a different wavelength including UV-A, UV- B and UV-C for 60 min (17), and extracted in the previous work by ammonium sulfate (18).

Medium preparation:

Murashige and Skoog (MS) medium components (19) were prepared (tables 1 and 2) and supplemented with sucrose, myo-inositol and growth regulators. The pH of the medium was adjusted to 5.8 using 0.1N NaOH or 0.1N HCl, then 8g/l agar was added to the medium. The medium was dispensed into 15x2.5cm tubes (10 ml/tube). The medium was sterilized by autoclaving.

Table (1): Effect of UV radiation type A, B and C on the mean % callus induction, after inoculating explants onto solid MS medium for four weeks, n=10.

UV (nm)	% Callus induction
0.0	88.7
A	72.1
B	98.6
C	54.9
LSD 0.05	13.46

Table (2): Effect of UV radiation type A, B and C on the mean callus fresh weight (mg), after inoculating explants onto solid MS medium for four weeks, n=10

UV (nm)	Callus fresh weight (mg)
0.0	113
A	97
B	123
C	102
LSD 0.05	26.92

Seed sterilization, germination and callus induction *in vitro*:

Treated and non-treated white bean seeds (*Phaseolus vulgaris L.*) were surface sterilized using 70% (v/v) ethanol, then rinsing with stirring in 2.5% sodium hypochlorite. The seeds were taken to the laminar airflow and washing three times with sterile distilled water. Callus cultures were initiated from hypocotyl of newly germinated embryos using MS medium containing 0.5mg/l BA and 1.5mg/l 2,4-D. All the cultures were incubated in a growth room under a 16 h photoperiod (cool, white fluorescent light) and the temperature was maintained at 25 ± 2°C with 2 - 7% relative humidity (7). Callus induction frequency (%) was calculated using the following formula (20).

Callus induction frequency (%) =
No. seeds produced callus/total seeds cultured x100**.**

Callus fresh weight was measured after 8 weeks of sub culturing into a callus growth medium (21).

Assay of amylase inhibitor activity:

Amylase inhibitory activity was measured according to (22).The total hydrolytic activity assay used to determinate the reduction in amylase activity when

the amylase inhibitor was extracted and added to the reaction mixture. A 3.5-Di Nitro salicylic acid used as an alkaline color reagent 1 ml of the incubation mixture (3ml soluble starch 2% and 3 ml extracted sample) after 30 min incubation in 30°C was added to an equal volume of alkaline color reagent, mixed thoroughly and heated for 5 min in boiling water bath. Samples (with their replication) including**:**

1. Alpha-amylase standard without inhibitor as a blank.
2. Alpha-amylase: Mixed with same volume (1:1) of alpha amylase inhibitor extracted and purified from Iraqi *Phaseolus vulgaris* samples, then cooled to room temperature and stored for at least 30 min. absorbance at 546nm was measured using Aquarius 7000 Series spectrophotometer against a reference and blank. One unit of inhibitor is the amount that suppressed the amylase activity under the assay conditions. Protein was estimated as described by (23) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Effect of UV radiation types A, B and C on mean % callus induction and callus fresh weight:

Results revealed that a significant increase resulted in the % callus induction in UV-B treatment with mean value 98.6% compared with UV-A and UV-C treatments, which recording72.1% and 54.9% respectively. While there was no significant differences recorded between UV-B and control (88.7%) in the mean %callus induction. While there was no significant differences obtained between treatments in the mean callus fresh weight compared with control, the highest fresh weights recorded in UV-B with mean value 123 mg. These results were in agreement with those obtained by (24), who reported that UV- B affected plant cells growth and development through its effects on a number of important physiological processes through different pathways including second messengers such as calcium, kinases and the catalytic formation of reactive oxygen species (ROS). The study conducted by (24) also concluded that high level of UV radiation causes cellular damage and oxidative stress, thus activating a general stress signal transduction pathway, which leads to a response similar to the one which occurs after pathogen attack and other stresses.

While the effect of tissue culture could be explained by (4), who reported that most cultured plant cells could produce somaclonal variation, which is another way of producing new and interesting plant phenotype. Besides variation, propagation of new plantlets through *in vivo* and *in vitro* systems could also cause mutation and the effects of mutations could be observed through the new plant phenotype produced**.** Figure (1) describes the differences of callus cultures which originated from hypocotyls germinated from seeds treated UV radiation type A, B, C in additional to the control and showing the changes in the callus mass of *Phaseolus vulgaris,* which grown on MS medium for four weeks.

Figure (1): *Phaseolus vulgaris* callus cultures originated from hypocotyle germinated from seeds treated UV radiation type A, B, C in additional to the control, showing the changes in the callus mass grown on MS medium for six weeks

Effect of UV radiation types A, B and C on Alpha-amylase Inhibitor activity:

Figure (2) exhibits that the percentage of inhibition is about 46% for intact plant tissue and 53% for callus tissue, and the highest percentage was obtained in callus cultures originated from UV-B treated seeds recording 65%. So that gave an inductor that the crude extracted working as amylase inhibitor and these results disagreement with those obtained by (25), who suggested that direct exposure of bean seedlings to visible light and UV-radiation, induced significant variable changes in the total amount and in the relative composition of the carbohydrate pool. Concurrently with carbohydrate changes, significant variable increases in the activitiesof both invertase and a-amylase of bean seedlings were maintained throughout the entire period of the experiment. The increase in α-amylase inhibitor activity could be explained as improved mechanism which developed and used by the plant cells to combat the stress of UV radiation against plant cells and to reduce the amounts of consumed carbohydrates and keeping energy.

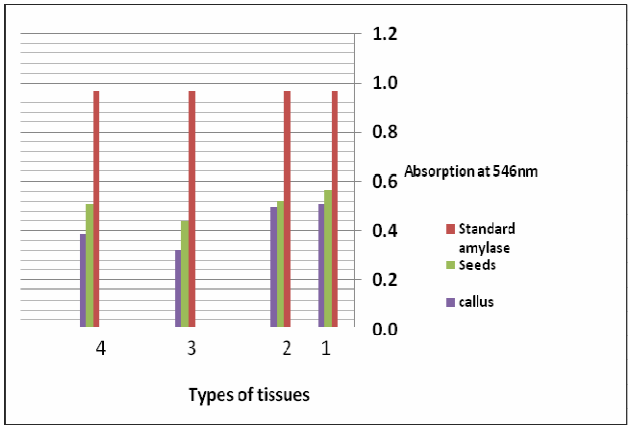

Figure (2): The effect of UV radiation and tissue culture technique on anti-amylase Inhibitor activity. 1= Non treated tissues (control), 2=UV- A, 3= UV-B, 4=UV-C

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Determination of some heavy metals and some chemical variables in the leaves of plants near of the diesel generators associations

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ABSTRACT

Pollution caused by heavy metals affects all froms of life. Plant has a remarkable ability to take up and accumulate heavy metals from their external environment such as electric generation .It is well known that high levels of heavy metals affect different physiological and metabolic processes. The effect of some heavy metals on growth and biochemical parameters of Zizyphusspina (Nebca) and Eucalyptus leaves after exposure to the smoke of the diesel generator were studied. It was found that heavy metals stress decreased chlorophyll content in leaves , the averages of chlorophyll (a, b, total) content (0.78, 0.124, 0.90 mg/g), in in Zizyphusspina leaves compared with un pollutant site the averages of chlorophyll (a ,b ,total) content in Zizyphusspina were(20.16, 7.14, 27.32 mg/g) . heavy metals stress decreased chlorophyll content in leaves ,the averages of chlorophyll (a, b, total) content (0.634, 0.18 ,0.81 mg/g), in Eucalyptus leaves compared with un pollutant site the averages of chlorophyll (a ,b ,total) content in Eucalyptus leaves were(17.6 ,8.2 , 25.8 mg/g) . The levels of four different heavy metals (Fe ,Ni ,Cu , Cd) were determined in leaves plant (Zizyphusspina and Eucalyptus) in Al-Saydiah and Al-Aalam regions. Atomic Absorption Spectrophotometer was used to determine the concentration of these metals .The average concentrations for Fe ,Cu ,Ni, and Cd were (23.2, 22.2, 2.1 ,0.7 ppm) respectively for un pollutant of Zizyphusspina ,but increased the levels of heavy metal nearby generation were the average concentrations for Fe ,Cu ,Ni, and Cd were (124 ,33 ,27 , 9.98 ppm) respectively of Zizyphusspina, while the average concentrations for Fe ,Cu ,Ni, and Cd were (89, 21, 1.6 ,0.3 ppm) respectively for un pollutant of Eucalyptus but increase the levels heavy metal nearby generators were the average concentrations for Fe ,Cu ,Ni, and Cd (118, 32.8, 24,9.8 ppm) respectively for Eucalyptus . Heavy metals –induced oxidative stress was evidenced by the generation of reactive radical MDA content increased lipid peroxidation increased significantly with heavy metals concentration, the higher concentration of metals in leaves corresponded with the higher concentration of MDA in the plant the followed by an increase in MDA production up to 8.07nmol compared with un pollutant was 1.014 nmol for Zizyphusspina while the average of Eucalyptus was 8.97 nmol for nearby generators compared with un pollutant was 1.32 nmol. These results indicated that heavy metals stress negatively impacted nearly all the parameters assayed , toxic levels of heavy metals contaminations threat to the plants . Plants exposed to heavy metals resulted injury in terms of chlorosis along with toxic effect in the form of reduced photosynthesis , growth inhibition and finally death.

Key words: Heavy metals, Zizyphusspina plant, Eucalyptus plant, diesel generators, MDA, chlorophyll (a, b, total)

الملخص باللغة العربية

يحدث التلوث الناجم عن المعادن الثقيلة تأثيرا على جميع أنماط الحياة ، ومن تلك الأنماط ما يرتبط بالنبات الذي لديه القدرة الهائلة على سحب و تراكم المعادن الثقيلة من المصادر البيئية الخارجية المختلفة ، والتي منها على سبيل المثال المولدات الكهربائية.

ومن المعروف أن وجود مستويات عالية من المعادن الثقيلة يحدث تأثيرا على عمليات الفسلجة والتمثيل الغذائي في النبات. في هذا البحث، تمت دراسة تأثير المعادن الثقيلة على خاصية النمو في نبات النبك واليوكالبتوس بعد التعرض لدخان مولدات الديزل، حيث تبين حدوث انخفاض في المحتوى الكلوروفيلي في الأوراق، وكان معدل الكلوروفيل نوع (أ، ب، الكلي) (0.78، 0.124، 0.90 ملغ / غ) على التوالي، في النبك مقارنة مع الموقع غير الملوث، حيث كان معدل الكلوروفيل(20.16, 7.14 , 27.32 ملغ /غ). كما بينت النتائج انخفاضا في المحتوى الكلوروفيلي في الأوراق، وكان معدل محتوى الكلوروفيل (أ، ب، الكلي) (0.634، 0.18، 0.81 ملغ / غ)، في أوراق الشجر اليوكالبتوس مقارنة مع الموقع غير الملوث، وكان معدل محتوى الكلوروفيل (أ، ب ، الكلي) (17.6، 8.2، 25.8 ملغ / غ). وقد تم تحديد أربعة مستويات من المعادن الثقيلة المختلفة (الحديد، النيكل، النحاس والكادميوم) في أوراق النبك واليوكالبتوس في مناطق السيدية والأعلام ، وقد استخدمت مطيافية الامتصاص الذري لتحديد تركيز كل من هذه المعادن، وكان متوسط تركيز الحديد، النحاس، النيكل، والكادميوم (23.2، 22.2، 2.1، 0.7 جزءا في المليون) للموقع غير الملوث ، ولكن زيادة مستويات المعادن الثقيلة بالقرب من المولدات الكهربائية كانت متوسطة التراكيز لكل من الحديد، النحاس، النيكل، والكادميوم (124، 33، 9،27.9 جزء في المليون) على التوالي في النبك، في حين كانت متوسطات تراكيز كل من الحديد، النحاس، النيكل، والكادميوم (89، 21، 1.6، 0.3 جزءا في المليون) على التوالي للموقع غير الملوث من اليوكالبتوس، ولكن الزيادة في مستويات المعادن الثقيلة القريبة من المولدات سببت زيادة في متوسطات تراكيز كل من الحديد، النحاس، النيكل، والكادميوم (118، 32.8، 9.8،24 جزءا في المليون) على التوالي في اليوكالبتوس .

وبهذا فإن المعادن الثقيلة تؤثر على مستوى الأكسدة الفوقية (الإجهاد التأكسدي) ، ومما دل على توليد جذور نشطة فعالة هو محتوىMDA الذي يزداد معنويا بزيادة بيروكسيد الدهون بشكل كبير مع تراكيز المعادن الثقيلة، كما يرتبط ارتفاع تركيز المعادن الثقيلة في الأوراق مع ارتفاع تركيز MDA. حيث كان متوسط إنتاج MDA قد بلغ 8.07 نانومول في الموقع القريب من المولدات مقارنة مع الموقع غير الملوث 1.014نانومول في أوراق النبك، في حين كان متوسط اليوكالبتوس 8.97 نانومول للمولدات القريبة مقارنة بالموقع غير الملوث 1.32 نانومول.

وتشير هذه النتائج إلى أن المستويات السامة من المعادن الثقيلة أثرت سلبا على المتغيرات الكيميائية، و تهدد وتشكل خطرا على النباتات المعرضة للمعادن الثقيلة لما تتسبب به من تأثير سام يتمثل في تخفيض التمثيل الضوئي، وتثبيط النمو وأخيرا موت النبات.

INTRODUCTION

Pollution of the environment with toxic metals has enriched very big since the onset of the industrial revolution (1). Industrial air pollutants are very serious and serious on plant, animal and human in the world. Industrial emissions in air are the main origin of air pollution. Many studies indicated dangerous effects on the plants grown near the industrial generations and factories (2). Anthropogenic pollutants like heavy metals enter our environment in a variety of ways and These include gas exhaust which emission from vehicles, generation ,mining, electroplating, metal smelting , energy and fuel production, down wash from power lines , power transmission, sludge dumping (3,4). Because of these activities ,the levels of heavy metals, such as cadmium ,copper and lead in the environment are currently of great concern (5). Heavy metals are increased in the environment by human activities of different types (6), and cause a lot of problems. Some of these metals such as copper, nickel and iron have known functions as micronutrients and are needed by plants as parts of coenzymes and enzymatic prosthetic groups(7) but lead, cadmium and mercury have unknown biological functions (7,8) if they are required for plant growth or not. All heavy metals are toxic to plants at high levels (4,7). Generator's fuel contains metallic elements of heavy metals such as Pb, Ni, Cd, Cu. Heavy metals are that elements having specific gravity that is at least five times of the specific gravity of water, which is expressed as 1 at 4°C and refers to metallic elements with an atomic weight greater than iron (55.8 g/mol) (9).Copper (Cu) is an important trace metal needed for proper human health in an appropriate limit , Pb and Cd are non-essential nutrients for plant but it owns a toxic effect. It is effectively absorbed by both the root and leaf systems (10) .These elements are stable and highly toxic, because they cannot be analyzes, the trace quantities of heavy metals are nutritionally main for a healthy life; they are commonly found naturally in plants. Therefore, plants are considered as a vital indicator for sensing the occurrence of pollution near generators. Heavy metals are also common in the electrical generation stations, batteries, alloys, fuel and medical industries, electroplated, transportation setting, refining oil stations, and hazardous waste sites. Similar to these electrical generation stations, factories are found in Baghdad as found in previous studies (11, 12). Heavy metals occurrence and toxicity for plants were viewed by (12). Heavy metals in edible green vegetables grown along the sites of the Sinza and Msimbazi rivers were found in Dares Salaam, Tanzania (13), market basket survey for some heavy metals in Egyptian fruits and vegetable (14) and bioavailability of heavy metals from polluted soils to plants (15).

The aims of the study:

1. Determination of heavy metals (Fe , Cd , Cu, Ni)in leaves of plants Zizyphusspina (Nebca) and Eucalyptus after exposure to the smoke of the diesel generators.
2. Determination of malondialdehyde (MDA) in leaves of plants Zizyphusspina (Nebca) and Eucalyptus after exposure to the smoke of the diesel generators.
3. Determination of chlorophyll content in leaves of plants Zizyphusspina (Nebca) and Eucalyptus after exposure to the smoke of the diesel generators.

MATERIALS AND METHODS

Sample preparation:

Samples (leaves of plant Zizyphusspina grown near generators with two sites (control and pollution) and leaves of plant Eucalyptus near generators with two sites (control and pollution) were placed in polyethylene bags and brought to laboratory for analysis. All samples were washed with tap water followed by DDI (double de-ionized distil water). Samples were cut into small pieces and dried at 105°C for18 hours (16). After drying, the samples were ground into powder form. Approximately 1.0 g of each sample in triplicate taken into digestion tubes, were soaked in 40 ml of nitric acid (HNO_3) from company BDH (England) and perchloric acid ($HCLO_4$) from company BDH (England) (3:1) and left over night for complete contact of material. After 24 hs. Samples were treated at 2hrs with heat at 120°C for 2 hrs and then180°C on heating digester) till the solution becomes transparent. Digestion stopped when sample solutions reduced to 2-3 ml. Cooled samples were transferred into100 ml. volumetric flask and volume raised up to the mark with. 0.1 M HNO_3 treated samples were analyzed for heavy metal by FAAS (flame Atomic Absorption Spectroscopy) (16-17).

Estimation of MDA (Malonedialdehyde) contents:

MDA in selected samples were analyzed according to (18). This method is based on the reaction with thiobarbituric acid TBA (Thiobarbituric acid) from company BDH (England). Fresh leaves (1.0 g) were ground properly in 20 ml of 0.1% tri-chloroacetic acid solution, TCA (Trichloro acetic acid) from company BDH (England), One ml of the supernatant was reacted with 4 ml of 20% tri-chloroacetic acid solution from company BDH (England) TCA solution comprising 0.5% thiobarbituric acid TBA (Thiobarbituric acid) from company BDH (England) and then it was heated for 30 min., at 95C in a water bath and then immediately cooled on ice. After the absorbance of the supernatant was read at 532 and 600 nm by SP8-100 UV –ViS spectrophotometer PYE –Unicom .

The contents of MDA were worked out using the formula:

MDA level (nmol) = Ä (A 532nm-A 600nm)/1.56. (18)

Determination of chlorophyll content:

Fresh biomass (leaves) of plant include Zizyphusspina and Eucalyptus were homogenized in 80% acetone solution as a common reagent used in the work from company BDH (England) in the dark and then filtration was done and the supernatant was determined by SP8-100 UV –ViS spectrophotometer PYE –Unicom at 663 , 645 nm. To determine chlorophyll a ,b and total contents, 80% of acetone was used according to the formula:

Total chlorophyll (mg/g) = 20.2 (A645) + 8.02 (A663)
Chlorophyll a (mg/g) = 12.7 (A663) -2.69(A 645)
Chlorophyll b (mg/g) =22.9(A 645) -4.68 (A663) (19).

RESULTS AND DISCUSSION

Effect of heavy metals resulted from diesel generators on different pigment chlorophyll in Zizyphusspina and Eucalyptus leaves:

Heavy metals had caused reductions of chlorophyll content via damage to chloroplast membrane. A decrease in chlorophyll content was observed depending on metal pollution . The effect of heavy metals on different pigments of chlorophyll in Zizyphusspina and Eucalyptus leaves at two locations for generators in the Al-Aalam and the Al-Saydiah (in Baghdad city) regions Respectively as shown in the table (1).
In the present study, the exposure of heavy metals affected Zizyphusspina plants by chlorophyll (a ,b and total) . Table (1) showed the high averages in chlorophyll a ,b and total in Al-Aalam region (20.16 , 7.14 and 27.32 mg/g) respectively for control (un polluted) , on one meter distance (pollutant). The high averages in chlorophyll a ,b and total in Al-Aalam region were (4.68, 0.94 and 5.63 mg/g) respectively and , on two meters distance (pollutant), the high averages in chlorophyll a ,b and total in Al-Aalam region were (0.78, 0.124 and 0.90 mg/g) respectively, but the low averages in chlorophyll a ,b and total in Al-Saydiah region were (13.07, 6 .15 and 19.2 mg/g) respectively for control (un polluted). On one meter distance (pollutant) the high averages in chlorophyll a ,b and total in Al-Aalam region were (3.47, 1.148 and 13.86 mg/g) respectively and , on two meters distance (pollutant) the high averages in chlorophyll a ,b and total in Al-Aalam region were (1.84, 0.072 and 1.91 mg/g) respectively . Exposure of heavy metals affected Eucalyptus plants including chlorophyll (a ,b and total). As shown in table (1), the high averages in chlorophyll a ,b and total in Al-Aalam region were (17.61, 8.27and 25.89 mg/g) respectively for control (un polluted) , on one meter distance (pollutant), the high average in chlorophyll a ,b and total in Al-Aalam region were (7.42, 6.6 and 14.09 mg/g) respectively and , on two meters distance (pollutant), the high averages in chlorophyll a ,b and total in Al-Aalam region were (4.85, 1.15 and 6.00 mg/g) respectively, but the low averages in chlorophyll a ,b and total in Al- region were (14.04,5.93 and 19.9 mg/g) respectively for control (un polluted). On one meter distance (pollutant), the high averages in chlorophyll a ,b and total in Al-Aalam region were (10.0, 4.07 and 14.07 mg/g) respectively and , on two meters distance (pollutant), the high averages in chlorophyll a ,b and total in Al-Aalam region were (6.94, 2.14 and 9.14 mg/g) respectively . But the values had decreased in Al-Saydiah region where the high averages in chlorophyll a ,b and total of Eucalyptus leaves in Al-Saydiah region were (16.15 , 7.47 and 23.62 mg/g) respectively for control (un polluted). on one meter distance, the high averages in chlorophyll a ,b and total in Al-Saydiah region were (9.02, 3.16 and 12.89 mg/g) respectively and , on two meters distance (pollutant), the high averages in chlorophyll a ,b and total in Al-Saydiah region were (2.14, 1.95 and 4.10 mg/g) respectively. The low averages in chlorophyll a ,b and total in Al-Saydiah region were (13.86, 6.07 and 19.9 mg/g) respectively for control (un polluted). On one meter distance (pollutant), the high averages in chlorophyll a ,b and total in Al-Saydiah region were (9.70, 4.16 and 13.86 mg/g) respectively and , on two meters distance (pollutant), the high averages in chlorophyll a ,b and total in Al-Aalam region were (4.55, 1.19 and 5.74 mg/g) respectively . The reason of reduction in Chlorophyll a, chlorophyll b and total chlorophyll content was due to heavy metal stress and that was argued with (20) in *Lemna polyrrhiza* L. a decrease in chlorophyll content associated with heavy metal stress may be the result of inhibition of the enzymes responsible for chlorophyll biosynthesis (21). The amount of chlorophyll in plants is often estimated to evaluate the effects of environmental tensions. These tensions may stop metabolic processes by preventing enzyme activity. The reduction of chlorophyll in plants under tension is probably either because of controlling chlorophyll synthesis enzymes activity or the increasing of chlorophyll pigment disintegration (22), and observed in Zizyphusspina leaves Contain on its surface Scales helped on the accumulation of heavy elements abundance while Eucalyptus leaves Contain Smooth layer . The toxicity of heavy metals on the plants depends on the plant species, age (age diesel generator ,age of trees), whenever increased age of the tree decreased response to plant absorption of heavy metals which emitted from diesel generators and number working hours have important role cause excess in emission pollutants), and element chemical composition and concentration (23) . Significantly reducing in photosynthetic rate and chlorophyll content in plant leaves as a result of the pollution with Cu, Ni, Cdand Pb were observed by

(24-25). These results correspond with (26, 27) respectively.

Table (1): Effect of heavy metals on different pigment chlorophyll in Zizyphusspina and Eucalyptus leaves

Regions	Plant name	Distance	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Total Chlorophyll (mg/g)
(1) Al-Aalam	Zizyphusspina Leaves	A	20.17± 0.034 *	7.14± 0.022*	.026*0 27.32±
		B	4.68 ± 0.032*	0.032*0 0.94 ±	5.63± 0.031*
		C	0.78±0.034*	0.124±0.33*	0.90 0.035*
(2) Al-Saydiah	Zizyphusspina Leaves	A	13. 07 ±0.042*	6.15 ±0.040*	19.22±0.042*
		B	3.47 ±0.040*	1.146 ±0.042*	13.86 ±0.042*
		C	1.846 ± 0.036*	0.072 ±0.035*	1.91 ± 0.035*
(3) Al-Aalam	Eucalyptus Leaves	A	17.61±0.003	8.27 ± 0.02*	25.890 ± 0.011*
		B	16.07 ± 0.04*	7.14± 0.03*	23.18 ± 0.037*
		C	7.42 ± 0.031*	6.6±0.022*	14.09 ± 0.041*
(2) Al-Aalam	Eucalyptus Leaves	A	14.72±0.02*	8.432 ± 0.023*	23.36 ± 0.031*
		B	10.72±0.027*	2.64 ± 0.021*	13.36 ± 0.032*
		C	4.85±0.031*	1.15 ± 0.023*	6.00 ± 0.017*
(4) Al-Aalam	Eucalyptus Leaves	A	16.94±0.03*	7.56 ± 0.021*	24.59 ± 0.026*
		B	10.64±0.022*	2.68 ± 0.021*	13.327± 0.03*
		C	6.99±0.031*	1.634 ± 0.004*	8.63 ± 0.03*
(5) Al-Aalam	Eucalyptus Leaves	A	15.395±0.02*	5.78 ± 0.022*	21.18 ± 0.025*
		B	12.18±0.024*	3.85 ± 0.022*	16.03 ± 0.03*
		C	0.634±0.04*	0.18 ± 0.034*	0.81 ± 0.035*
(6) Al-Aalam	Eucalyptus Leaves	A	14.04 ± 0.032*	5.933± 0.042*	19.9 ± 0.032*
		B	10.0 ± 0.04*	4.07± 0.031*	14.07± 0.032*
		C	6.945± 0.03*	2.19± 0.025*	9.14± 0.040*
(7) Al-Saydiah	Eucalyptus Leaves	A	16.15± 0.025*	7.47± 0.020*	23.62± 0.030*
		B	9.028± 0.022*	3.164± 0.032*	12.87± 0.033*
		C	2.14± 0.028*	1.95± 0.028*	4.10± 0.030*
(8) Al-Saydiah	Eucalyptus Leaves	A	15.72± 0.022*	4.52± 0.025*	20.25± 0.033*
		B	8.34± 0.021*	3.70 ± 0.026*	12.0 ± 0.034*
		C	5.01± 0.034*	1.30± 0.042*	6.32± 0.022*
(9) Al-Saydiah	Eucalyptus Leaves	A	13.86± 0.035*	6.07± 0.044*	19.9± 0.031*
		B	9.70± 0.034*	4.16± 0.04*	13.87± 0.028*
		C	4.55± 0.02*	1.19± 0.032*	5.74 ± 0.025*

The average (mean) of three replicates + SD (*) statistically significant at $p < 0.05$ level . Distance (A:control,B: Pollutant by close near generator 1 meter distance, C: Pollutant by near generator 2 meter distance). 1, 2, 3, 4…etc. refer to number sites in locations

Effect on lipid peroxidation levels (MDA) content on Zizyphusspina and Eucalyptus leaves:

Malondialdehyde (MDA) is an end product of membrane lipid peroxidation and high MDA levels in plants are used as indicator of oxidative stress (28). Heavy metals such as Cu , Cd , Pb , Co , Fe , and Ni caused increase in MDA content in plants (29, 30) in Zizyphusspina and Eucalyptus leaves in two locations for generators in Al-Saydiah and AL-AaLam regions. As shown in table (2), for Zizyphusspina plants MDA showed the average in Al-Saydiah region of value (1.34 nmol) for control (un pollutant) , on one meter distance (pollutant), the high averages in Al-Aalam region was (6.41nmol) and , on two meters distance (pollutant), the high average in Al-Aalam region was (1.39 nmol), but the average in Al-Aalam region was (1.014 nmol) for control (un pollutant) , on one meter distance (pollutant), the high average in Al-Aalam region was (8.07 nmol) and on two meters distance (pollutant), the high average in Al-Aalam region was (1.60 nmol). Eucalyptus plants including MDA showed the average in Al-Aalam region (1.017 nmol) for control (un pollutant) , on one meter distance (pollutant), the high average in Al-Aalam region was (8.205 nmol) and , on two meters distance (pollutant), the high average in Al-Aalam region was (1.215 nmol) and another location where the average in Al-Aalam region was (1.32 nmol) for control (un pollutant) , on one meter distance (pollutant), the high average in Al-Aalam region was (8.97 nmol) and , on two meters distance (pollutant), the high average in Al-Aalam region was (1.98 nmol). Malondialdehyde is an end product of membrane lipidperoxidation and high MDA levels in plants are used as an indicator of oxidative stress (31). Heavy metals such as Cu, Cd, Pb, Co, Hg and Mn caused increases in MDA content in plants (32).The results showed that there were significant differences between two sites generators (control and pollutant). These differences attributed to reason for pollutants emitted (heavy metal such as Ni, Cu, Cd and Fe) from diesel generators, which caused an increase in malondialdehyde (MDA) (table 2). These results correspond with (29-31).

Table (2): Effect on lipid peroxidation levels (MDA) contents on Zizyphusspina and Eucalyptus leaves

Plant name and location	Distance	MDA content (n mol)fw
Zizyphusspina (1) AL-Saydiya	A	1.34 ± 0.024*
	B	6.41 ± 0.027*
	C	1.39 ± 0.026*
Zizyphusspina (2) Al-Aalam	A	1.014 ± 0.025*
	B	8.07 ± 0.026*
	C	1.60 ± 0.025*
Eucalyptus (1) Al-Aalam	A	1.017 ± 0.03*
	B	8. 205 ± 0.03*
	C	1.215 ± 0.022*
Eucalyptus (2) Al-Aalam	A	1.032 ± 0.032*
	B	8.26 ± 0.032 *
	C	1.643 ± 0.030 *
Eucalyptus (3) Al-Aalam	A	1.055 ± 0.033*
	B	8.33 ± 0.03 *
	C	1.66 ± 0.035 *
Eucalyptus (4) Al-Aalam	A	1.064 ± 0.04*
	B	8.37 ± 0.04 *
	C	1.677 ± 0.032*
Eucalyptus (5) Al-Aalam	A	1.070 ± 0.042*
	B	8.46 ± 0.03*
	C	1.68 ± 0.042 *
Eucalyptus (6) Al-Aalam	A	1.076 ± 0.036*
	B	8.4 ± 0.04*
	C	1.71 ± 0.03*
Eucalyptus (7) Al-Aalam	A	1.12 ± 0.031 *
	B	8.52 ± 0.031*
	C	1.76 ± 0.03 *
Eucalyptus (8) Al-Aalam	A	1.264 ± 0.032*
	B	8.715 ± 0.042 *
	C	1.97 ± 0.032 *
Eucalyptus (9) Al-Aalam	A	1.32 ± 0.042*
	B	8.97 ± 0.04 *
	C	1.98 ± 0.043 *

The average (mean) of threereplicate + SD (*) statistically significant at p < 0.05 level . fw: fresh weight. Distance (A: control, B: Pollutant by close near generator 1 meter distance, C: Pollutant by near generator 2 meter distance) . 1,2,3,4…etc refer to number site in Al-Saydiya and Al-Aaalam (shabab) regions

Heavy metal contents in the Zizyphusspina and Eucalyptus leaves obtained from different sites:

Heavy metal pollution affects biosphere in many places around the world. Heavy metals make a significant contribution to environmental pollution as a result of human activities such as smelting, mining, electroplating, energy and fuel production, power transmission, sludge dumping and other industrial activities. Data obtained in the study showed that the concentrations of Pb , Cd , Ni, Cu, Zn and Fe were considerably higher in plant tissues. Heavy metal contents in Zizyphusspina and Eucalyptus leaves in two locations for generators in the Al-Saydiah and Al-Aalam regions are shown in the table (3).

Fe (Iron) in leaves :

For Zizyphusspina plants, results showed that the average in Al-Aalam region was (23.2) ppm for control (un polluted) , on one meter distance (pollutant), the average in Al-Aalam region was (124) ppm and , on two meters distance (pollutant) the average in Al-Aalam region was (121) ppm, but the average in Al-Saydiah region was (23.0) ppm for control (un polluted) , on one meter distance (pollutant), the high average in Al-Saydiah region was (121) ppm and , on two meters distance (pollutant) in Al-Saydiah region was (120) ppm.

For Eucalyptus plants, the average in Al-Aalam region was (89.8) ppm for control (un polluted) , on one meter distance (pollutant), the average in Al-Aalam region was (118.8) ppm and , on two meters distance (pollutant), the average in Al-Aalam region was (112.8) ppm. Average in Al-Aalam region was (82) ppm for control (un polluted) , on one meter distance (pollutant) the average in Al-Aalam region was (112) ppm and , on two meter

distance (pollutant) the in Al-Aalam region was (110) ppm (table 3)..

Cu (Copper) in leaves:

For Zizyphusspina plants, Cu showed that the average in Al-Aalam region was (22.2) ppm for control (un polluted) , on one meter distance (pollutant) the average in Al-Aalam region was (33.8) ppm and , on two meter distance (pollutant) the average in Al-Aalam region was (31.2) ppm but the average in Al-Saydiah region was (21.9) ppm for control (un polluted) , on two meters distance (pollutant) the high average in Al-Saydiah region was (33.0) ppm and , on two meters distance (pollutant) in Al-Saydiah region was (30.0) ppm .

For Eucalyptus plants, Cu showed the average in Al-Aalam region that was (21.2) ppm for control (un polluted) , on one meter distance (pollutant), the average in Al-Aalam region was (32.8) ppm and , on two meters distance (pollutant), the average in Al-Aalam region was (30.7) ppm. At the other location the average in Al-Aalam region was (18.2) ppm for control (un pollutaned) , on one meter distance (pollutant) the average in Al-Aalam region was (30.4) ppm and , on two meters distance (pollutant) the in Al-Aalam region was (27.9) ppm (table 3).

Ni (Nickle) in leaves:

For Zizyphusspina plants, Ni showed that the average in Al-Aalam region was (2.1) ppm for control (un polluted) , on one meter distance (pollutant), the average in Al-Aalam region was (27.4.8) ppm and , on two meters distance (pollutant), the average in Al-Aalam region was (24.2) ppm, but the average in Al-Saydiah region was (1.8) ppm for control (un polluted) , on one meter distance (polluted), the high average in Al-Saydiah region was (24.7) ppm and , on 2 meter distance (pollutant) in Al-Saydiah region was (21.6) ppm.

For Eucalyptus plants, Ni showed that the average in Al-Aalam region was (1.6) ppm for control (un pollutant) , on one meter distance (pollutant), the average in Al-Aalam region was (24.5) ppm and , on two meters distance (pollutant), the average in Al-Aalam region was (21.4) ppm, but the other location the average in Al-Aalam region was (0.07) ppm for control (un pollutaned) , on one meter distance (pollutant), the average in Al-Aalam region was (17.1) ppm and , on two meters distance (pollutant) the in Al-Aalam region was (16.8) ppm (table 3).

Cd (Cadmium) in leaves:

For Zizyphusspina plants, Cd showed that the average in Al-Aalam region was (0.7) ppm for control (un pollutaned) , on one meter distance (pollutant), the average in Al-Aalam region was (9.98) ppm and , on two meters distance (pollutant) the average in Al-Aalam region was (8.56) ppm but the average in Al-Saydiah region was (0.5) ppm for control (un pollutaned) , on one meter distance (pollutant), the high average in Al-Saydiah region was (9.9) ppm and , on two meters distance (pollutant) in Al-Saydiah region was (8.3) ppm .

For Eucalyptus plants, Cd showed that the average in Al-Aalam region was (0.3) ppm for control (un pollutant) , on one meter distance (pollutant), the average in Al-Aalam region was (9.8) ppm and , on two meters distance (pollutant) the average in Al-Aalam region was (8.18) ppm but the other location the average in Al-Aalam region was (0.009) ppm for control (un pollutaned) , on one meter distance (pollutant) the average in Al-Aalam region was (9.48) ppm and , on 2 meter distance (pollutant) the in Al-Aalam region was (6.6) ppm (table 3).

Markedly, the accumulation of heavy metals with high concentrations in Baghdad city is attributable to the pollution of leaves. The descending order of heavy elements in leaves as well as the both types of plant tissues; leaves as Fe > Cu >Ni > Cd indicate a systematic uptake of trace elements from leaves .On the basis of plants are able to accumulate trace elements (especially heavy metals) above established background concentrations in or on their tissues (32) . Obviously, the leaf plant tissues store the greater quantity of heavy metals. The reason of increased Iron in leaves plants : protein structure (hemeproteins) was increased because micronutrient plant which play important role for plant life (33) and diesel generators emission iron (Fe) from fuel because using as improver for the combustion of fuel may also be contributed from engine wear over time .Cadmium (Cd) the reason increased in Cadmium leaves plants : The vehicle exhausts in heavy traffic are the main source of Cd (32) Cd are non-essential nutrient for plant and toxic; it is effectively absorbed by both the root and leaf systems, and is also highly accumulated in soil organisms (33), is a byproduct of diesel fuel smelting of lead and zinc; it can be found in nickel-cadmium batteries and diesel fuel contain on Cadmium.

Nickel (Ni) the reason increased Nickel in leaves plants is micronutrient plant which play important role for plant life (33) can be found in nickel-cadmium batteries, diesel fuel. The cause change in nickel was nickel product of stainless steel, nonferrous alloys also using in manufacture of batteries, nickel used alloys and cause lung cancer.

Copper (Cu) the reason increased Copper in leaves plants : is an essential trace element for plants. Bothdeficiency Industrial and mining .These reasons attributed to the presence of heavy metals in Diesel fuel derived from crude oil, which contains the heavy elements(Fe, Ni, Cu and Cd)(34) These results correspond with researcher (35).

Table (3): Heavy metals contents in the Zizyphusspina and Eucalyptus leaves obtained from different sites

Plant name / location	Sample No.	Distance	Fe ppm	Cu ppm	Ni ppm	Cd ppm
Zizyphusspina Al-Aalam	1	A	23.2±8.5	22.2±2.3	2.1± 0.12	0.7±0.08
		B	124 ± 17.0	33.8±2.51	27.4 ±1.40	9.98±0.11
		C	121±9.05	31.2±3.2	24.2 ±1.31	8.56±0.12
Zizyphusspina Al-Saydiah	2	A	23.0±8.2	21.9 ±2.1	1.8 ±0.11	0.5±0.01
		B	121±16.2	33.0±2.11	24.7 ±1.52	9.9±0.12
		C	120±9.5	30.1±3.21	21.6±1.43	8.3±0.13
Eucalyptus Al-Aalam	1	A	89.8±9.2	21.2 ± 2.1	1.6 ± 0.01	0.3 ± 0.01
		B	118.8±15.12	32.8± 2.15	24.5 ± 1.3	9.8 ± 0.12
		C	112.8±10.21	30.7 ± 3.2	21.4 ± 1.44	8.18 ± 0.14
Eucalyptus Al-Aalam	2	A	89.1±8.4	21.0 ± 2.12	1.4 ± 0.021	0.2 ± 9.82
		B	117.8±12.14	32.5 ± 2.11	24.2 ± 2.56	9.8 ± 0.14
		C	112.0±11.4	30.3 ± 2.76	20.9 ± 2.66	8.12 ± 0.16
Eucalyptus Al-Aalam	3	A	88.8 ±7.14	20.8 ± 2.11	1.2 ± 0.012	0.15 ± 0.001
		B	114.2± 11.2	32.1 ± 2.17	23.7± 2.71	9.77 ± 0.16
		C	111.6±10.1	29.8 ± 2.41	20.3 ± 1.81	8.10 ±0.17
Eucalyptus Al-Aalam	4	A	87.7 ± 8.11	20.4 ± 2.46	0.8 ±0.01	0.08 ± 0.11
		B	113.0±10.2	31.8 ±2.44	23.2 ± 2.11	9.72 ± 0.18
		C	111 .4±11.6	29.5 ± 2.14	19.8 ± 1.8	8.8 ± 0.17
Eucalyptus Al-Aalam	5	A	86.9 ±7.90	20 ± 2.41	0.5 ± 0.01	0.06 ± 0.01
		B	112.5 ± 11.4	31.6 ± 2.16	22.7 ± 3.11	9.68 ± 0.15
		C	111± 11.5	29.4 ± 3.11	19.2 ± 2.51	8.4 ± 0.17
Eucalyptus Al-Aalam	6	A	85.2 ± 8.21	19.7 ± 2.3	0.3 ± 0.01	0.04 ± 0.01
		B	112.0 ± 10	31.2 ± 2.44	22.3 ± 2.41	9.64 ± 0.17
		C	110.1± 11.4	28.9 ± 2.91	18.7 ± 2.11	7.9 ± 0.13
Eucalyptus Al-Aalam	7	A	83.5 ±8.10	19.4 ±2.5	0.2 ± 0.01	0.02 ±0.01
		B	111.3 ± 11.4	31 ± 3.10	19.7 ± 2.43	9.60 ± 0.12
		C	110±10.8	28.7 ±2.4	18.2 ± 2.33	7.7 ± 0.13
Eucalyptus Al-Aalam	8	A	83.0 ±8.10	19.0 ±2.9	0.1 ±0.01	0.01 ±0.011
		B	111.2 ± 11.2	30 ±2.41	19.1 ± 2.61	9.5 ±0.11
		C	110 ± 12.1	27 ± 2.22	17.8 ±2.11	7.2 ±0.14
Eucalyptus Al-Aalam	9	A	82.9 ±8.17	18.3 ±2.11	0.09 ± 0.01	0.01 ± 0.11
		B	114.3 ± 11.7	30.6 ±3.4	17.8 ±1.9	9.52 ± 2.82
		C	110 ±11.2	28.3 ± 2.12	16.2 ±2.11	6.8 ± 1.71
Eucalyptus Al-Aalam	10	A	82 ±8.11	18.2 ±2.9	0.07 ±0.011	0.009 ± 0.001
		B	112 ±11.2	30.4 ± 3.5	17.1 ±2.71	9.48 ± 1.7
		C	110 ±10.7	27.9 ± 2.4	16.8 ±2.16	6.6 ± 1.8

The average (mean) of three replicate + SD (*) statistically significant at p < 0.05 level .fw: fresh weight Distance(A: control, B :Pollutant by close near generator 1 meter distance, C: Pollutant by near generator 2 meter distance) . 1, 2, 3, 4…etc refer to number site in the AL-Saydiya and AL-Aaalam (shabab) regions . Mean of three replicates ± SD

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Immunomodulatory effect of Candida albicans cell wall mannoprotien on mice immunized with Hepatitis B virus (HBs) vaccine

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ABSTRACT

Candida albicans cell wall mannoprotein immunomodulation innate and adapt immune response in mice immunized with Hepatitis B virus (HBs) vaccine. The purpose of this study was to determine the effect of the *Candida albicans cell wall mannoprotein* on phagocytic activity: antibody production: serum gammaglobuline percentage and lymphocyte transformation index after vaccination with hepatitis B surface (HBs) antigen.

Eight groups of BALB/c mice were included in the current study. The first three groups were injected with distilled water, moderate and high dose of mannoprotein as negative and positive controls, while group four was immunized with a HBs antigen vaccine only. Groups five and six were immunized with combination of HBs vaccine and cell wall mannoprotein. The last two groups were injected with dose of prednisone prior to immunizing with combination of HBs vaccine and cell wall mannoprotein. Blood samples were collected for 10 days to measure phagocytic activity by NBT test reading by ELIZA. Two weeks were estimated for lymphocyte transformation measurement by MTT test and 3, 4 weeks for post-vaccination, and anti-HBs antibodies in the serum were measured by indirect immunoflouresnt. The results indicated that mice in groups were immunized with combination of vaccines and candida albicans cell wall mannoprotein revealed higher serum anti-HBs level and significance increase in phagocytic activity and lymphocyte proliferation percentage occured. This study concluded that mannoproteins isolated from *Candida albicans* cell wall are important immunomodulatorts in the development of immune response against *HBs antigen vaccine.* The results demonstrated a clear immunomodulatory effect of the mannoproteins of Candida albicans cell wall (improvement of non-specific, cellular and humoral immune response) of the treated mice

Keywords: HBs antigens vaccine, Candida albicans cell wall mannoprotein, NBT, MTT and serum anti HBs antibodies level

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الملخص باللغة العربية

تمت دراسة تأثير المحورات المناعية (Mannoproteins) المستخلصة من جدار خلايا المبيضات (*Candida albicans*) على الاستجابة المناعية للفئران الممعنة بلقاح مستضد فيروس التهاب الكبد الفيروسي من النوع ب Hepatitis B virus (HBs) vaccine . هدفت الدراسة إلى تحديد تأثير مستخلص جدار خلايا المبيضات على فعالية خلايا البلعمة ، وإنتاج الأجسام المضادة ، والهجرة الكهربائية لبروتينات المصل ومعامل التحول للخلايا اللمفاوية.

أجريت التجربة على ثماني مجموعات من الفئران البيضاء نوع BalB/c ، تم حقن المجموعات الثلاثة الأولى بالماء المقطر وجرعة متوسطة وعالية من مستخلص جدار خلايا المبيضات كمجاميع سيطرة سالبة وموجبة. وحقنت المجموعة الرابعة بلقاح فيروس (HBS) Hepatitis B virus منفردا ، وحقنت المجموعتان الخامسة والسادسة بخليط اللقاح مع الجرعتين المتوسطة والعالية من مستخلص جدار خلايا المبيضات ، بينما حقنت المجموعتان السابعة والثامنة بمحلول مادة البريسلون Prednisolone كمثبط مناعي قبل حقن خليط اللقاح ومستخلص جدار خلايا المبيضات المتوسطة والعالية بمدة خمسة أيام.

جمعت نماذج الدم في اليوم العاشر، وتم قياس فعالية خلايا البلعمة باستخدام (معامل اختزال ملون Nitro blue tetrazolium; NBT) بجهاز القراءة في الآليزا ، وفي اليوم الرابع عشر تم قياس فعالية خلايا التحول باستخدام مادة MTT بجهاز قراءة الآليزا وبعد مرور ثلاثة وأربعة أسابيع تم قياس عيارية الأضداد ضد فيروس HBS بالطريقة غير المباشرة للمجهر.

أظهرت النتائج تأثيرات واضحة للمحورات المناعية المستخلصة من جدار خلية المبيضات (Candida albicans) في تقوية الاستجابة المناعية في الفئران الممنعة بلقاح فيروس ب Hepatitis B، حيث أظهرت النتائج تحسنا ملحوظا في المناعة المتأصلة ،والمناعة الخلطية والمناعة الخلوية.

INTRODUCTION

Hepatitis B virus (HBV) is a serious public health problem and major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. It was estimated that approximately 2 billions people have serological evidences of past or present HBV infection and there are 350 millions carriers of virus worldwide (1). The World Health Organization (WHO) strategy for effective control of HBV infection and its sequel is mass vaccination of neonates and children within the framework of Expanded Program on Immunization (EPI) and recommended that hepatitis B vaccination should be included in national Immunization system in all countries by 1997 (2). In a series of studies, it has been demonstrated that 90-99% of healthy neonates, children, adolescents and adults developed protective levels of anti-HBs antibody following a standard vaccination course with hepatitis B vaccine (3-4). The effectiveness of routine infant hepatitis B immunization in significantly reducing the prevalence of chronic HBV infection has been demonstrated in a variety of countries (1). Accordingly, some investigators have suggested the need for a booster dose after 5-15 years (5, 6). Hepatitis B virus (HBV) is an enveloped, double-stranded DNA virus belonging to the Hepadnaviridae family and is recognized as the major cause of blood transmitted hepatitis together with hepatitis C virus HCV(7). Hepatitis B surface antigen or HBsAg, previously described as Australia antigen, is the most important protein of the envelope of Hepatitis B Virus. The surface antigen contains the determinant "a", common to all known viral subtypes and immunologically distinguished in two distinct subgroups (ay and ad). HBV has 10 major serotypes and four HBsAg subtypes have been recognized (adw, ady, ayw, and Ayr) (8). The serological detection of HBsAg is a powerful method for the diagnosis and prevention of HBV infection and ELISA has become an extensively used analytical system for screening of blood donors and clinical diagnosis of HBV in infected individuals (9).

Polysaccharide immunomodulators were first discovered over 40 years ago. Mannan and mannoprotein fractions are derived from digested surface cell walls of C. albicans, and their role in the immunization was determined (10). These polymers can influence innate and cell-mediated immunity through interactions with T cells, monocytes, macrophages, and polymorphonuclear lymphocytes. The ability to modulate the immune response in an appropriate way can enhance the host's immune response to certain infections (11).

Immunization with C. albicans mannoproteins (MAN) in mice showed immunopotentiator effects on the three cell types (antigen presenting cells, T cells, and B Cells) that are involved in immune responses (12 ,13) . The purpose of this study was to determine the effect of the Candida albicans cell wall mannoprotein on phagocytic activity: antibody production: serum gammaglobuline percentage and lymphocyte transformation index after vaccination with hepatitis B surface (HBs) antigen.

MATERIALS AND METHODS

Media:

culture media were used in the experiments were: Agar agar, Sabouraud dextrose agar, Sabouraud dextrose broth, Tryptase soya agar, Tryptase soya broth (Difco, USA). FITC-Rabbit Anti-Mouse IgG (H+L), Trypan blue stain (The Institute of Sera and Vaccines, Baghdad, Iraq), Hellabio agarose gels (Hellabio, Spain), Nitro blue tetrazolium (Sigma, USA), MTT (Sigma, USA).

Fungal cells:

Candida albicans was isolated, cultured, and maintained from women with vaginitis. The isolated strain was identified by using Candida check (14). Identification of Candida albicans was performed according to the method of (15), by conducting biochemical test (germ tube) which is considered as specific test for identification the Candida albicans microscopically and crossly. Microbiological observations of pseudohyphae, hyphae and chlamydospores were made on cornmeal tween 80 agar incubated at 35°C for 3 days. Culture medium GYEP containing 2% glucose, 0.3% yeast extract and 0.1% peptone (supplemented with penicillin 100 IU/mL and streptomycin 100 µg/mL) were used for *C. albicans* (15).

To Prepare Candida *albicans* Cell Wall Mannoproteins, Candida colonies were harvested by washing method (18), 2 liters of culture medium were subjected to further purification including ultra-centrifugation to prepare mannoproteins, which had a final weight of 2.8 grams. Total protein was estimated by UV spectrophotometry method and glucose was estimated by method (16).

Experimental animals:

Two-hundred mice were divided into eight groups (25 each) used in this study.

Group I: mice were injected subcutaneously with a single dose (0.2 ml) of deionized distilled water at 1st day.

Group II: mice were injected subcutaneously with a high dose (200 ug/ml) of mannoproteins in a total volume (0.2 ml) at 1st day.

Group III: mice were injected subcutaneously with a moderate dose (300 ug /ml) of mannoproteins in a total volume (0.2 ml) at 1st day.

Group IV: mice were injected subcutaneously with a single dos*e HBs* vaccine at 1st day.

Groups V and VI: mice were injected subcutaneously with a single dose of *combination of HBs vaccine* vaccinated moderate and high dose respectively at 1st day.

Groups VII and VIII: mice were injected subcutaneously with a single dose of prednisone 5 days prior to the combination of the *HBs* vaccine and moderate and high dose of mannoprotein at 1st day.

Laboratory method used in the present study was Nitro blue Tetrazolium (NBT) index. The assay was carried out on peripheral blood of immunized mice according to a method presented by (18). The procedure of MTT assay (3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) measured by Elisa to assess the lymphocytes transformation percentage after an *in vitro* stimulation with specific antigen (19) . The IFAT was used to assess anti-*HBs* antibody titer in the sera of mice that were immunized with *HBs* vaccine in different treatment regimens. The procedure of WHO (1997) was adopted to determine such titer (20). Serum electrophoresis was carried out using a commercially available kit (Hellabio, Spain). Statically analysis the values of the investigated parameters were given in terms of means ± standard errors (S.E.), and differences between means were assessed by conducting analysis of variance (ANOVA), least significant difference (LSD) and Duncan test, using the computer programmer SPSS (Statistical Package of Social Sciences) version 7.5. The difference was considered significant when the probability value was equal or less than 0.05.

RESULTS AND DISCUSSION

The prepared solution of mannoproteins revealed that it was 82 mg/ml, while glucose content was 78 mg/m estimated.

After the tabulated procedures and calculations the LD_{50} of *C. albicans* cell wall mannoproteins range a widely between (from 100 - 600 ug /mouse) (Table 1). Based on these findings, dose of 200 ug /mouse was considered as the moderate dose and 300 ug / mouse as the high dose in the present study (21).

Table (1): Doses of *C. albicans* cell wall mannoproteins that were used in the assessment of LD50

	Dose/mouse	Dose/Kg	Number of Animals	Mortality Rate (%)
***C. albicans* Cell Wall Mannoproteins**	100 µg	4 mg	6	0.0
	200 µg	8 mg	6	0.0
	300 µg	12 mg	6	0.0
	400 µg	16 mg	6	0.0
	500 µg	20 mg	6	0.0
	600 µg	24 mg	6	0.0

Safety assessments of mice treated with different materials (mannoproteins, prednisolone and HBs vaccine) in the present study revealed no major alternations in the general activities of the animals. Their weights showed no significant changes between pre-and post-treatments and their food consumption were normal.

Furthermore, there were no clinical signs, which may reflect a deleterious effect of the treatment.

The results of NBT index were given in table (2). All groups of mice showed different significant increases in the NBT index which represented the phagocytic activity% as compared to group I (0%), which was injected with deionized water (control negative group). The best NBT index was recorded in group VI (268%), which included mice that were treated with combination of 300 µg/ kg of Candida albicans mannoproteins and *HBs* vaccine while lowest NBT index was recorded in group II (105.8%) included mice that were treated with 200 µg/ kg of Candida albicans mannoproteins only.

Table (2): Nitro blue tetrazolium (NBT) index in treated mice

Groups	NBT OD (mean ± S.E.)*	phagocytic activity%
I	1.03±0.15 ^{c}	0%
II	2.12±0.84 ^{b}	105.8 %
III	2.36±1.03 ^{b}	129.13 %
IV	3.02±1.02 ^{b}	1.93.2 %
V	3 .35±1.02 ^{a}	225.25 %
VI	3 .80±1.04 ^{a}	268.93%
VII	2.88±1.11 ^{b}	276.69 %
VIII	2.90±1.09 ^{b}	181.55 %

**a, b, c: Significant differences (P≤0.05) between means of the same column*

Results of NBT index showed a significantly increase percentage in immunized mice and are also in favour of such agreement.

Phagocytic activity by reduction of nitro blue tetrazolium (NBT) to insoluble blue Formozan granules occurred during the stimulus-induced respiratory burst of mature granulocytes. Nitro blue tetrazolium (NBT) test addition of the yellow NBT dye to plasma results in the formation of a NBT–heparin or NBT–fibrinogen complex, which may be phagocytosis by neutrophils (22).

Normal neutrophils showed little incorporation of the complex unless they are 'stimulated' to phagocytic activity, e.g. by the addition of endotoxin. This technique was used to measure the degree of 'stimulation' of untreated cells or their capacity for phagocytosis after stimulation. Stimulated neutrophils incorporated the dye complex into phagosome and, after lysosomal fusion, intracellular reduction results in the formation of blue insoluble crystals of formazan.

The percentage of phagocytic cells may be determined using a light microscope or, as described below, the total dye reduction may be quantified spectrophotometrically after alkaline DMSO, which reacts with NBT to produce coloured diformazan (23).

Although macrophages and monocyte possess killing mechanisms in the resting state, these mechanisms can be enhanced, and new mechanisms can be expressed when they are activated. Activation can occur through exposure to microbial products (i.e. *C. albicans* cell wall mannoproteins and HBs antigen). Such immunomodulators can cause a direct activation of phagocytes, or indirect activation through triggering cytokine release from them to induce macrophage for killing intracellular bacteria (24). Once the organism is internalized, it is exposed to an array of killing mechanisms; oxygen-dependent killing mechanisms

(this pathway is also called reactive oxygen intermediates; ROIs) and reactive nitrogen intermediates (RNI) (25).
Oxygen-independent killing mechanisms are also a further pathway, which may be more important than was previously thought, because many organisms can be killed by cells from patients who cannot produce ROIs (10). It is expected that the isolated mannoproteins are effective immunomodulators. These are in agreement with this conclusion, several researchers suggested the potentianal use of *C. albicans* cell wall mannoproteins in this line of experimental immunology by using different laboratory approaches and animals (13, 25- 27).

Lymphocyte transformation index by MTT assay:
Table (3) showed the results of lymphocyte transformation index. Mice showed different significant increases in the MTT index, which represent the lymphocyte transformation index % as compared to group I(control negative group) (0%), which was injected with deionized water (control group). The best MTT index was recorded in-group VI (271.79%), which included mice that were immunized with combination of 300 µg/ kg Candida albicans mannoproteins with *HBs* vaccine while the lowest index was recorded in-group II (117.9 %) included mice that were injected with 200 µg/ kg of Candida albicans mannoproteins only.

Table (3): Lymphocyte transformation index in treated mice

Groups	Lymphocyte Transformation OD (mean ± S.E.)*	Lymphocyte Transformation activity index %
I	0.078 ± 0.02 ^{f}	0 %
II	0.17 ± 0.02^{e}	117.9 %
III	0.19 ± 0.01^{d}	143.6 %
IV	0.28 ± 0.01 ^{a}	258.97 %
V	0.18 ± 0.01^{d}	130.76 %
VI	0.29 ± 0.01 ^{a}	271.79 %
VII	0.19 ± 0.01^{d}	143.59 %
VIII	0. 20 ±0.1^{c}	156.41 %

a, b, c: Significant difference (P≤0.05) between means of the same column

The lymphocyte transformation test (LTT) has been an *in vitro* test of the lymphocytes, which have been sensitized by a certain antigen, transform into blasts and proliferate when they are again exposed to this antigen. This proliferation is determined by MTT (3-[4, 5-dimethyl-2-thiazolyl] -2, 5-diphenyl -2H-tetrazolium bromide)-reduction method measured by ELISA. The test has the advantage over skin tests of avoiding re-exposure of individuals (28). However, the LTT measures only the sensitization of lymphocytes, but not the effector reaction, i.e., there may be positive results in exposed individuals even in the absence of clinical symptoms. Different research groups for the evaluation of various cell-mediated immune reactions have applied the test. The principle of the LTT is based on the fact that lymphocytes, which have been sensitized by a certain antigen (memory cells), transform into blasts and proliferate when they are again exposed to this antigen, (29). The MTT [3-(4, 5-dimethyl-2-thiazolyl) -2, 5-diphenyl -2H- tetrazolium bromide] were based on the capacity of viable cells to reduce MTT to formazan that was assayed by spectrophotometric quantitation of optical density (OD) after its extraction with acid-propanol, with the OD taken as a measure of the metabolic status and the total, viable mass of the *Candida* cells. Development of the protective immune response to HBsAg is T-cell dependent and is associated with the production of specific neutralizing antibodies The immunobiology mechanism may be due to increase of T-lymphocytes CD receptors; MHC 1 and enhance cytokines production result in stimulates TH1 cells and macrophages, and then causes an elevation of both immunoreactive and bioactive TNF-alpha and gamma interferon in serum and mesenteric lymph nodes (30).

Indirect Fluorescent Antibody Test (IFAT):
The sera of mice in groups II, III, and I showed no anti-*HBs* antibodies at the start titer 1:16 after 21 days off vaccination, while the other groups showed some variations. All mice of group VI showed a higher positive immunoflourescent reaction at the titer 1:512, while the other groups IV and VIII showed a positive reaction which was observed at the titer 1:64. After 28 days the sera of mice in groups II, III, and I showed no anti-*HBs* antibodies at the start titer 1:16, while the highest anti HBs antibodies titer was recorded in mice of group VI at the titer I: 512. After that positive Immunoflourescent reaction at the titer 1:128 was observed in mice of groups V after 28 day of vaccination (tables 4 and 5).
Immunofluorescence is the visualization of antigens within cells using antibodies as fluorescent probes. Anti-HBs antibodies showed an increased titer in all immunized groups treated with the immunomodulators used in the study, especially groups V1 and IV as compared to the control group that received vaccine only. Such observation suggests that the immunomodulation also involved the humoral immune response, although the pathway may be through the modulation of macrophages and T lymphocytes as both types of cells are required to enhance the B-lymphocytes to produce immunoglobulin (10). Development of the protective immune response to HBsAg is T-cell dependent and is associated with the production of specific neutralizing antibodies. Previous studies in nonresponsive but otherwise healthy people did not find defects in antigen uptake or processing by antigen-presenting cells. However, the different cell surface glycoproteins responsible for presenting protein antigens to $CD4^{+}$ T cells, largely contributes to the human antibody response to HBV vaccine. These are in agreement with conclusions that were conducted by several researchers, who suggested the potentianal use of C. albicans cell wall mannoproteins in this line of experimental immunology by using different laboratory approaches and animals (16 and 27).

Table (4): Anti-*HBs* antibody titer in sera of treated mice after 21 days

Groups	AntiHBs antibodies titer after 21 days								
	16	32	64	128	256	512	1024	2048	4096
I	0	0	0	0	0	0	0	0	0
II	16	32	0	0	0	0	0	0	0
III	0	0	0	0	0	0	0	0	0
IV	16	32	64	0	0	0	0	0	0
V	16	32	64	128	0	0	0	0	0
VI	16	32	64	128	256	512	0	0	0
VII	16	32	64	0	0	0	0	0	0
VIII	16	32	64	0	0	0	0	0	0

Table (5): Anti-*HBs* antibody titer in sera of treated mice after 28 days

Groups	AntiHBs antibodies titer after 28 days								
	16	32	64	128	256	512	1024	2048	4096
I	0	0	0	0	0	0	0	0	0
II	16	32	64	0	0	0	0	0	0
III	0	0	0	0	0	0	0	0	0
IV	16	32	64	128	0	0	0	0	0
V	16	32	64	128	256	0	0	0	0
VI	16	32	64	128	256	512	0	0	0
VII	16	32	64	128	0	0	0	0	0
VIII	16	32	64	128	0	0	0	0	0

Gamma globulin serum fraction:

The results of gamma globulin fraction are shown given in (table 6).

The highest significant increase in the percentage of gamma globulin fraction was observed in groups VI and V (46.53%) and (34.54 %) as compared to group I (24.06%) at 21 days after vaccination, while the highest percentage of gamma globulin fraction was observed in VI and IV groups (35.50 % and 32.55%) after 28 days of vaccination of mice. The lowest percentage of gamma globulin fraction was showed in-group II (24.24 %) after 28 days of vaccination.

Table (6): Gamma globulin serum fraction in treated mice

Groups	Gamma Globulin Serum Fraction (mean ± S.E.) %)*		Probability** ≤
	After 21 days	After 28 days	
I	24.06 ± 0.15 ^{d}	22.63 ± 0.12 ^{e}	0.05
II	25.23 ± 0.23 ^{c}	24.24 ± 0.12 ^{d}	0.05
III	27.23 ± 0.23 ^{c}	26.34 ± 0.12 ^{c}	0.05
IV	33.86 ± 0.25 ^{b}	32.50 ± 0.06 ^{b}	0.05
V	34.53 ± 0.12 ^{b}	29.55 ± 0.06 ^{c}	0.01
VI	46.84 ± 0.35 ^{a}	35.37 ± 0.09 ^{b}	0.01
VII	30.36 ± 0.25 ^{b}	29.16 ± 0.25 ^{c}	0.05
VIII	31.36 ± 0.25 ^{b}	30.26 ± 0.25 ^{b}	0.05

** Different letters: Significant difference (P≤0.05) between means of the same column.*

*** The comparison is between means of the two columns (horizontal comparison*

Serum electrophoresis was carried out using a commercially available kit (Hellabio, Spain). The Hellabio Agarose Gels for protein electrophoresis are intended to be used for *in vitro* diagnosis, and they enable quantitative and qualitative estimation of proteins in serum and other biological materials. After serum gel electrophoresis, five fractions (albumin, α1, α2, β and γ globulin) were recognized, which were given as percentages of the total. These result was supported the effectiveness role of Candida albicans cell wall mannoprotein on humoral immune response in mice vaccinated with HBs vaccine.

The evaluation of *C. albicans* cell wall mannoproteins LD_{50} demonstrated a dose of a wide range safety (100 to 600 µg/kg), also was effective in terms of toxicity and immunomodulatory backgrounds. The *C. albicans* cell wall is essential to nearly every aspect of the microorganism biology and pathogenicity, because it contains materials that are able to mediate interactions with the host immune response (11,13). These contents are mainly polysaccharides in addition to proteins and minor amounts of lipids (26, 27). Therefore, it was expected that the isolated mannoproteins are effective immunomodulators. In agreement with this conclusion, several researchers enhanced the potentional use of *C.*

albicans cell wall mannoproteins in this line of experimental immunology using different laboratory approaches and animals (11-13, 24- 26, 31, 32). It was shown that the extraction of mannoprotein from the intact cell wall of yeast using chemical method. Using this procedure, it was expected that purified antigens should retain main epitope features and conformational characteristics such that they may be successfully used as immunogenic and antigen base for assay development (16).

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Performance of molecular and serological methods for hepatitis C virus diagnosis in patients from Anbar Governorate, Iraq: A comparative evaluation

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ABSTRACT

Hepatitis C virus (HCV) infection is a significant health problem throughout the world. HCV is a causative agent for acute, chronic and fulminate hepatitis. This study was designed to evaluate any correlation between 3rd generations ELISA positivity, Rapid immunochromatographic assay for anti-HCV antibody and Real-time PCR based detection among various categories of patients in Anbar Province for establishing the diagnosis of hepatitis C virus (HCV) infection.

Fifty serum samples collected from HCV adult patients (male and female) were collected and analyzed for anti-HCV antibodies using Rapid immunochromatographic assay and enzyme-linked Immunosorbent assay (ELISA) methods. Positive samples were selected to real time polymerase chain reaction (RT-PCR) for the quantitative detection of hepatitis C virus in human plasma and the simultaneous detection of HCV. Twenty apparently healthy individuals were included as control group.

Out of fifty seropositive patients by rapid immune chromatography assay (Strip test), 46 (92%) were found to be seropositive by ELISA ($P < 0.05$). Out of them, 30 (65.2 %) patients were showing positive viral load (copies and IU/ml) and 40 % of them were showing negative viral load results. Sixteen 16(32%) of the negative viral load patients were considered as non-detectable (ND) viral load patients because they were showing positive ELISA test for hepatitis. Regarding RT-PCR as Gold test, the study interprets the following findings for RT-PCR :-Sensitivity = 60%, Specificity = 100%, Accuracy = 71%, Negative predictive value= 50%. The range of viral load in patients was 100.000- 426.225.60 IU/ml.

The current study concluded that there is no correlation between ELISA and viral load in hepatitis C virus infection ($P>0.05$), also indicated that seropositivity does not reveal the presence of active HCV infection. On the other hand, Real Time PCR is diagnostic confirmatory test and considered as the golden test for the diagnosis and follow up of hepatitis c virus infection.

Keywords: HCV, Rapid immune chromatography assay, ELISA, RT-PCR

الملخص باللغة العربية

يعتبر التهاب الكبد الفيروسي من النوع سي من أكثر المشاكل الصحية خطورة في العالم، إذ إن الفيروس يسبب التهاب الكبد الحاد والمزمن وتليف الكبد مع ارتفاع الوفيات بالإضافة إلى تسببه بسرطان الخلية الكبدية . صممت هذه الدراسة لتقويم الترابط بين الجيل الثالث لاختبار الاليزا الموجب والاختبار المصلي السريع لأضداد فيروس الكبد من النوع سي واختبار البلمرة المتسلسل الكمي لمختلف المرضى المصابين في محافظة الأنبار لإرساء طريقة تشخيص التهاب الكبد الفيروسي من النوع سي.

تم جمع عينات مصل دم من خمسين مريض من كلا الجنسين لتشخيص إصابتهم بالتهاب الكبد الفيروسي من النوع سي، وقد أجريت اختبارات الانتشار السريع المناعي واختبار الاليزا من الجيل الثالث، وبعدها تم فحص العينات الموجبة باستخدام فحص البلمرة المتسلسل الكمي(RT-PCR) للكشف الكمي لفيروس الكبد في بلازما المرضى. وكانت المجموعة الضابطة مكونة من عشرين شخص بأعمار متماثلة مع أعمار وأجناس المرضى.

من بين (50) مريضا كانت نتيجة اختبار الانتشار المناعي موجبة لهم ، فقد ظهرت نتائج 46 منهم موجبة لاختبارالاليزا ($P< 0.05$). وكانت لثلاثين شخص منهم 65.5 موجبة، فيما تم اعتبار (16) مريضا الباقين منهم بحكم الحالة غير المشخصة (ND) وذلك لأن اختبار الإليزا كان لديهم موجبا عند اعتبار RT-PCR هو الاختبار الذهبي، وقد كانت نسبة الحساسية له 60 % والخصوصية 100% ، الدقة 71%، والقيمة التنبؤية السالبة 50%. وكان مدى معيار الفيروس في المرضى 100000-426.225.26 وحدة دولية / مل . ينتج من هذه الدراسة عدم وجود ترابط بين اختبار الإليزا ومعيار فيروس الكبد من النوع سي ($P<0.05$) وتبين أيضا أن ايجابية الاختبار المصلي لا تبين وجود التهاب الكبد الفعال، وكذلك فإن اختبار البلمرة المتسلسل الكمي هو اختبار تأكيدي ويعتبر الاختبار الذهبي لتشخيص ومتابعة التهاب الكبد الفيروسي من النوع سي.

INTRODUCTION

HCV is a causative agent for human chronic, acute and fulminant hepatitis (1,2). Hepatitis-C virus (HCV) belongs to the family Flaviviridae. It is a spherical, 30- 60nm in diameter, enveloped, single stranded RNA virus. Hepatitis C virus (HCV) spreads parentally, either through intravenous drug use or, in lesser-developed countries, through blood and its products, contamination during medical procedures and infected syringes (3-5).

Several assays are used to diagnose HCV infected patients, but measurement of HCV RNA levels has become an important part of the management of patients. Real Time PCR test allows for detection of PCR amplification products during the early phase of the reaction and provides a distinct advantage to detect precise PCR products at the end-point of the reaction (6-8). Qualitative and quantitative methods for HCV RNA viral load investigations are used to diagnose chronic HCV infection, identify patients who need antiviral therapy, monitor the virological responses to antiviral therapy, and document treatment failure (9,10). The high diversity of viral isolates will probably make it very difficult to develop a vaccine and therapeutic modalities are still limited (11). This study was designed to evaluate any correlation between third generations ELISA positivity, Rapid immunochromatographic assay for anti-HCV antibody and Real-time PCR based detection among various categories of patients in Anbar Province for establishing the diagnosis of hepatitis C virus (HCV) infection.

PATIENTS AND METHODS

Fifty HCV patients from both sexes were included in this study, they were attending Ramadi Teaching Hospital , Ramadi Maternity and Child Teaching Hospital , Hit General Hospital and private Clinics. The study was conducted during the period extended from April to December 2014. All inclusion and exclusion criteria were applied for patients; they were examined by senior physician to diagnose their cases. A total sample of 20 sera were collected from healthy volunteers having no history of any liver complications or hepatic disorder was included as negative controls. Informed consent was from each patient was applied.

Five ml of fresh blood was taken from each patient by vein puncture; three ml were collected in sterile EDTA tube to pool plasma. Plasma was transferred to sterile Eppendorf tubes and immediately stored at -20 ^{o}C till be used for molecular Investigations (Real-time PCR). Serum was pooled from 2 ml fresh blood in sterile plastic tube and immediately stored at -20 ^{o}C until used for serology**.**

Serological investigations:

Rapid immunochromatographic assay: The detection of antibodies of HCV in the samples was performed by one step cassette style anti-CV device as per instructions from the manufacturer (AponBiopharm, China) The presence or absence of anti-HCV antibodies in the samples was determined by appearance of specific colored line on the cassette.

Detection of Hepatitis C antibody by ELISA: All serum samples were tested for the presence of antibodies to HCV with a commercial ELISA kit (Biotech, U.S.A). Serum or plasma samples are added to these wells. If antibodies specific for HCV are present in the sample, they will form stable complexes with the HCV antigens on the well. Excess sample is removed by a wash step and a rabbit anti-human IgG conjugated with peroxidase is then added and allowed to incubate. The conjugate will bind to any antigen-antibody complexes formed. After a second wash, a solution of enzyme substrate and Chromogen is added. This solution will develop a blue color if the sample is positive. The blue color changes to yellow after blocking the reaction with sulphuric acid. The intensity of color measured by (ELISA reader, Awareness, USA) at 450 nm and it is proportional to anti HCV antibodies concentration in the sample. Wells containing negative samples remain colorless (12).

Molecular investigation:

A-HCV RNA Extraction: HCV RNA was isolated from plasma samples with (SACACE Ribo-Sorb kit, Italy) using the silica based technology as mentioned by (Buckingham and Flaws., 2007) (7). With SACACE Ribo-Sorb Virus kit, Italy, RNA viruses are lysed quickly and efficiently by lysis buffer RAV1 which is highly concentrated solution of GITC. Lysis buffer and ethanol create appropriate conditions for binding of nucleic acids to the silica membrane in the Ribo virus columns. Carrier RNA improves binding and recovery of the low- concentrated viral RNA. Contaminations (potential PCR inhibitors) like salts, metabolites and soluble macromolecular cellular components are removed in simple washing steps with ethanolic buffers RAW and finally RAV3. The nucleic acids can be eluted in low salt buffer or water and are ready-for use in subsequent reactions. The prepared nucleic acids are suitable for applications like RT-PCR. The detection limit for certain viruses depends on individual detection procedures e.g. in – house nested (RT- PCR). It was highly recommended the use of internal standards as well as positive and negative controls in order to monitor the purification, amplification and detection processes. The real time amplification must be performed on the same day of extraction.

B- HCV real-time quantification (Amplification): Kit HCV Real-TM Quant is a real-time test for quantitative detection of hepatitis C virus in human plasma. HCV RNA is extracted from plasma, amplified and detected using fluorescent reporter dye probes specific for HCV or HCV IC. Internal control serves as an extraction and amplification control for each individually processed specimen to identify possible inhibition. IC is detected in a channel other than the HCV RNA. Monitoring the fluorescence intensities during real time allows the detection and quantification of the accumulating of the accumulating product without having to re-open the reaction tube after the real time amplification (7,13).

Reagent for amplification was prepared as the follows:

1- One set of reagents was thawed, the tubes were vortexed and centrifuged briefly.
2- Reaction tubes or PCR plate were Prepared.
3- Reaction Mix preparation: In to the tube with DTT,300µl of RT –PCR-mix-1, 200µl of RT –PCR-mix-2,20 µl of host start Taq polymerase and 10µl of M-MLV Revertase was added. The contents were vortexed thoroughly and centrifuged briefly. This mix is stable for 1 month at -20 ^{o}C. Then each sample (N) was added in the new sterile tube 12.5*N µl of mix,0.5 *N µl of Taq F polymerase and 0.25* N µl of M-MLV.
4- 12.5 µl of reaction mix added into each tube.
5- 12.5 µl of extracted RNA sample was added to the appropriate tube with reaction mix and mixed by pipetting if the Ribo-Sorb isolation kit , re-centrifuged all the tubes with extracted RNA for 2 min at maximum speed (12000-16000g) and supernatant was taken carefully .N.B. we do not disturb the pellet, sorbent inhibit reaction.
6- For each run 6 standards and 1 negative control were prepared:
*12.5 µl of quantitation standards HCV (QS1 HCV, QS2 HCV, QS3 HCV)were added into tubes. 12.5 µl of quantitation standards HCV (QS1 IC, QS2 IC, QS3 IC) were added in to labeled tubes.
*12.5 µl of TE-buffer was added to the tube labeled negative control.
Tubes were closed and transferred to real-time PCR instrument.

Protocol Name

Sacace HCV Real-TM Quant

Stage 1

Hold

Temp	Secs	Optics
95.0	900	Off

Stage 2

Repeat times: 42

2-Temperature Cycle

Deg/Sec	Temp	Secs	Optics
NA	95.0	20	Off
NA	60.0	40	On

** smart cycler RT-PCR program used for amplification of the target DNA for HCV RNA gene according Sacace Biotechnologies kit.*

Test interpretation:

In the menu (Analysis settings), the value 20 was chosen for the channels Fam and CY3. In the table of results (Results Table) appear the values of Ct (threshold cycle) for Fam and CY3 channels. The calculation of HCV RNA concentration in the clinical specimens sample and standards can be performed in the same experiment, but with the Smart Cycler software it is possible to calculate the samples concentration by importing the experiment with standard curve in the experiment with clinical samples. The curve was improved from another experiment clicking on Import Std. Curve. In any case, if the calibrators were inserted with the clinical samples in the same experiment or after the importation of standard curves from another experiment, in the table of results, in the column FAM Std./Res for IC HCV and in the column CY3 Std./Res for cDNA HCV reveals the calculated values.

Results interpretation:

The internal control(IC) was detected on the FAM channel and HCV RNA on the CY3 channel. For each control and patient specimen, the concentration of HCV RNA was calculated by using the following formula:

$$\frac{\text{HCV RNA copies/specimen(the CY3 channel)}}{\text{IC RNA copies/specimen (FAM channel)}} \times \text{coefficient} = \text{IU HCV/ml}$$

Where, coefficient is specific for each lot and it is reported in the HCV TM RG Quant Data Card provided in the kit**.**

Results may also be calculated using (HCV Quant Result calculation sheet) provided with the kit. To obtain the results in copies/ml multiply the IU HCV/ml value by 4)
IU RNA HCV/mL × 4 = copies RNA HCV / ml

Analytical specificity:

The analytical specificity of the primers and the probes was validated with 80 negative samples. They did not generate any signal with the specific HCV primers and probes. The specificity of the kit HCV Real-Tm Quant was 100%. The potential cross-reactivity of the HCV Real-Time Quantitation was also tested against the group control. Any cross-reactivity with these pathogens was not observed**.**

Analytical sensitivity:
The kit HCV Real-Tm Quant allows to detect HCV RNA in 100% of the tests with a sensitivity not less than 200 IU/ml (value obtained using the "Magno-Virus" extracted kit Sacace REF K-2-16 and Rotor Gene 6000). The detection was carried out on the control standard and its dilutions by negative plasma**.**

Statistical analysis:
Statistical analysis was done using Student's t test, significance ,P value used was (P<0.01). Chi-square (X2) test for significance was done , P value was (P<0.05). Standard deviation (SD) was done for molecular and serological parameters. The statistical significance of difference in mean of variable between more than two groups was assessed by ANOVA test. Probability values of P<0.05 were considered statistically significant (14).

RESULTS

Rapid immunochromatographic assay result:
All study patients revealed positive rapid immunochromatographic assay while all the individuals within the control group showed negative results with high statistical difference (P < 0.0005).

Enzyme linked immunosorbent assay result:
Regarding ELISA test, all individuals of control group revealed negative ELISA tests results for anti-hepatitis C virus antibody in their sera while 46 (92%) of patients showed positive ELISA results (Figure 1). So high significant difference (P < 0.0005) was found between results of ELISA of control individuals and patients.

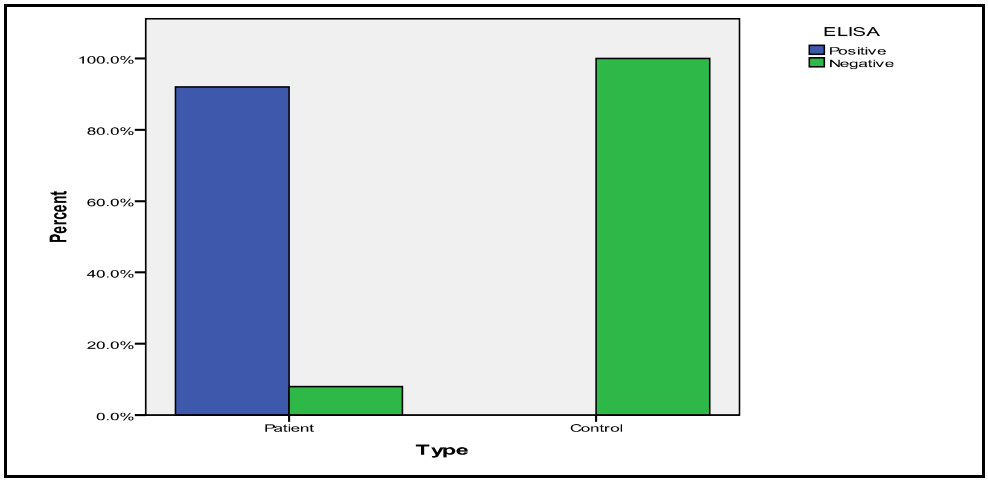

Figure (1): ELISA test results in patients and control group

Molecular RT-PCR result:
The mean of viral load in patients was 42.622.560 IU/ml.

Viral load versus rapid immunochromatographic assay results:
High significant difference (P<0.0005) was found between number of positive viral load patients and patients with positive rapid immunochromatographic assay. Sixty percent (60%) of patients with positive rapid immunochromatographic assay showed positive viral load (Figure 2).

Viral load versus ELISA results:
Among 46 ELISA positive patients,30 (65.2 %) patients were showing positive viral load (copies and IU/ml).While all ELISA negative patients were showing negative viral load results (100%). High significant difference (P< 0.00001) was found between results of patients and control group, all control individuals were showing negative viral load results while 60 % of patients were showing positive viral load results and 40 % of them were showing negative viral load results. Among negative viral load patients,16 (32%) patients were considered as non-detectable (ND) viral load patients because they were showing positive ELISA test for hepatitis C Ab.(Figure 3).

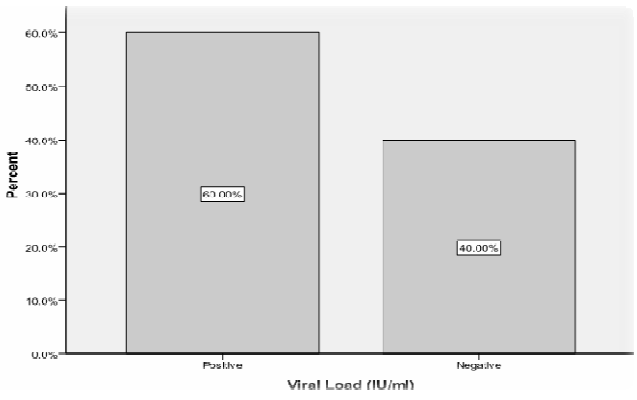

Figure (2): Viral load rapid immunochromatographic assay results

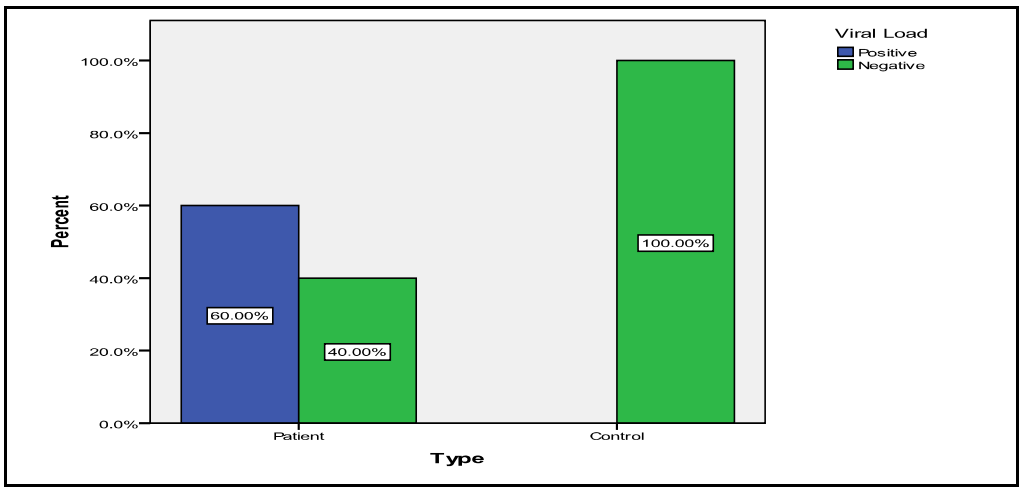

Figure (3):Viral load results in patients and control group

Concerning ELISA as a golden test, the results showed the following findings for Real-time PCR:-
Sensitivity = 60%
Specificity = 100%
Accuracy = 71%
Positive predictive value = 100 %
Negative predictive value= 50% (figures 4a, 4b).

Viral load correlation with Elisa test result:
There was no correlation between ELISA and viral load Viral Load/IU/ml or viral load/copy with both the P value was 0.419 (P>0.05). Although both the correlations were negative but they were not significant (Figure 5).

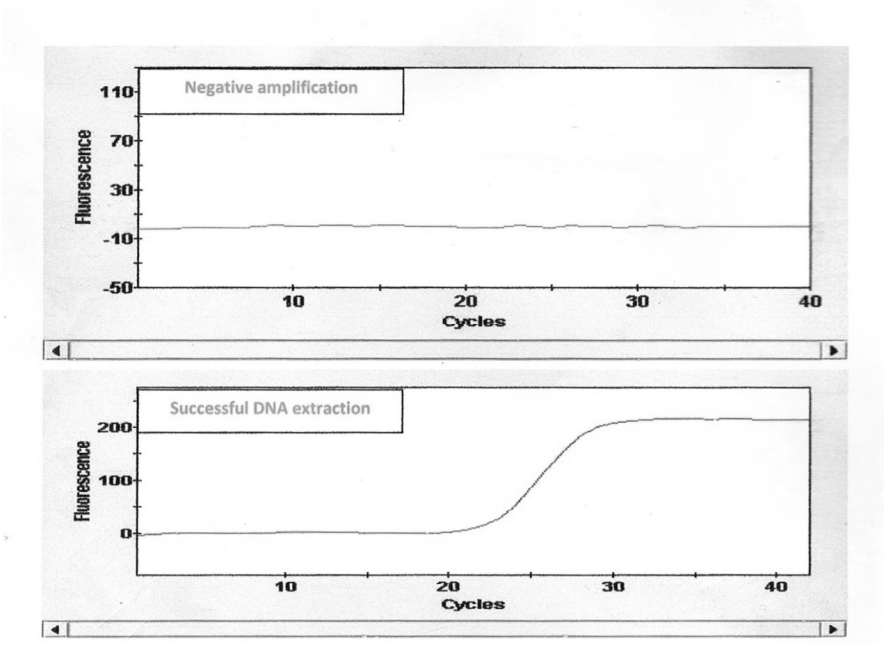

Figure (4 a): Result of successful DNA extraction and negative amplification of the target gene

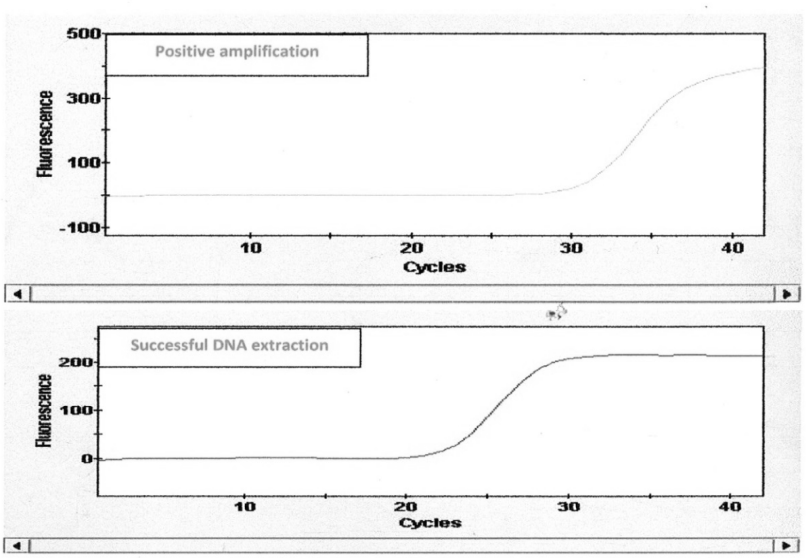

Figure (4 b): Result of successful DNA extraction and positive amplification of the target gene

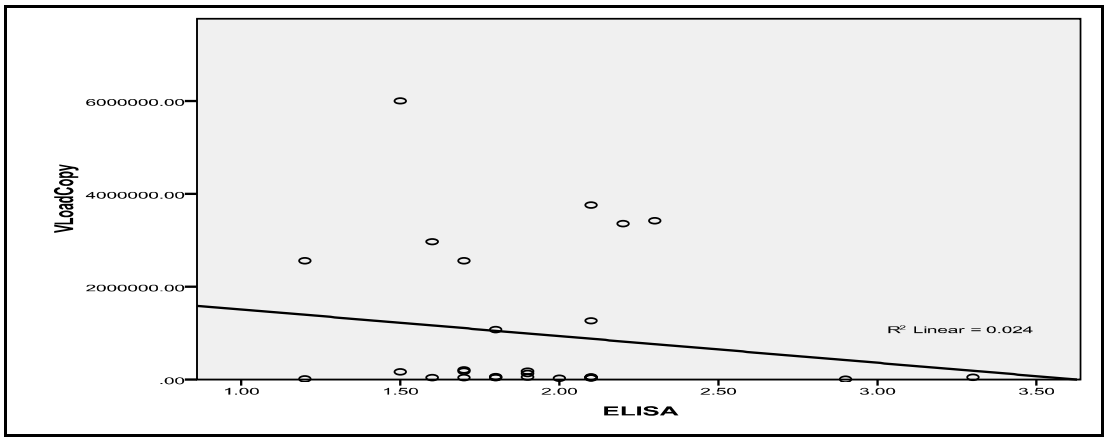

Figure (5): Correlation between ELISA test and Viral load results

DISCUSSION

Immune-chromatographic assay versus ELISA:

In the present study, it was found that the results of rapid immune chromatography assay (paper chromatographic method or strip test) agreed with those of ELISA in 46 cases out of 50 (92% of all cases). These results were in accordance with the findings of (15, 16).

Reverse transcriptase -PCR versus ELISA results:

Out of 50 positive samples by rapid immune-chromatographic assay, 46 (92%) were positive by ELISA. 30(65.2%) of them showed positive PCR 16 (32%). However, the results of the two techniques should be interpreted with caution because during the course of infection, when the virus is cleaned up, only the antibody remains positive, and the nucleic acids are generally not detected. Previous report by (17) indicated that HCV may persist in the liver in the absence of serum positive PCR test. Therefore, it appears that initially a patient may be HCV positive by all tests but clearing up of virus from the serum later and becomes serum PCR negative, yet remains antibody positive and liver PCR positive or these patients may suffer from hepatitis C at the chronic phase of infection, existing antibody without any viruses (18, 19).

In the current study, among negative RT-PCR result, 16 (34.8%) revealed non-detectable results (below threshold limit, 200IU/ml). Wang *et al* . (15) concluded that a possibility of PCR false negative results considering the sensitivity of the assay or this might indicate the resolution of HCV, acute HCV during the period of low-viremia, or false anti-HCV positive. The sensitivity of RT-PCR in our study 60% when the ELISA used as gold standard, The sensitivity of the RT-PCR increased more than 10-fold than ELISA detection. It could be due to either fluctuation of HCV RNA levels or an emergence of an HCV mutant. Hence, the detection rate of PCR was lower when ELISA was used as a gold standard. The present study was consistent with another study that showed false positive ELISA tests for anti HCV which can be seen in

patients who have cleared the virus after acute infection by therapy and this may be positive on ELISA which may indicate past infection (19). Patients with autoimmune hepatitis and other hyperglobulinemic states give false positive tests, false positive cases have been noted in 23% of patients. This could be due to nonspecific antibodies detected by ELISA or more likely due to previously cleared virus after acute attack (20, 21). In low-risk population (e.g., healthy blood donors), the false positivity of ELISA has been recorded up to 25% in one study while in another study it was 20.21% (20,21).

Some disorders linked to HCV infections, e.g., autoimmune hepatitis, Sjögren's syndrome, Lichen planus, thyroiditis, membranous, and polyarthritis nodosa, and the essential mixed cryoglobulinemia may be screened and if positive, it should be conformed for HCV RNA by RT-PCR (22). Results of positivity of anti-HCV by 3rd generation ELISA of negative PCR cases are in accordance with (23), who have also recommended the use of the polymerase chain reaction for improving the specificity of HCV detection. Few other authors have also concluded that further refinement of antibody screening and confirmatory assays and standardization of molecular testing are necessary to optimize testing and fully characterize the diagnosis of HCV infection (24). The present study agreed with a study mentioned that the false positivity of ELISA has been well documented in the healthy population where the prevalence of HCV infection is low (23, 25). Results of both ELISA and RT-PCR revealed significant difference between control individuals and HCV patients. These findings indicate that both are sufficient for the diagnosis of HCV infection in clinical laboratories in Iraq. In this study, PCR helps to resolve weakly positive or negative ELISA results when clinical signs and/or risk factors are compatible with HCV infection. Here, the antibody was only detected 1-2 weeks after infection, which reflected the immune response of the host, but could not explain the virus replication (20). The sensitivity, specificity and accuracy of 3rd generation ELISA in the current study were 92%, 100%, and 94%, respectively. The positive predictive value, negative predictive values were 100 %, 50%, respectively. These are good enough for a diagnostic assay. Similarly, the specificity of RTPCR was absolute at high sensitivity indicating that it is not only suitable for clinical diagnosis but also suitable for the screening of HCV to prevent the transmission of this disease. Interestingly, when authors combined both techniques, the sensitivity and the specificity were absolute and the diagnostic index was 200% indicating that it is advisable to confirm reactive samples using the two methods, and their combination can be useful in epidemiological studies.

In spite of occasional false positive results of ELISA tests, it has many advantages in the diagnostic setting including ease of automation, ease to use, relative cost-effectiveness, and low variability. Additional confirmatory testing is often helpful and it is better like RT- PCR (26,27).

RT-PCR should be used in all cases of ELISA positive patient to assess recent or past infection and before initiating antiviral therapy. HCV infection clearance/persistence should be assessed by RT-PCR, as the antibody persists for longer duration after the virus is cleared. Similarly, immune suppressed patient should be tested by RT-PCR at some regular intervals, as such high risk patient may have co-infection of HCV. It is suggested that the detection of HCV RNA in serum by RT-PCR has better diagnostic value than the anti-HCV antibody tests alone and that HCV RNA detection in liver tissue is possible even when it is absent in the serum (28,29). In the present study it can be concluded that the RT-PCR was a confirmatory test with higher sensitivity and specificity and considered a rapid, accurate, and reproducible method. This conclusion was in accordance with that of (16), who suggested that the sensitivity of the real time PCR method was the highest with a high dynamic range for determination of HCV viral loads in the clinical laboratory settings. Also it was in accordance with that of (29), who found that real-time PCR system for HCV RNA quantification is sensitive, specific, and precise.

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Serological tests to confirm the diagnosis of toxoplasmic retinochorditis infection

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ABSTRACT

Toxoplasmic retinochoroiditis had failed to determine a detectable systemic immune response. A correct diagnosis of the disease is an essential basis for estimating its clinical burden. This study tries to confirm the clinical presentation. Fifty six patients with active retinal infection were attended Ibn Al-Haitham Teaching Eye Hospital in Baghdad, from July to November 2014. Patient serum samples were screened using anti- Toxoplasma IgG and IgM antibodies, and then IgG avidity test by ELISA technique was used to differentiate between the recent and the more distant toxoplasmic infection. 39 (69.64%) of the patients had infection in one eye, most of them between (21-30) year, while 17 (30.36%) of them had the infection in both eyes, most of them over 40 year. All patients showed negative anti- Toxoplasma IgM antibodies, while positive serum anti-toxoplasma IgG antibodies recorded in 23 (41.07%) patient, most of them below 30 year, 18 (78.26%) of them had low IgG avidity while other 5(21.73%) showed high IgG avidity. In atypical cases, serologic tests such as serum anti-Toxoplasma titers of IgM and IgG may be helpful to support the clinical diagnosis.

Keywords: ocular toxoplasmosis, IgG avidity, serological tests, atypical cases.

الملخص باللغة العربية

لم يتمكن مرض التهاب الشبكية والمشيمية الناتج عن الإصابة بالمقوسات الكونديه من تحديد الاستجابة المناعية الجهازية، ويعتبر التشخيص الدقيق للمرض أساسا لتقدير العبء السريري والعلاجي، ولذلك، تحاول هذه الدراسة تعزيز التشخيص السريري بالاختبارات المصلية، حيث جرى اختبار خمسة وستين مريضا يعانون من إصابات حادة بالشبكية، والذين كانوا من ضمن المراجعين لمستشفى ابن الهيثم التعليمي للعيون في بغداد للفترة من شهر تموز إلى شهر تشرين الثاني من العام 2014. وقد تم فحص مصول المرضى باستخدام اختبار الإليزا للكشف عن الأجسام المضادة للجلوبيولينات المناعية ، ثم اختبار الألف للجلوبيولين المناعي (IgG) لتحديد الفرق بين الإصابة القديمة والحديثة.

أظهرت نتائج الدراسة أن تسعة وثلاثين من المرضى بما نسبته (69.64%) كانت لديهم إصابة في عين واحدة معظمهم ضمن الفئة العمرية (20−30) عاما، بينما كان سبعة عشر منهم بما نسبته (30.36%) لديهم إصابة في كلا العينين ومعظمهم بعمر يقل عن 40 عاما،. وقد أظهر كل المرضى نتيجة سلبية للأجسام المناعية نوع (IgM) ، بينما كانت النتيجة إيجابية للأجسام المضادة (IgG) في ثلاث وعشرين حالة بما نسبته (41.07%) مريضا معظمهم بعمر أقل من 30 عاما، منهم 18(78.26%) أظهر انخفاضا في اختبار الألفا للأجسام المناعيه (IgG) والباقي خمسة مرضى (21.73%) أظهر ارتفاعا في اختبار الألفا للأجسام المناعية من النوع ذاته.

وبذلك تخلص الدراسة إلى أن الفحوصات المصلية مثل قياس معيار الأجسام المضادة للجلوبيولينات المناعيه (IgG) و(IgM) قد تكون مفيدة في تأكيد التشخيص السريري في الحالات غير التقليدية.

INTRODUCTION

Ocular toxoplasmosis is a disease caused by *Toxoplasma gondii* infection through congenital or acquired routes. Once the parasite reaches the retina, it proliferates within host cells followed by rupture of the host cells and invasion into neighboring cells to make primary lesions. Sometimes the restricted parasite by the host immunity in the first scar is activated to infect another lesion nearby the scar (1).

Ocular toxoplasmosis most often presents as a focal necrotizing retinitis. It is generally associated with vitritis and often with anterior uveitis. Less commonly, it may present as a papillitis (2).

Some studies had suggested a possible route of infection from the brain to the eye through the optic nerve; however, now ocular infection is most likely mediated via the bloodstream (3). Norose *et al.* (4) described that the kinetics of parasite load in various areas of the eye revealed that parasite detection in the retina and choroid precedes detection of parasites in the optic nerve, arguing against the optic nerve theory as the main port of entry into the eye. In addition, a hematogenous route of dissemination into the eye is supported by the fact that ocular toxoplasmosis can occur in the absence of toxoplasmic encephalitis (5). Smith *et al*. (6) have found that retinal vascular endothelial cells are more readily infected with *T. gondii* compared with endothelial cells from the other sites of the body, which suggests a preferential infection of the retina by the parasite.

The diagnosis of ocular toxoplasmosis is made by ophthalmic examinations and a variety of clinical presentations that are consistent with *T. gondii* infection of the retina. When this clinical diagnosis cannot be made definitely by a fundoscopic examination, detection of increased *T. gondii* antibody titers in blood and ocular fluids or amplification of *T. gondii* DNA have been used successfully to confirm the diagnosis (3).

The clinical diagnosis of ocular toxoplasmosis may be supported by laboratory tests in 60–85% of cases, depending on the time of sampling (7).

PATIENTS AND METHODS

A retrospective study of 56 consecutive patients with active retinal infection was reviewd by Ophthalmology specialist physicians in Ibn Al-Haitham Teaching Eye Hospital, from July to November 2014.

Information was collected from patients using a questionnaire sheet. All serum samples were screened using anti- *Toxoplasma* IgG and IgM antibodies.

Enzyme linked immunosorbent assay (ELISA-IgM:

According to the manufacturer's instructions of (bioChek Toxoplasma IgM (BC-1085) kit) IgM antibodies to Toxoplasma were determined quantitatively. The test was done by filling the microtiter plate with diluted serum. The microtiter well coated with mouse anti-human IgM antibodies. All the IgM class antibodies present in the sample will bind to the immobilised antibodies.

Enzyme linked immunosorbent assay (ELISA-IgG):

The bioCheck *Toxoplasma* IgG ELISA (BC-1085) kit was used .The *Toxoplasma* IgG ELISA is intended for use in evaluating a patient's serologic status to *T.gondii* infection. Diluted patient serum was added to the micro wells which are coated with purified *T.gondii* antigen (Ag). Antibodies to *T. gondii*, if present in the specimen, will combine with the antigens attached to the well.

The IgG avidity test by ELISA technique:

This test is used to differentiate between the recent and the more distant infection with Toxoplasma gondii in patient serum (GenWay Biotech,Inc, USA).

Microtiter strip coated with Toxoplasma antigen were incubated with diluted serum specimen. After washing, one well was incubated with avidity reagent and the corresponding well with washing buffer. In this step the low avidity antibodies were removed from the antigens whereas the high avidity ones were still bound to the specific antigens. Anti-human IgG labeled with peroxidase was added. The immuncomplex bonded with TMB/substrate gives a blue reaction product. Stopping solution was added to stop the reaction and changing the color of the reaction product into yellow. Absorbance at 450 nm was read using an ELISA microtiter plate reader.

The avidity % was calculated according to the following equation:

$$\text{Avidity \%} = \frac{\text{Absorbance of sample with avidity reagent}}{\text{Absorbance of sample with washing buffer}} \times 100$$

Statistical analysis:

The Statistical Analysis System- SAS (2012) (8) was used to analyze the effects of different factors .Chi-square test was used to compare between percentages in this study**.**

RESULTS

Thirty-seven patients were females and 19 were males. Their ages were between 8-62 years. All patients had retinal infection, 39 (69,64%) of them had infection in one eye, most of them between (21-30) years, while 17 (30.36%) of them had the infection in both eyes, most of them over 40 years. There were high significant ($p<0.01$) differences between age groups and infected eye. (table 1).

Positive serum anti-toxoplasma IgG antibodies were recorded in 23 (41.07%) patients, most of them below 30 years, with high significant ($p<0.01$) differences between age groups and between positive and negative anti- Toxoplasma IgG antibodies (table 2).

All patients showed negative anti- Toxoplasma IgM antibodies as shown in table (3).

After determination the IgG avidity of the positive anti- Toxoplasma IgG antibodies samples, which recorded 23(41.07%), the results showed 18 (78.26%) of them had low IgG avidity while other 5(21.73%) showed high IgG avidity.

Table (1): Retinal infected patients according to age groups and infected eyes

Age group	Total No.	Infected eye (%)		Chi-square
		One eye	Two eyes	
<20	9	8 (88.89%)	1 (11.11%)	13.69 **
21-30	20	16 (80.00%)	4 (20.00%)	11.95 **
31-40	12	9 (75.00%)	3 (25.00%)	11.36 **
>40	15	6 (40.00%)	9 (60.00%)	7.25 **
Total	56	39 (69.64%)	17 (30.36%)	10.29 **
Chi-square	---	9.17 **	9.17 **	---

*** (P<0.01)*

Table (2): Anti-*Toxoplasma* IgG Abs. with difference age groups

Age group	Total No.	Anti-Toxoplasma		Chi-square
		IgG-Positive	IgG-Negative	
<20	9	8 (88.89%)	1 (11.11%)	13.69 **
21-30	20	7 (35.00%)	13 (65.00%)	9.88 **
31-40	12	5 (41.67%)	7 (58.33%)	6.03 **
>40	15	3 (20.00%)	12 (80.00%)	11.95 **
Total	56	23 (41.07%)	33 (58.93%)	6.15 **
Chi-square	---	9.93 **	9.93 **	---

*** (P<0.01)*

Table (3): Anti-*Toxoplasma* IgM Abs. with difference age groups

Age group	Total No.	Anti-Toxoplasma		Chi-square
		IgG-Positive	IgG-Negative	
<20	9	0 (0.00%)	9 (100.0%)	15.00 **
21-30	20	0 (0.00%)	20 (100.0%)	15.00 **
31-40	12	0 (0.00%)	12 (100.0%)	15.00 **
>40	15	0 (0.00%)	15 (100.0%)	15.00 **
Total	56	0 (0.00%)	56 (100.0%)	15.00 **
Chi-square	---	0.00 NS	0.00 NS	----

*** (P<0.01), NS: non- significant*

DISCUSSION

Ocular toxoplasmosis is usually a clinical diagnosis. Laboratory investigations are undertaken to support the clinical diagnosis or help when the clinical presentation is atypical. Ocular toxoplasmosis is usually considered to be due to infection acquired congenitally but may be the result of postnatal infection. Less commonly ocular symptoms may be associated with acute Toxoplasma infection as shown by (9).

Recognition of this clinical spectrum of toxoplasmic retinochoroiditis is crucial, but other infectious, noninfectious, and neoplastic entities should also be considered in the differential diagnosis. Investigations such as serological tests, polymerase chain reaction of blood, ocular fluids are useful. Ocular toxoplasmosis has multiple clinical manifestations, which partially overlap with those of other entities and these should be carefully considered when making the differential diagnosis, particularly in less typical cases (10).

In atypical cases, serologic tests such as serum anti-Toxoplasma titers of IgM and IgG may be helpful to support the diagnosis. In the present study negative results are of importance to exclude atypical ocular toxoplasmosis, and this in compatible with the results obtained by (11).

The seropositivity for *T. gondii* infection is relatively high worldwide and the presence of antibodies to *T. gondii* is useful only to confirm previous exposures to the parasite. These seropositive findings, however, can confirm the diagnosis of ocular toxoplasmosis with recognition of a variety of clinical presentations as shown by (12).

The present results showed that most retinal infection and positive anti-*Toxoplasma* IgG antibodies occur in the second and third decades. Cochereau-massin, *et al.* (2) recorded that the age of the first attack of ocular toxoplasmosis is typically in the second decade and during a long-term follow-up, 5-year recurrence rate was 79%, and some patients had multiple recurrences.

In acute infection antibody levels are raised and specific IgM will be detected. In reactivated infection (usually congenital) antibody levels are often not raised and IgM is not detected (13). Antibody levels may be low even during episodes of acute ocular disease (14).

Determination of IgG avidity relies on the progressive increase of the affinity of the antibody for its target antigen during the course of natural immunity following infection (15).

Recently, it has been discovered that IgG avidity tests can provide confirmatory evidence of an acute infection and they can distinguish reactivations from primary infections with a single serum specimen. This is of particular value for pregnant and immunosuppressed patients (16). Thus, according to this study, samples of IgG seropositive and IgM seronegative that had low avidity results indicating reactivation of latent infection of *Toxoplasma*.

It was recorded by (17), that *T. gondii* was found in the peripheral blood of acutely and chronically infected patients regardless of the presence of toxoplasmic retinochoroiditis. This indicates that the parasite may circulate in the blood of immunocompetent individuals and the parasitemia could be associated with reactivation of the ocular disease (18).

The diagnosis of ocular toxoplasmosis is made by ophthalmic examinations and a variety of clinical presentations that are consistent with *T. gondii* infection of the retina. When this clinical diagnosis cannot be made definitely by a fundoscopic examination, detection of increased *T. gondii* antibody titers in blood or ocular fluids or amplification of *T. gondii* DNA have been used successfully to confirm the diagnosis (19, 20).

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Evaluation of clinical status of patients admitted to Cardiac Care Unit (CCU) in Diyala province at 2013

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ABSTRACT

Cardiovascular diseases remain the main cause of death in the developed world, although a decrease in their mortality has been achieved during the last few years. The coronary heart disease is most common cause of heart disease and single most important cause of premature death in the world .Thus, the current study aimed to evaluate the clinical status of patients admitted to cardiac care unit and follow their medical conditions.

The clinical and epidemiological data from those patients admitted to the cardiac care unit (C.C.U.) of Baqubah Teaching Hospital during the year 2013 were recorded and analyzed; we record day of admitted ,month ,age, time, sex of patients, and cause of admission and fate of patients. The results of this study a (3192) patients were admitted and managed by the staff of the first department of cardiology in the year 2013. 52.2 % of them had suffered from angina (stable or unstable angina), 15% were admitted due to cardiac arrhythmias (mainly aterial fibrillations), 13.2 % because of myocardial infarction, 8.7% dueto heart failure, 5.1% due to pulmonary embolism and deep venous thrombosis and 5.8% due to other disease .We conclude a cardiac care unit is one of very important unit for treatment of cardiac disease emergently; very important unit for decrease the mortality and morbidity of patients by early diagnosed and treatment of cardiac diseases.

Keywords: patients, C.C. U., Diyala province

الملخص باللغة العربية

تعتبر أمراض القلب والأوعية الدموية السبب الرئيس للوفاة في الدول المتقدمة، على الرغم مما تحقق من انخفاض في نسبة حالات الوفيات خلال السنوات القليلة الماضية، وتعتبر أمراض قصورالشرايين التاجية من أكثر الأمراض القلبية وأخطرها وأكثر الأسباب للوفاة في سن مبكرة.

أجريت هذه الدراسة في مستشفى بعقوبة التعليمي بالرجوع إلى سجلات المرضى الداخلين لوحدة العناية القلبية خلال عام 2013 ، وتم تحليل المعلومات سريريا بالكامل ، وتم تسجيل عدد المرضى والجنس والعمر ويوم الدخول وشهر الدخول وأسباب الدخول وحالة المريض النهائية، حيث أدخل 3192 مريضا منهم 1611 مريضا من الإناث (50.4%) و1581مريضا من الذكور(49.4 %)، حيث وجد أن أكثر المرضى دخولا هي الذبحة الصدرية المستقرة وغير المستقرة كانت تشكل 52.2% من كل المرضى ثم يأتي بعدها الارتجافات الأذينية القلبية، وتشكل 15% من كل الحالات ثم احتشاء العضلة القلبية، وتشكل 13.2%، ثم عجز القلب ويمثل 8.7% من حالات الدخول ثم أمراض الخثرة الرئوية وخثرة الأوردة العميقة تمثل 5.1% وبقية الأمراض الباطنية، وتحتوي مضاعفات قلبية تمثل 5.8% من جميع حالات الدخول، كما بينت الدراسة أن أكثر الأعمار دخولا هم من 40 سنة إلى 60 سنة، وتأتي الأعمار بعد عمر 60 سنة أقل منها قليلا وأقل الأعمار دخولا قبل سن 40 سنة.

ونستنتج من هذه الدراسة أن وحدة العناية القلبية لها دور كبير في تقليل الوفيات وتقليل المضاعفات من الأمراض للمرضى الداخلين للوحدة من خلال التشخيص المبكر والعلاج السريع والسليم.

INTRODUCTION

Diseases of cardiovascular system are the most common cause of mortality and morbidity in the world (1). The coronary heart disease is most common cause of heart disease &single most important cause of premature death in the world (2) over all death due to cardiovascular system account 26 % and from them the death due to myocardial ischemia account 32% (1). Most chronic heart disease are initially asymptomatic and this silent phase last for years, cardiac disease may be diagnosed during routine examination or because development of complication and symptoms developed due to heart disease depend on many factors includes (patient age, sex, family history, social history, physical finding) (3).

Most symptoms of heart disease result from myocardial ischemia or rhythm disorder or impaired pump action, one-third of patients with acute myocardial infarction do not have chest pain (4).

Some patients have nonspecific symptoms such as tiredness, easy fatigability and anorexia but the two main most symptoms are chest pain and breathlessness (5).

Chest pain is most common reason for referral of patients for acute medical admission, prompt and accurate diagnosis is very important but our ability to differ between patients with life threatening cardiac condition and some time self limiting musculoskeletal discomfort still depend on clinical presentation plus interpretation E.C.G., chest X-ray, cardiac enzymes and echocardiograph (6).

Ischemic heart disease almost always due to atheroma and it's complication (2). Atherosclerosis is disruptive team for thickened and hardened lesion of medium and large muscular and elastic arteries, this lesion is lipid rich in contrast or arteriosclerosis which is the genetic term for thickened and stiffened arteries of all sizes, in atherosclerosis lesion occur in inner most layer of artery "intimae" and are largely confined to this region of the vessels; if they become complicated by thrombosis can occluded artery and cause ischemia and necrosis (7).

Atherosclerosis leads to angina and myocardial infarction (5), and the acute myocardial infarction occurs due to death of myocardial tissue because of inadequate blood flow (8).

Myocardial infarction occur when myocardial necrosis caused by ischemia have crushing, central substernal chest pain, stabbing in nature associated with shortness of breath, nausea and vomiting (9). The pain of myocardial infarction usually sudden in onset and radiated to left arm, neck and back brought on exercise, emotion and fright, pain last more than 30 minutes or for several hours while the pain of angina last less than 30 minutes and may relieved by rest (5).

Angina pectoris is transient myocardial ischemia occur when imbalance between oxygen demand and oxygen supply (2).

Various classifications of angina have been inspired by considerations of etiology, assessment of severity, prognosis and treatment (7). They are classified into stable and unstable angina. Stable angina has substernal chest pain radiated to the arm, jaw and relieved by rest and nitrate, while unstable angina occurs more frequently unrelieved by rest or nitrate (10). Various classifications of angina have been inspired by considerations of etiology, assessment of severity, prognosis and treatment (7). They are classified into stable and unstable angina. Stable angina has substernal chest pain radiated to the arm, jaw and relieved by rest and nitrate, while unstable angina occurs more frequently unrelieved by rest or nitrate (10).

Various classifications of myocardial infarction:

Non Q-wave MI (NSTEMI): more severe plaque damage result in more persist thrombotic occlusion perhaps last up to one hour.

Q-wave MI(STEMI): plaque larger and result from frequent of fixed and persistent thrombus which lead to abrupt cessated of myocardial perfusion more than one hour (10).

Silent myocardial ischemia should not be regarded as separated disease entity, but rather as one of several possible manifestations of myocardial ischemia and coronary heart disease, it may be defined as objective evidence of transient myocardial ischemia without chest pain or other evidence of angina and other manifestations of silent ischemia include recognized silent myocardial infarction, ischemic cardiomyopathy and sudden cardiac death (11).

Cohn's had identified three different types of silent ischemia to important as prevalence, management and prognosis:

Type 1: silent myocardial ischemia: patient totally asymptomatic coronary artery disease and detected by screening exercise test (11).

Type 2: silent myocardial ischemia: patient asymptomatic after myocardial infarction and defined as early post infracted exercise test.

Type 3: silent myocardial ischemia: patient with angina and have silent episode of myocardial ischemia (11).

Prevention of coronary heart disease important because coronary heart disease will become one of killer disease in the world in 21st century, wide spread strategies will be essential for both developed and development countries, research have made a great strides in identify a large numbers of life style, biochemical and genetic factors associated with coronary heart disease, the process of disease prevention must be pushed beyond understanding disease mechanism and identify risk factors toward established intervention strategies that definitively reduce risk, weighing the benefit of given interventions against these risk and cost has led to establishment of guidelines for health providers and general public, implementing these guidelines however remain a difficult task (12).

Aim of the study:
The aim of the present study was to evaluate patients with and without ischemic heart disease admitted to coronary care unit in Diyala province at 2013 to identify the common causes of admission , common age groups, sex ,dates of admission to cardiac care unit and analyze the condition of the patients and follow up until discharge from cardiac care unit.

PATIENTS AND METHODS

The present study is a retrospective study conducted in Baqubah teaching hospital, Coronary Care Unit to evaluate the patients admitted to cardiac care unit during period between 1st of January 2013 to the end of December 2013 (12 months).
Those patients admitted to CCU were referred from emergency unit, private clinic and referred from other hospitals were recorded and analyzed the (age, sex, date of admission, causes of admission and outcomes of patients). Number of patients evaluated in this study were (3192).

RESULTS

Out of the (3192) cases of admitted to cardiac care unit (C.C.U.) at 2013, (1611) of cases were females (50.4%) and (1581) of cases were males (49.5%) (tables 1 and 2).
The causes of admission were:
1. First common cause is unstable angina 40.7%.
2. Second common cause is a trial fibrillations and cardiac arrhythmias 15%.
3. Third cause is myocardial infarction 13.2 %.
4. Forth cause is stable angina 11.5 %.
5. Fifth cause is heart failure and its complications 8.7 %.
6. Sixth cause is other diseases which include (non specific chest pain, respiratory failure, renal failure, stroke) with some ischemic changes of ECG: 5.8%.
7. Last cause of admission is deep venous thrombosis and pulmonary emboli 5.1% (table 3).
The common age group affected is that between 40-59 years old , then age group above 60 years old and less likely affected age group is between 20-39 years old (table 4).
In this study most cases improved with treatment either discharges from C.C.U. or referred to medical word, other cases referred for cardiac catheterization or died (table 5).

Table (1): The total number of patients monthly in 2013 and number of males and females

Months	No. of patients	No. of male	No. of female
January	306	149	157
February	272	137	135
March	307	163	144
April	253	128	125
May	286	136	150
June	263	129	134
July	286	139	147
August	230	108	122
September	266	136	130
October	250	124	126
November	253	125	128
December	220	107	113
Total	3192	1581	1611
Percentage %	100%	49,54%	50,46%

P value 0.0001 by convential criteria this difference is considered to be extremely statistically significant

Table (2): Number of cases admitted to C.C.U. daily in 2013

Months	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
January	58	53	32	27	43	42	51
February	32	49	40	54	35	26	36
March	39	39	44	43	50	47	45
April	32	52	34	45	28	38	24
May	35	40	44	40	47	40	40
June	40	45	40	40	30	35	33
July	57	46	46	38	37	31	31
August	29	23	33	28	41	33	43
September	57	33	31	29	26	30	60
October	36	43	31	41	32	31	36
November	33	21	35	43	47	33	41
December	31	34	24	35	37	29	30
Total	479	478	434	463	453	415	470
Percentage %	15%	14.9%	13.6%	14.5%	14.3%	13%	14.7%

Table (3): The common causes of admission of patients to cardiac car unit(C.C.U.)

Months	Unstable angina	Stable angina	Myocardia infarction	Atrials fibrillation and c. arrhythmia	Heart failure	Pulmonary emboli and DVT	Others
January	83	86	39	28	20	30	20
February	85	49	35	33	35	20	15
March	128	21	49	59	20	10	20
April	84	34	25	40	34	10	26
May	155	23	29	39	20	10	10
June	128	20	29	46	20	12	8
July	122	20	63	31	30	10	10
August	107	20	20	42	20	14	77
September	133	25	30	48	20	15	15
October	96	22	40	32	30	15	15
November	102	24	28	49	20	12	18
December	98	22	36	34	10	5	15
Total	1301	366	423	481	276	163	179
Percentage %	40.7%	11.5%	13.2%	15%	8.7%	5.1%	5.8%

Table (4): Number of patients related to their age group monthly in 2013

Months	20 -39 years	40 – 59 years	Above 60 years
January	30	136	140
February	37	111	124
March	45	127	135
April	33	128	92
May	41	141	104
June	25	133	105
July	33	151	102
August	27	116	87
September	40	116	110
October	42	91	117
November	33	108	112
December	30	100	90
Total	416	1458	1318
Percentage %	13%	45.6%	41.4%

P value is less than 0.0001 by conventional criteria this difference is considered to be extremely statistically significant

Table (5): The fate of patients monthly in 2013

Months	Improved & discharge	Referred to medicine report	Referred to Iraqi catheter center	Died	Discharge on their responsibility
January	96	122	20	18	50
February	102	80	15	24	51
March	110	19	10	88	80
April	100	65	10	15	63
May	120	81	10	11	64
June	100	70	15	11	67
July	98	90	12	19	67
August	91	61	4	9	65
September	110	82	5	12	57
October	100	50	10	16	74
November	79	74	6	24	70
December	81	61	8	23	47
Total	1187	855	127	270	755
Percentage %	37%	27%	4%	8.5%	23.5%

DISCUSSION

Out of the (3192) cases of admitted to cardiac care unit (C.C.U.) at 2013 a (1611) of cases are female (50.4%) & (1581) of cases are male (49.5%) we analyze the data from these patients include age, sex, date of admission, causes of admission and outcomes of patients. The obtaine results throughout the current study were being compared with the previous studies.

Gender:

The results of the current study showed that the admission to C.C.U. is more common in females than males, the main cause for this is that the number of females in Diyala province are more than males, as well as some male patients prefer treatment out side the hospital**.**

These results were in agreement with a study done by (13), who showed that Women (4 million visits/year) were hospitalized more frequently than men (2.4 million visits/year) for the evaluation of chest pain**.**

Another study confirmed these results, that the heart disease is the leading cause of death for both men and women, and women are just as likely as men are to have a heart attack. However, more women than men have died from heart disease each year for the past 30 years. Furthermore, women are more likely than men to die after their first heart attack possibly because their doctors misdiagnosed them. Or, women ignored or misinterpret their heart attack signs (14).

However, a study performed in U.S (Survey: 2009–2012) showed that the incidence of heart attack or fatal and coronary heart disease (CHD) were more common in males than females (15). Another study conducted in Nitherlands found that the incidence of ischemic heart diseases in females before menopause age was lower than in males, but cardiovascular diseases develop 7 to 10 years later in women than in men and is still the major cause of death in women (16).

Age:

The current study showed that the most age group admitted to C.C.U. was those between 40-59 years old, which is considered to have more risk factors of heart diseases, where the second common age group admitted was those above 60 years, which is slightly less than first group and the less likely admitted age group was those between 20-39 years old. However, these results were disagreed with those obtained by (15), which showed that the common affected age group is that between 55-64 years old, followed by the range of 75-84 years old, where these results were reflected from s urvey between years 2005- 2011.

Another disagreed results from previous study was concluded by (13), who found that cardiovascular diseases are most frequent diagnosed and the leading causes of death in both men and women older than 65 years old in USA.

These results might be due to that the proportion of people older than 65 years old in USA and other developed countries is subjected to increase from 12.4% of the population in 2000 to 19.6% in the following ten years (13), while in developing countries, especially Arab world , the common age group is ranging from 45- 65 years old.

Causes of admission:

In the current study, the most common cause of admission of patients in C.C.U. was the case of unstable angina. The second common causes were atrials fibrillations and cardiac arrhythmia. The third cause was myocardial infarction. The forth cause was stable angina. The fifth cause was heart failure, while the sixth cause were other diseases which include (non specific chest pain, musculoskeletal pain, respiratory failure, renal failure and stroke), and the last cause of admission is pulmonary emboli.

The current study agreed with many previous studies conducted in different countries to determine the most common causes of admission to CCU, but the incidence of each disease is differed from one study to another, so when comparing the current study with a study conducted in USA by (14), which showed that 44% of patients had suffered an ST-elevation acute myocardial infarction, 18% were admitted due to a non-ST-elevation myocardial infarction or unstable angina, 21% because of significant arrythmias and in 17% the reason of admission was decompensated heart failure and/or pulmonary edema. The mean length of stay was 2.36 days. We found our results in agreement with other European reports although certain differences were noted in comparison with registries from the USA, where heart failure prevails in admission ,diagnoses and there is a slightly longer duration of stay in the CCU (14).

When compering the current study with three studies conducted in three different countries, namely (Greece, United States and Norway), it can be concluded that the most important causes of heart disease cases admitted at CCU were similar but differed regarding higher incomes (17) (figure 1).

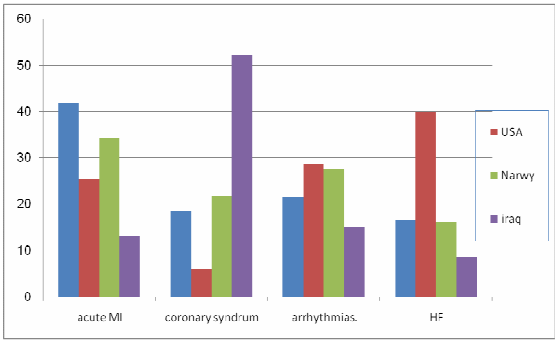

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Figure (1): comparison of 3 studies in a common causes of diseases admitted to CCU from (Greece, USA ,Norway) with the current study

However, a study conducted by (18) revealed that most common cause of admission in C.C.U. is myocardial infarction followed by unstable angina and angina.
Another study conducte in General Athene Hospital, Greece had revealed that most common cause of admission in C.C.U. was myocardial infarction 44%, then Atrial fibrillation 21%, angina 18%, and heart failure 17% (18).

Fate of patients:
In the current study, results showed that the most cases were improved by treatment in C.C.U. and discharged or referred to medical reports. These results were in agreement with studies conducted by (14-19).

Admission according the occurrence (daily and monthly):
It was revealed that March is the optimistic month for cases being admitted at CCU, and among week days, Sunday was the day of the peak for the admission.

CONCLUSION

The acute coronary syndromes and other cardiac diseases are one of the common causes of mortality and morbidity in the world **.**The cardiac care unit C.C.U. is considered one of very important units for reception and treatment of emergency cardiac disease cases. As earlier the dignosis is occurred, a decrease in mortality and morbidity will occur.

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Synthesis, pharmacological and modeling study of new sulphathiazole derivative

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ABSTRACT

Condensation of 4-amino-*N*-(1,3-thiazol-2-yl) benzenesulfonamide (sulphathiazole drug) with 3,4-dihydroxy benzaldehyde afforded Schiff base derivative in good yield. The new compound was characterized by elemental analysis, IR, ^{1}H, ^{13}C, and 2D (HSQC and HMBC- NMR) spectroscopy. It was screened for antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus, Streptococcus spp, Klebsella spp, Salmonella spp, proteus spp* and *Pseudomonas spp* as well as fungicidal activity against *Aspergillus multi, Aspergillus niger*, *Candida albicans, Candida trobicalis and Candida krusi*. It exhibited also low to moderate activity against *Bacillus cereus, Salmonella spp* and *Psedumonas spp* and good active against *Aspergillus multi, Aspergillus niger*, *Candida albicans* and *Candida krusi*. The toxicity of the compound was also assayed by the determination of its LD_{50} value by using Dixon's up and down method, which exhibited an LD_{50} of 418.6 mg / kg of body weight. The *in silico* molecular modeling study of the synthesized Schiff's base was studied.

Keywords: Sulphathiazole, 3,4-Dihydroxy benzaldehyde, 2D- NMR, Antimicrobial, molecular modeling

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الملخص باللغة العربية

لقد أعطت عملية تكثيف المركب الكيميائي (4-amino-*N*-(1,3-thiazol-2-yl)benzenesulfonamide) المعروف بعقار السلفاثيازول مع المركب الكيميائي (3,4-dihydroxy benzaldehyde) مشتقا جديدا لقاعدة شيف بحصيلة إنتاجية جيدة.

تم تشخيص المركب الجديد بواسطة التحليل العنصري الدقيق وأطياف الأشعة تحت الحمراء والرنين النووي المغناطيسي للبروتون والكربون−13 ، والرنين النووي ثنائي المحور، وجرى اختبار المركب المحضر كمضاد بكتيري ضد كل من: (*Staphylococcus aureus*، *Escherichia coli*، *Bacillus cereus*، *Streptococcus* ، *Klebsella* ، *salmonella spp*، *proteus spp* ، و *Psedumonas spp*).

كما جرى اختبار المركب كمضاد فطري ضد كل من: (*Aspergillus multi* ، *Aspergillus niger* ، *Candida albicans* ، *Candida trobicalis* ، و *Candida krusi*).

وقد أظهر المركب فعالية متوسطة إلى منخفضة كمضاد للبكتيريا ضد كل من : (*Psedumonas spp* ، *salmonella ssp* ، و *Bacillus cereus*)، وفعالية جيدة ضد الفطريات: (*Aspergillus multi, Aspergillusniger, Candida albicans, Candida krusi*). كما تم دراسة السمية الحادة لتحديد قيمة السمية LD50 باستخدام طريقة ديكسون، والتي بلغت قيمتها 418.6 ملجم / كجم من وزن الجسم، وتم دراسة النمذجة الجزيئية الحاسوبية لمركب قاعدة شيف الجديد المحضر.

INTRODUCTION

Sulfa drugs, developed in the 1930s, were the first medications effective against bacterial diseases. They appeared as the first "miracle drugs" at a time when death from bacterial infections such as pneumonia and blood poisoning were common (1). Moreover, sulfa drugs had attracted special attention for their therapeutic importance as they were used against a wide spectrum of bacterial ailments (2,3). Sulfathiazole is an organosulfur compound used as a short-acting sulfa drug. It is an organic compound. Formerly, it was a common oral and topical antimicrobial, until less toxic alternatives were discovered. However, It is still occasionally used, sometimes in combination with sulfabenzamide and sulfacetamide, and in aquariums. Figure (1) below shows Sulfathiazole chemical structure.

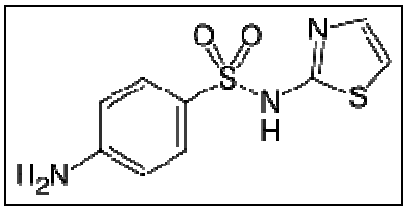

Figure (1): Chemical structure of sulphathiazole drug

Sulfa Schiff bases have been subjected to thorough studies, where a wide diversity of these derivatives were prepared and used in various biological and pharmacological fields (4-6). Schiff base compounds, which contain the azomethine (imine) group (–RC=N–) are usually prepared by the condensation of a primary amine with an active carbonyl compound (7). Schiff bases derived from sulfa drug and aromatic and hetero aromatic aldehydes are the most studied sulfonamide derivatives. These type of derivatives are very important because of their varied structures and biological activities (8-12). The Schiff bases are also known as anticancer and antiviral agents (13). The condensation of sulfa drugs with aldehyde gives biologically active Schiff bases. Keeping in view of the pronounced biological activity of the Schiff bases derived from sulfa drug, the aim of current study was to synthesize, characterize and investigate the antimicrobial ability and toxigenicity of Schiff bases derived from 3,4-dihydroxy benzaldehyde with sulfathiazole drug.

MATERIALS AND METHODS

Infrared spectra (IR) was recorded as KBr discs in the range of 4000-400 cm^{-1}using FT-IR spectrophotometer Shimadzu model IR. Affinity-1 at the department of Chemistry, College of Education for pure sciences, University of Basrah, Iraq. ^{1}H, ^{13}C NMR and 2D NMR (HSQC and HMBC NMR spectra) were measured on a Brucker at 600 MHz, with TMS as internal reference at Konstanz University, Germany. Microanalysis for carbon, hydrogen and nitrogen were carried out by a Perkin-Elmer 240B Elemental Analyzer. Melting points were measured by a Philip Harris melting point apparatus.

Antimicrobial activity:

The *in-vitro* biological screening of the 4-[(*E*)-(3,4-dihydroxybenzylidene)amino]-N-(1,3-thiazol-2-yl) benzenesulfonamide was investigated against various bacterial species: *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus, Streptococcus, Klebsella, salmonella spp, proteus spp* and *Psedumonas spp* and fungicidal activity against *Aspergillus multi, Aspergillusniger*, *Candida albicans, Candida trobicalis and Candida krusi* using the disc-agar diffusion technique (14). Muller Hinton agar was used as culture media for antibacterial activity. The antifungal activities were tested against selecte fungus by disk diffusion method. Recommended concentrations 50, 100 and 200 µg/ml of the test samples in DMSO solvent were introduced in the respective methods. Antibiotic drugs Gentamycin (10 mg) were used as control for bacteria and Flurazol (10 mg) for fungi, respectively. Petri plates containing 20 ml of Mueller Hinton Agar were used for all the bacteria tested. *Aspergillus multi, Aspergillus niger*, *Candida albicans*, *Candida trobicalis and Candida krusi* strains were cultivated in Sabouraud's dextrose agar. Sterile Whatman no.1 filter paper disks (6mm in diameter) impregnated with the solution in DMSO of the test were placed on the Petri plates. A paper disc impregnated with dimethylsulfoxide (DMSO) was used as negative control. The plates were incubated for 24 hrs. in the for bacteria and 72 hrs. for fungi at 28 °C. The inhibition zone diameters were measured in millimetres using a calliper vernia.

Acute toxicity (LD_{50}):

Animals. All experiments were performed on 10-14-weak old male and female Balb/c mice weighing 22-25 gm at the time of treatment by using up-and-down method formed by Dixon(15).

Male and female mice were injected intraperitonially with different doses of the Sulphathiazole derivative after conducting series of test levels. With equal spacing between doses, a series of trails were carried out using this method: increased dose following a negative response and decreased dose following a positive response. Testing continued until chosen "nominal" sample size was reached. LD_{50} were determined after reading final results (response-dead (X) or non response alive (O), then the following equation was applied:

$$LD_{50} = XF + Kd.$$

The estimate of LD_{50} is XF + Kd, where (XF) is the final test level and (K) is the interval between dose levels. (d) is the tabulated value (table 1).

Table (1): Data represented Dixon values (15).

	K represented serial tests started with				
	O	OO	OOO	OOOO	
XOOO	0.157-	0.154-	0.154-	0.154-	OXXX
XOOX	0.878-	0.861-	0.860-	0.860-	OXXO
XOXO	0.701	0.747	0.741	0.741	OXOX
XOXX	0.084	0.169	0.181	0.182	OXOO
XXOO	0.305	0.372	0.380	0.381	OOXX
XXOX	0.305-	0.169	0.144-	0.142-	OOXO
XXXO	1.288	1.500	1.544	1.549-	OOOX
XXXX	0.555	0.0897	0.985	1.000	OOOO
	X	XX	XXX	XXXX	

Synthesis of Schiff base:

4-[(E)-(3,4-dihydroxybenzylidene) amino]-N-(1,3-thiazol-2-yl) benzenesulfonamide (3):

A solution of 4-amino-*N*-(1,3-thiazol-2-yl)benzenesulfonamide (sulphathiazole)(1) (2.0 g, 7.83 mmol) in EtOH (25 ml) was added to a hot ethanolic solution of 3,4-dihydroxy benzaldehyde(2) (1.08g, 7.83 mmol) followed by addition of three drops of glacial acetic acid. The mixture was heated under reflux for 3 hrs. and then left at refrigerator overnight.

The solid was filtered and washed with acetone and the final product was recrystallized by $CHCl_3$-EtOH(4:1) to give 3 as a brown-dark crystals (79%), m.p.=137-140^oC. FT-IR (KBr,cm^{-1}): 3466 (O-H), 3356(N-H), 3065 (C-H aromatic), 2900, 2810 (C-H aliphatic), 1668 (C=N), 1598 (C=C), 1192 (C-O). ^1H NMR (DMSO-d_6); δ9.71(s, 2H,OH); 8.38 (s,1H,CH=N) (7.80) m, 7H, Ar-H); 5.82 (s,2H, $H_{thiazole}$). ^{13}C NMR(DMSO-d_6); δ152-1112 (C-Ar), 162(C-CH=N), 168 (C-C=N). Analytical calculated for $C_{16}H_{13}N_3O_4S_2$ (375.4): C, 51.14; H, 3.46; N, 11.18. found: C, 50.94; H, 3.12; N, 11.4. (figure 2).

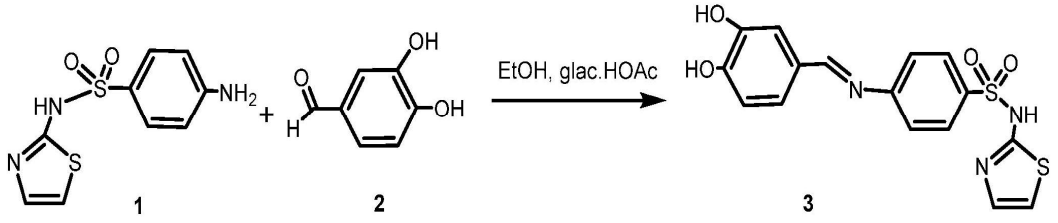

Figure (2): Preparation of new Schiff base 3 derived from sulphathiazole derivative 1

RESULTS AND DISCUSSION

Chemistry:

Isolated yield, melting point, color and spectral data IR and ^1H NMR of synthesized new compound 3 were reported. The present work describes the synthesis of new Schiff base derived from sulphathiazole and aldehyde to produce bioactive Schiff base. Thus, treatment of 4-amino-*N*-(1,3-thiazol-2-yl) benzenesulfonamide (sulphathiazole) with 3,4-dihydroxy benzaldehyde in 1:1 mole ratio gave the new organic compound in good yield. IR spectra for the synthesized compound displayed common features in certain regions and characteristic bands in the fingerprint and other regions. The IR spectra of new prepared compound showed strong bands in the rang 3466-3356 cm^{-1} due to ν(O-H) and ν(N-H) respectively. The IR spectra of the synthesized compound showed bands at 1668, 1598 due to(C=N) and(C=C) cm^{-1} respectively (figure 3).

The ^1H NMR spectra of studied synthesized compound was recorded in DMSOd_6 solution and show all the expected protons with proper intensity ratio, It is worthy to note that the proton of Ar-OH resonate as a single at 9.7 ppm which is in agreement with previously reported data (16). The aromatic protons of the compound appeared within the range 7.80-6.75 ppm. The proton of azomethine (CH=N) resonate as a singlet at 8.38 ppm (figures 4 and 5). The ^{13}C NMR spectra of synthesized compound showed the expected resonance signals and is consistent with their structures. The large variation of carbon atoms bearing sulphar can be explained by the polarity of the C-S bond in thiazole ring (figure 6). HSQC and HMBC NMR showed the correlation of protons and carbon in aromatic rings which support the chemical structure of synthesized new compound (Figures 7, 8)

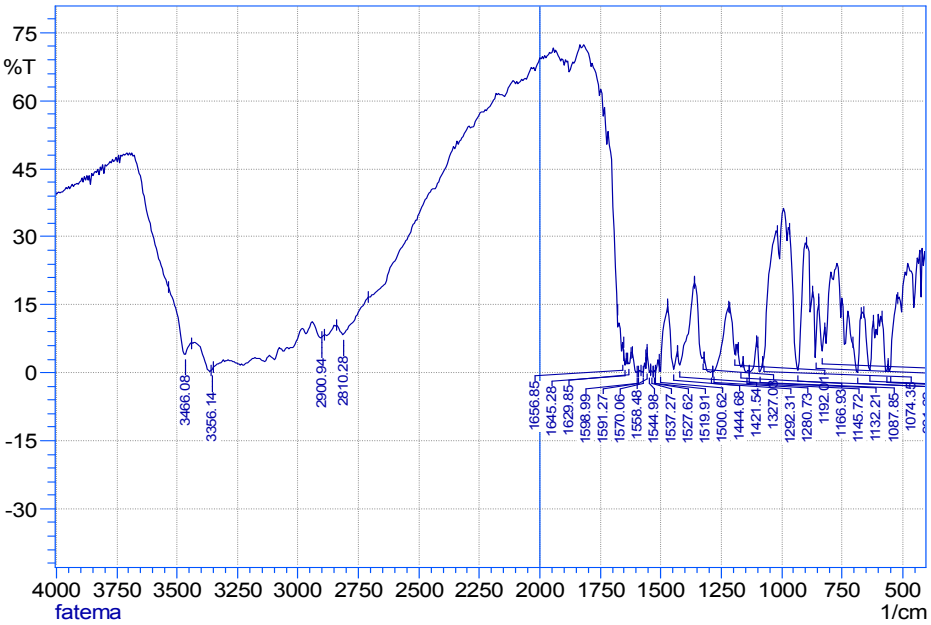

Figure (3): Infra red spectrum of the new derivative of suphathiazole

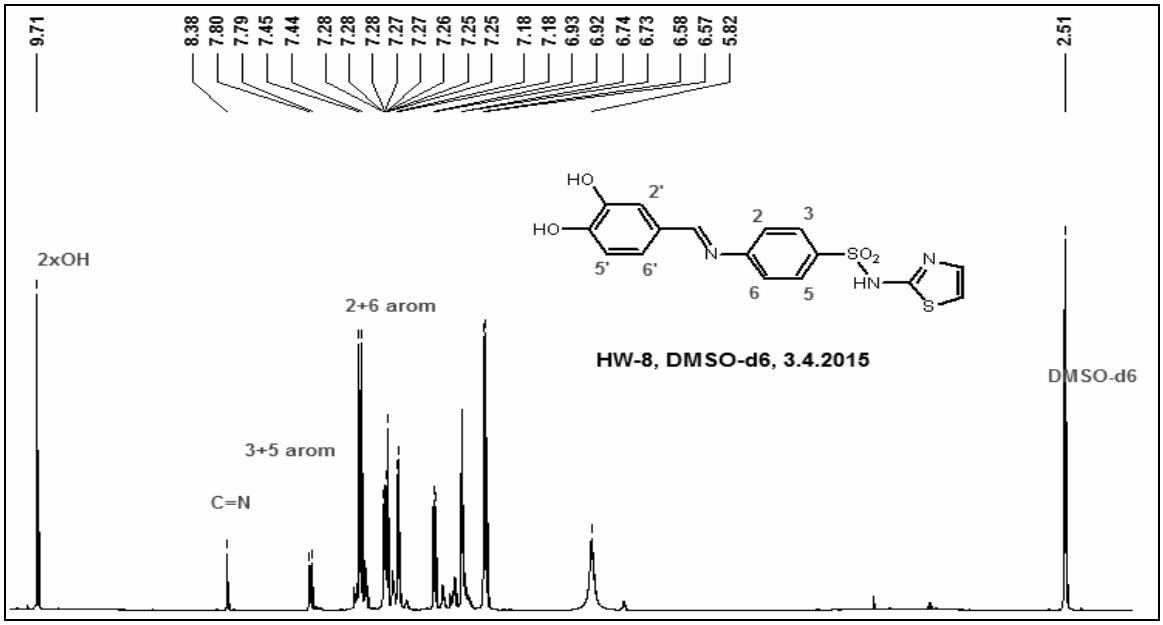

Figure (4): ^{1}H NMR spectrum of the new derivative of suphathiazole

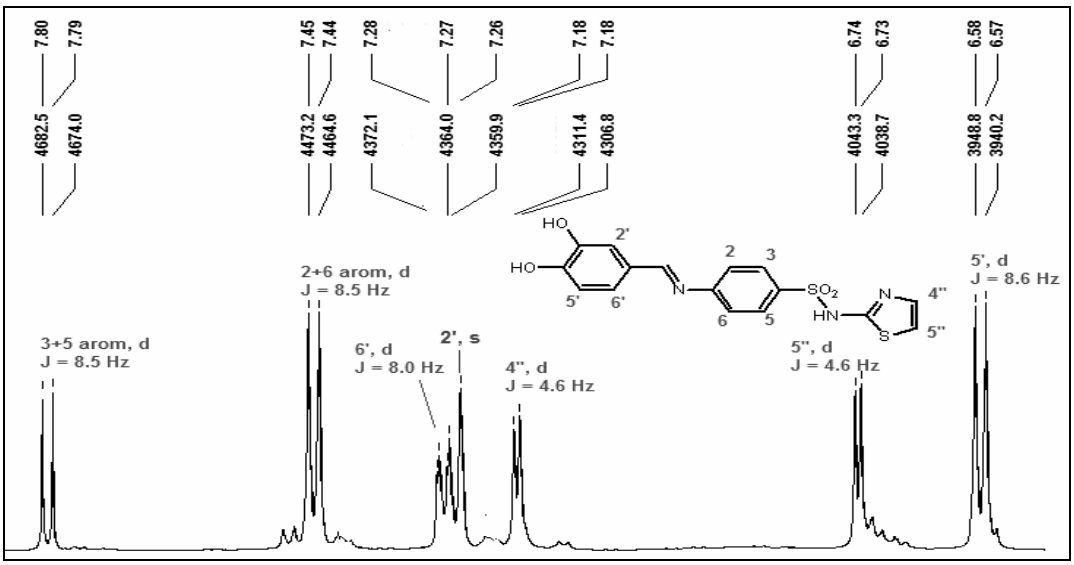

Figure (5): ^{1}H NMR expansion spectrum of the new derivative of suphathiazole

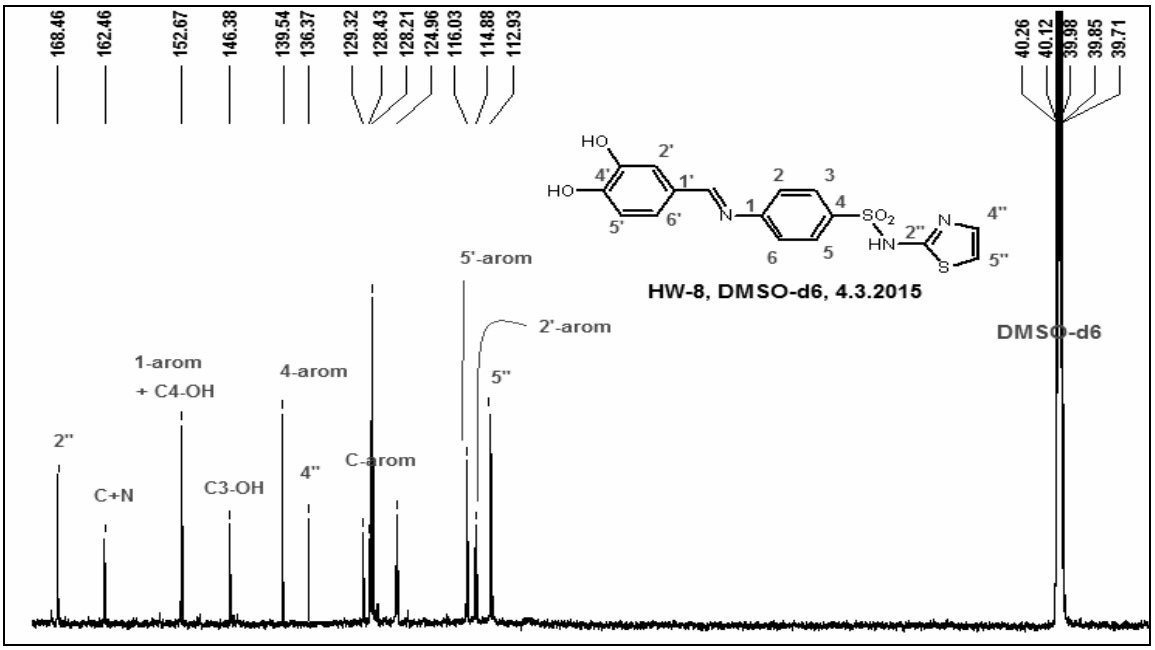

Figure (6): ^{13}C NMR spectrum of the new derivative of suphathiazole

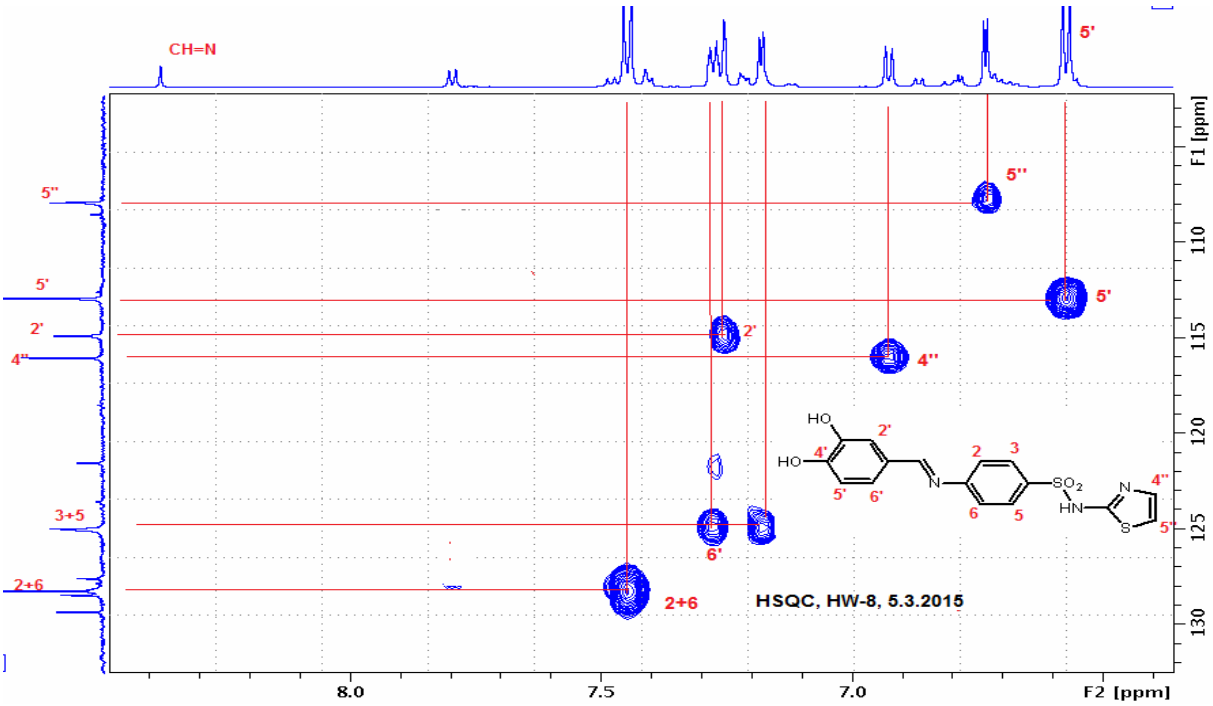

Figure (7): HSQC NMR spectrum of the new derivative of suphathiazole

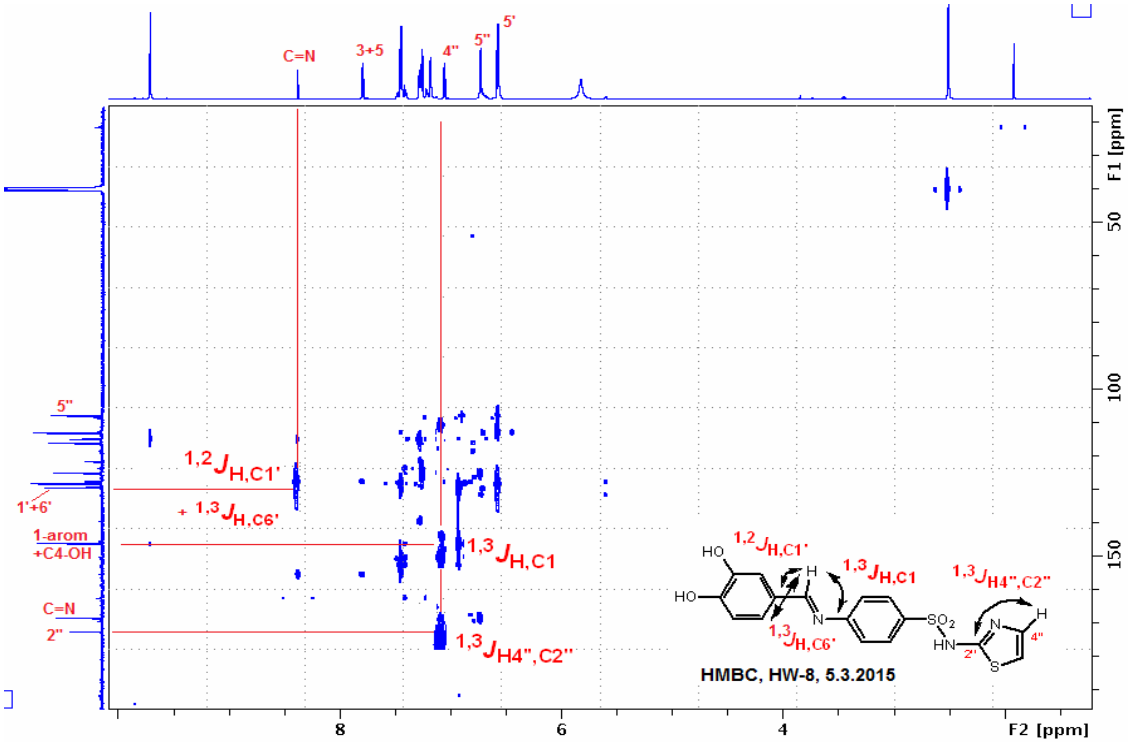

Figure (8): HMBC NMR spectrum of the new sulphathiazole derivative

Pharmacological study:

1. Median lethal dose (LD_{50}): Determination of the 50% of lethal dose (LD_{50}) of the studied compound *in vivo* was detected in the mice by using the "up-and-down" procedure described by (15). In the experiment we using 10 animals of white mice 10-14 weeks in age, Graded doses of injection to each one animal, a series of concentrations (250, 300, 350, 400 mg/k.gb.w) in 0.1 ml (dimethyl sulphoxide) DMSO, were administered and chosen with equal spacing (concentrations) between doses. Mortality was recorded after 24 hrs. that each one animal treated with one dose and after 24 hrs. was recorded as O if the animal lives and then increased the treated dose. While X recorded for the death of animal and then decreased the dose according for the result of the animal the code which formed as being (OOXX) and according for Dixon value was get and the LD_{50} was determined according to the formula employed by (15).

LD_{50} =X*f*+ Kd
LD_{50} = 400 + 0.372 x 50
LD_{50} = 418.6 mg / kg b.w
1/10 LD_{50}= 41.86 mg / kg (1 kg = 40 mice depending on the weight mice 25 g.
1/10 LD_{50}=1.0465 mg /mice depending on the weight mice 25 g

2. Antimicrobial study: The results of the antimicrobial activity are shown in table (2). The studied compound showed no activity against *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus ,Klebsella spp,* and *proteus spp,* but low active in *Bacillus cereus* at 200 µg/ml and moderate activity in *Salmonella spp* and *Psedumonas spp.* The results of antifungal activity of the compound showed no active towards *Candida trobicalis* , but good active against *Aspergillus multi, Aspergillus niger*, *Candida albicans* and *Candida krusi* compared with controls (table 2). The bacteria and fungi were supplied from department of Microbiology, College of Veterinary Medicine, University of Basrah**.**

Table (2): Microbial activities of the Schiff-base derivatives of sulphathiazole drug (Diameter of inhibition zone in mm for different microbial species)

Microorganism	50µg/ml	100µg/ml	200µg/ml	Gentamycine (10 µg)	Flurazol (10 µg)
E. coli	-	-	-	22	-
S.aureus	-	-	-	22	-
Sreptococcus	-	-	-	20	-
Klebsella	-	-	-	22	-
Bacillus	-	-	7	13	-
Salmonella	-	9	9	25	-
Psedumonas	-	-	9	22	-
Proteus Spp	-	-	-	20	-
Candida albicans	-	-	-	-	12
Candida trobicalis	8	10	10	-	12
Candida krusi	7	8	10	-	12
Apergillusmulti	10	10	12	-	12
Aspergillus niger	9	9	10	-	12

Molecular modelling analysis: The molecular docking was performed by using SYBYL- X 1.1 and the docking result was shown by PyMol (17). Our molecular docking analysis of the new analogue 3 based on the modelling study, which was performed to understand the binding mode of this compound with the *Candida tropicalis* amino acids binding pocket (PDB code: 1N9G (18).
Compound 3 showed binding energy score -8.3, indicating as electivity of substituted thiazole-Schiff base analogue in its binding to the enzyme pocket (figure 9). As shown in figure (9), proton of OH group of aromatic moiety of Tyr274 with *O* atom of OH proton at C-4 of aromatic ring of compound 3, Ser178 with *O* atom of OH group at C-3 of aromatic residue as well, in addition to the interaction between terminal NH_2 proton of Gln280 with sulphur atom of the thiazole scaffold. Overall, non-bonded of Gly175, Met277, Asn174 and Gly276 of *Candida tropicalis* amino acid residues were observed surrounded the synthesized molecule.

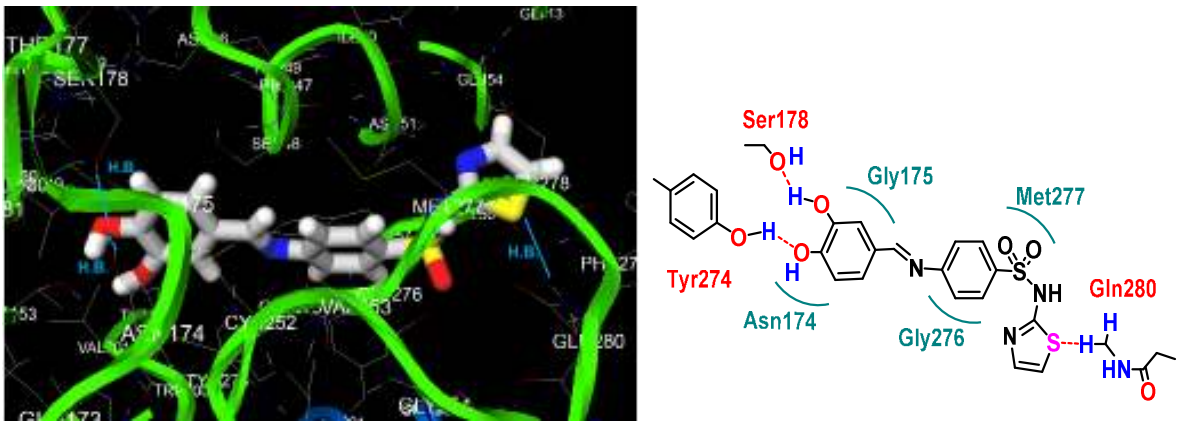

Figure (9): Docked conformation of 3 showing three hydrogen bonds: Proton of OH group of aromatic moiety of Tyr274 with *O* atom of OH proton at C-4 of aromatic ring of compound 3, Ser178 with *O* atom of OH group at C-3 of aromatic residue as well, in addition to the interaction between terminal NH2 proton of Gln280 with sulphur atom of the thiazole scaffold. Besides, non-bonded interaction of Gly175, Met277, Asn174 and Gly276 of *Candida tropicalis* amino acid residues were observed.

CONCLUSION

In conclusion, the present study reported the synthesis of new sulphathiazole analogue namely 4-[(*E*)-(3,4-dihydroxybenzylidene)amino]-*N*-(1,3-thiazol-2-yl)benzenesulfonamide, which revealed moderate *in vivo* toxic effects by LD_{50} measurement. In addition, the *in vitro* antibacterial and antifungal activities against some bacterial and fungi were studied, for further future biological studies.

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Detection of *Escherichia coli* in Asymptomatic *bacteriuria* in Al- Ramadi General Teaching Hospital

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ABSTRACT

Asymptomatic *bacteriuria* is the presence of bacteria in the urine without causing symptoms. It occurs in a small number of healthy people and it affects women more often than men. *Escherichia coli* is the most common organism associated with asymptomatic *bacteriuria* (ABU).Urine samples were collected from the patients for isolation and identification of *Escherichia coli* with serum sample to detect antibody against *Escherichia coli* by enzyme linked immunosorbent assay (ELISA)**.** One hundred and twenty five samples were obtained from urine specimens .Patients were attending to Al-Ramadi General Teaching Hospital during the period from March to September 2013. A quantitative urine culture for isolation and identification of bacterial species were used. The isolation of bacterial species were carried out on ordinary and selective media. The identification of bacterial species depended on morphological and biochemical reactions as API 20. Serological test of *E.coli* was done. Different bacterial species were isolated: *Escherichia coli*, *Pseudomonas aeruginosa, Klebsiella spp.*, *Streptococcus pyogenes, Streptococcus agaltactiae, Staphylococcus aureus* ,and *Staphylococcus saprophyticus* from patients. Different ages of patients from 20 – 89 years old were classified into seven groups, while diabetic patients were 6 (28.5 %) in female and 3 (30.0 %) in male patients. The current study also examined the relationship between antibiotic uses and patients and reveled that 10 (22.8 %) in females and 7 (36.8 %) in male patients. 42 patients showed positive reaction to enzyme linked immunosorbent assay (ELISA)**.** This study concluded the investigation of the specificity of an enzyme-linked immunosorbent assay (ELISA) for detection of *Escherichia coli* in *bacteriuria*. This technique has high sensitivity and will be suitable than other routine laboratory tests.

Keywords: Asymptomatic *bacteriuria, Escherichia coli*, Urine sample

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الملخص باللغة العربية

تعرف البكتيريا بأنها عديمة الأعراض من وجودها في الإدرار دون إحداث عرضية مرضية تظهر على المريض، وتحدث مثل تلك الحالات في عدد محدود من الأشخاص الأصحاء وتصيب النساء أكثر من الرجال. وتعد بكتيريا (*Escherichia coli*) الأكثر إحداثا لمثل تلك الحالات.
في الدراسة الحالية، تم جمع عينات إدرار من أشخاص لا يعانون من التهاب المجاري البولية، بهدف عزل وتشخيص بكتيريا (*Escherichia coli*) ، وتم جمع عينات مصل الدم للكشف عن الأجسام المضادة لهذه البكتيريا بواسطة تقنية الإليزا، وقد تم الحصول على (125) عينة إدرار من مراجعي مستشفى الرمادي التعليمي العام خلال الفترة من شهر آذار إلى شهر أيلول عام 2013. وقد تم إجراء الزراعة العددية لعينات الإدرار بهدف تحديد أنواع البكتيريا الموجودة فيها، وتم التعرف على الأنواع البكتيرية اعتمادا على الصفات المورفولوجية والبيوكيميائية مع التشخيص بواسطة API 20 والتشخيص المناعي للبكتيريا وقد تم عزل أنواع بكتيرية مختلفة: الشريكية القولونية و الزائفة الزنجارية و كليبسيلا و المكورات المسبحية نوع ب، والمكورات العنقودية الذهبية، والمكورات العنقودية *saprophyticus* من المرضى، وتراوحت أعمار المرضى ما بين 20–89 سنة، وتم تصنيفها إلى سبع مجموعات، في حين سجلت حالات مرضى السكري 6 (28.5%) من الإناث و 3 (30.0%) من المرضى الذكور، وتم دراسة العلاقة بين استخدامات المضادات الحيوية والمرضى، حيث تبين أن 10 (22.8%) من الإناث و 7 (36.8 %) من الذكور يستخدمون المضادات الحيوية، كما أظهر 42 مريضا رد فعل إيجابيا لفحص (ELISA). وينتج من هذه الدراسة أن التشخيص بتقنية (ELISA) للكشف عن الإشريكية القولونية يفيد في مثل هذه الحالات بسب ما تتمتع به من حساسية عالية، وأن هذه التقنية مناسبة أكثر من غيرها من الفحوصات المخبرية الروتينية.

INTRODUCTION

In medicine, *bacteriuria* denotes the presence of bacteria in urine. It is more common in women, in the elderly, in residents of long-term care facilities, and in patients with diabetes, bladder catheters and spinal cord injuries. Patients with a long-term Foley catheter uniformly show bacteriuria. Chronic asymptomatic *bacteriuria* without Urinary tract infection symptoms is prevalent in as high as 50% of the population in long-term care (1). Asymptomatic *bacteriuria* is the occurrence of bacteria in the urine without causing symptoms. The condition may not need treatment. This makes it different from a urinary tract infection that is caused by bacteria (2). Asymptomatic *bacteriuria* occurs in a small number of healthy people. It affects women more often than men. The reasons for the lack of symptoms are not well understood. Most people who have this condition do not need treatment because the bacteria are not causing any harm. People who have urinary catheters often will have *bacteriuria*, but most will not have symptoms (3). Certain people are at a higher risk for kidney infections if they develop this problem. The following increases the risk: diabetes, infected kidney stones, kidney transplant, older age ,pregnancy up to 40% of pregnant women with untreated asymptomatic *bacteriuria* will develop a kidney infection ,and Vesico ureteral reflux (backward movement of urine from the bladder into ureters or kidneys) in young children (4). Asymptomatic *bacteriuria* causes no symptoms. The symptoms of a urinary tract infection include burning during urination, an increased urgency to urinate, and increased frequency of urination. A urine culture is taken from a urine sample (5). Asymptomatic *bacteriuria* is diagnosed if there is a large overgrowth of bacteria in the urine culture. Some people are more likely to be given antibiotics. These include pregnant women, people who have received a kidney transplant, children with vesico ureteral reflux, and those with infected kidney stones (6). Giving antibiotics to persons who have long-term urinary catheters in place may cause additional problems. The bacteria may be harder to treat and a yeast infection may develop (4). This condition should be treated if it is discovered before a urinary tract procedure. This may help prevent complications. The type of treatment will depend on the person's risk factors. If it is not treated, asymptomatic *bacteriuria* can lead to a kidney infection in people at high risk (7).

MATERIALS AND METHODS

Urine samples were collected from patients attended to Al-Ramadi General Teaching Hospital, during the period between March to September 2013. Urine samples were obtained from the patients for isolation and identification of different bacterial species for asymptomatic *bacteriuria* by microbiological methods. A total of 125 patients with urine specimens were included in this study taken from 89 females and 36 male patients. A urine culture was used to identify bacterial species by obtaining a sample of midstream clean catch patient voids first portion of urine, then collects urine specimen midstream and discards the latter portion.

Catheterization: Urine was collected directly from an indwelling urethral catheter or from intermittent catheterization. Suprapubic aspiration - Urine collected from needle aspiration through suprapubic abdominal wall into the bladder.

Cystoscopy and other invasive procedures: Sample can also be obtained during this type of procedure**.**

Specimen handling: Urine should be processed within 2 hrs. after collection. If it cannot be processed in a timely manner, then either (1) refrigerate the specimen at 2-8°C (specimen will be stable for 24 hours) or (2) place the sample in preservative fluid and store at room temperature for up to 24-72 hours; boric acid is the most common preservative fluid used for culture (8).

All urine samples were inoculated on blood agar (Mast) MaCcnckey agar (Mast), Mannitol salt agar (Mast) and incubated at 37°C for 24 hrs. A specimen was considered positive for bacteriuria in the light of the number of yielded colonies ($\geq$105 cfu/ml) and the cytology of the urine through microscopic detection of bacteriuria and PMNs ($\geq$8 leukocytes/mm3) (9).

Escherichia coli is Gram – negative rods shape, facultatively anaerobic bacteria, no motile, non-spore forming ,and lactose fermenting .*Escherichia coli* cultivated on MacConkey agar (Mast). Lactose positive colonies. Cultivation for 24 hrs. in an aerobic atmosphere, 37°C. *Escherichia coli* on MacConkey agar (Mast), pink colony pigment is due to lactose fermentation (10).

Klebsiella spp. is a Gram-negative, non-motile, encapsulated, lactose fermenting, facultative anaerobic, rod shaped bacterium found in the normal flora of the mouth, skin, and intestines and *Klebsiella spp.* is a mucous, lactose positive colony of *Klebsiella spp.* on MacConkey agar (Mast). Cultivation 37°C, 24 hrs. (10).

Pseudomonas aeruginosa is Gram-negative, aerobic rod shape belonging to the family *Pseudomonadaceae* ,motile by means of a single polar flagellum, non spore forming, capsulated , aerobic. *Pseudomonas aeruginosa* Cultivation is occurred in 48 hrs. in an aerobic atmosphere, 37°C. *P. aeruginosa* secretes a variety of pigments, including pyocyanin (blue-green), pyoverdine (yellow-green and fluorescent), and pyorubin (red-brown).On Nutrient agar the Colonies are surrounded by bluish green coloration. On MacConkey agar pale yellow colonies lactose non fermenters and oxidase test positive (10).

The IMViC tests are a group of individual tests used in microbiology lab testing to identify an organism in the coliform group. A coliform is a gram negative, aerobic or facultative anaerobic rod which produces gas from lactose within 48 hrs. IMViC

stands for one of these tests. "I" is for indole test; "M" is for methyl red test; "V" is for Voges-Proskauer test, and "C" is for citrate test. These tests are useful in distinguishing members of *Enterobacteriaceae* (10).

Indole test :In this test, the organism under consideration is grown in peptone water broth. It contains tryptophan, which under the action of enzyme tryptophanase is converted to an Indole molecule, pyruvate and carbon dioxide. The indole is then extracted from the broth by means of xylene. To test the broth for indole production, Kovac's reagent is added after incubation. A positive result is indicated by a pink/red layer forming on top of the liquid (11).

Methyl red and Voges–Proskauer test :These tests both use the same broth for bacterial growth. The broth is called MRVP broth. After growth, the broth is separated into two different tubes, one for the methyl red (MR) test and one for the Voges-Proskauer (VP) test (10).

The methyl red test detects production of acids formed during metabolism using mixed acid fermentation pathway using pyruvate as a substrate. The pH indicator Methyl Red is added to one tube and a red color appears at pH's lower than 4.2, indicating a positive test (mixed acid fermentation is used). The solution remaining yellow (pH = 6.2 or above) indicates a negative test, meaning the butanediol fermentation is used (10).

The VP test uses alpha-naphthol and potassium hydroxide to test for the presence of acetylmethylcarbinol (acetoin), an intermediate of the 2,3-butanediol fermentation pathway. After adding both reagents, the tube is shaken vigorously then allowed to sit for 5-10 minutes. A pinkish-red color indicates a positive test, meaning the 2,3-butanediol fermentation pathway is used (10).

Citrate test: This test uses Simmon's citrate agar to determine the ability of a microorganism to use citrate as its sole carbon source. The agar contains citrate and ammonium ions (nitrogen source) and bromothymol blue as an indicator (10). The citrate agar is green before inoculation, and turns blue as a positive test indicator, meaning citrate is utilized in table (1) (10).

Table (1): The IMViC results of some important species

Species	Indole	Methyl Red	Voges-Proskauer	Citrate
Escherichia coli	Positive	Positive	Negative	Negative
Klebsiella spp.	Negative	Negative	Positive	Positive
*Pseudomonas aeruginos*a	Negative	Negative	Negative	Positive

Oxidase test: Basically, this is a test to see if an organism is an aerobe. It is a check for the presence of the electron transport chain that is the final phase of aerobic respiration. Normally, oxygen is the final electron acceptor for this system. In the oxidase test, an artificial final electron acceptor (N,N,N',N'-tetramethylphenylenediaminedihydrochloride) is used in the place of oxygen. This acceptor is a chemical that changes color to a dark blue/purple when it takes the electron from the last element (cytochrome oxidase) in the electron transport chain (11).

Gram-positive bacteria were identified with the corresponding recommended laboratory tests as the following *S.aureus* and other staphylococci should be blue, purple or violet under the light microscope, what is noted as Gram-positive. *S.aureus* is gram positive coocci clusters shape, non- motile , non-spore formin , facultative anaerobic and it is coagulase and catalase positive. On blood agar plates, colonies of *Staphylococcus aureus* are frequently surrounded by zones of clear beta-hemolysis, the golden appearance of colonies (12).

Mannitol salt agar or MSA is a commonly used selective and differential growth medium in microbiology. It encourages the growth of a group of certain bacteria while inhibiting the growth of others. This medium is important in medical laboratories by distinguishing pathogenic microbes in a short period of time. It contains a high concentration (~7.5%-10%) of salt (NaCl), making it selective for gram positive bacterium *Staphylococci* (and *Micrococcaceae*) since this level of NaCl is inhibitory to most other bacteria. It is also a differential medium for mannitol-fermenting staphylococci, containing carbohydrate mannitol and the indicators phenol red and a pH indicator for detecting acid produced by mannitol-fermenting staphylococci. *Staphylococcus aureus* produce yellow colonies with yellow zones, whereas other *Staphylococci* produce small pink or red colonies with no color change to the medium (4). If an organism can ferment mannitol, an acidic byproduct is formed that will cause the phenol red in the agar to turn yellow. It is used for the selective isolation of presumptive pathogen (pp) *Staphylococci* (13).

Identification of *Staphylococcus aureus:*

a- Catalase test is performed by adding 3% hydrogen peroxide to a colony on agar. Staphylococci contain catalase, and break down peroxide, produces O_2 and bubble, so they are catalase positive, what distinguish them from streptococci. Catalase test is done only when a culture is not typical (13).

b- Coagulase test is used to differentiate *Staphylococcus aureus* from coagulase-negative staphylococci. *S.aureus* produces two forms of coagulase (i.e., bound coagulase and free coagulase). Bound coagulase, otherwise known as "clumping factor", can be detected by carrying out a slide coagulase test, and free coagulase can be detected using a tube coagulase test.*Staphylococcusaprophyticus* coagulase negative (14).

Slide test*:* A slide coagulase test is run with a negative control to rule out autoagglutination. Two drops of saline are put onto the slide labeled with

sample number, Test (T) and control (C). The two saline drops are emulsified with the test organism using a wire loop, straight wire, or wooden stick. A drop of plasma (rabbit plasma anticoagulated with EDTA is recommended) is placed on the inoculated saline drop corresponding to test, and mixed well, then the slide is rocked gently for about 10 seconds. If 'positive', macroscopic clumping would be observed in the plasma within 10 seconds, with no clumping in the saline drop. If 'negative', no clumping will be observed. If the slide coagulase test is negative, a tube test should follow as a confirmation. Clumping in both drops is an indication of autoagglutination, so a tube test should be carried out. Tube test is not performed each institutions but most of the result depends on blood cultures from lab (15).

Tube test: The tube test uses rabbit plasma that has been inoculated with a staphylococcal colony (i.e., Gram-positive cocci which are catalase positive). The tube is then incubated at 37°C for 1.5 hrs. If negative, then incubation is continued up to 18 hrs .If 'positive', the plasma will coagulate (6), resulting in a clot (sometimes the clot is so pronounced, the liquid will completely solidify). If 'negative', the plasma remains a liquid. The negative result may be *S. epidermidis* but only a more detailed identification test can confirm this, using biochemical tests as in analytical profile index tests methods. A false negative can be perceived if the sample is not allowed to cool for about 30 minutes at room temperature or 10 minutes in the freezer, given that the serum can melt. If truly negative, the serum will remain liquid after cooling (16).

The API-20E test kit is used for the identification of *Enterobacteriaceae* (bioMerieux) provides an easy way to inoculate and read tests relevant to members of the Family *Enterobacteriaceae* and associated organisms. A plastic strip holding twenty mini-test tubes is inoculated with a saline suspension of a pure culture .This process also rehydrates the dessicated medium in each tube. A few tubes are completely filled (CIT, VP and GEL as seen in the photos below), and some tubes are overlaid with mineral oil such that anaerobic reactions can be carried out (ADH, LDC, ODC, H_2S, URE).After incubation in a humidity chamber for 18-24 hrs. at 37°C, the color reactions are read (some with the aid of added reagents), and the reactions (plus the oxidase reaction done separately) are converted to a seven-digit code which is called the Analytical Profile Index, from which name the initials "API" are derived. Note especially the color reactions for amino acid decarboxylations (ADH through ODC) and carbohydrate fermentations (GLU through ARA).The amino acids tested are (in order) arginine, lysine and ornithine. Decarboxylation is shown by an alkaline reaction (red color of the particular pH indicator used).The carbohydrates tested are glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin and arabinose. Fermentation is shown by an acid reaction (yellow color of indicator).Hydrogen sulfide production (H2S) and gelatin hydrolysis (GEL) result in a black color throughout the tube.A positive reaction for tryptophan deaminase (TDA) gives a deep brown color with the addition of ferric chloride; positive results for this test correlate with positive phenylalanine and lysine deaminase (17).

According to American Society for microbiology (9), API system (Bio Merieux) test kits is used for identification of Gram positive and Gram negative bacteria. API 20E presented herein is a biochemical panel for identification and differentiation of members of the family *Enterobacteriaceae*. Other API panels for other groups of bacteria, such as *staphylococci* and *streptococci*, are also available in the same format, but are not included in this presentation. In API 20E for identification of members of the family *Enterobacteriaceae*, the plastic strip holds twenty mini-test chambers containing dehydrated media having chemically-defined compositions for each test. These include**:**

1. ONPG: test for b-galactosidase enzyme by hydrolysis of the substrate *o*-nitrophenyl-b-D-galactopyranoside
2. ADH: decarboxylation of the amino acid arginine by arginine dihydrolase
3. LDC: decarboxylations of the amino acid lysine by lysine decarboxylase
4. ODC: decarboxylations of the amino acid ornithine by ornithine decarboxylase
5. CIT: utilization of citrate as sole carbon source
6. H_2S: production of hydrogen sulfide
7. URE: test for the enzyme urease
8. TDA: detection of the enzyme tryptophan deaminase
9. IND: production of indole from tryptophan by the enzyme tryptophanase. Indole is detected by addition of Kovac's reagent.
10. VP: the Voges-Proskauer test for the detection of acetoin (acetyl methylcarbinol) produced by fermentation of glucose by bacteria utilizing the butylene glycol pathway
11. GEL: test for the production of the enzyme gelatinase which liquefies gelatin
12. GLU: fermentation of glucose (hexose sugar)
13. MAN: fermentation of mannose (hexose sugar)
14. INO: fermentation of inositol (cyclic polyalcohol)
15. SOR: fermentation of sorbitol (alcohol sugar)
16. RHA: fermentation of rhamnose (methyl pentose sugar)
17. SAC: fermentation of sucrose (disaccharide)
18. MEL: fermentation of melibiose (disaccharide)

19. AMY: fermentation of amygdalin (glycoside).
20. ARA: fermentation of arabinose (pentose sugar)

The oxidase test is a test for cytochrome oxidase, which is performed separately from the above tests. It is done using a portion of a bacterial colony on a paper strip impregnated by the oxidase reagent N,N,N',N'-tetramethylphenylenediamine, which turns blue if cells possess oxidase enzyme (17).
Enzyme-linked immunosorbent assay (ELISA) test used to detect and measures antibodies in serum patients. This test can be used to determine the antibodies which related to certain infectious conditions. Antibodies are proteins that the body produces in response to harmful substances (antigens). An ELISA test may be used to diagnose different infections (18). Five ml of venous blood sample was collected from 15 patients with *bacteriuria* who showed *Escherichia coli* positive for ELISA test .Serum taken into a sterile capped plastic tubes then centrifuged at 3000 rpm for 5 minutes, their serum was then collected into another sterile tube and was kept at deep freeze (- 18C) for estimation of antibody titer and for further study (19). *Escherichia coli* was harvested from broth by centrifugation (Chilspin) at 2500xg for 15 min. they were washed three times in PBS, pH 7.2. The antigen disrupted by Sonication (Sondovest) for one hour and half, with a break of one minute in between the runs. The antigen was kept cool by surrounding the container with crushed ice. The sonicated antigen and the suspension were centrifuged at 4000xg for 10 minutes to remove cell wall debris. The antigen was stored at 4◦C until used. The antigen was stored -20◦C until used (20).Polystyrene plates with 96 flat - bottomed wells (linbro) were used. Antigen was first adsorbed on the well surface of microtiter plates. Serum samples suspected case of *Escherichia coli*were inoculated in separate antigen coated wells. Antigen - specific antibody present in the samples will bind to the antigen. Unbounded serum components are washed a way and horesradish peroxidase - anti -mice IgG conjugate (Dako) is added. Excess conjugate is washed away, and substrate solution (Bioelisa), is added to each well. The amount of color developed as the enzyme of the conjugate acts upon the substrate is directly proportional to the amount of antibody in the serum cleared by the enzyme storage, the plates were washed with distilled water (Bioelisa), and used stopping solution by 1 N Sulphuric acid (Bioelisa), finally dried at room temperature, sealed, and stored at-20◦C until used (21). Ten patients showing negative control against ELISA reaction and 10 patients showing positive reaction by ELISA test due to serum antibody against *Escherichia coli* .

RESULTS

The cultured urine samples (89 female and 36 male patients) showed the following isolate: *Escherichia coli* 32(35.9%), *Pseudomonas aeruginosa* 18(20.2%), *Klebsiella spp.* 14(15.7%), *Streptococcus pyogene*s 8(8.9%) , *Streptococcus agaltactia*e 6(6.7%), *Staphylococcus aureus* 4(4.4%) and *Staphylococcus saprophyticus* 3(3.3%) from females and *Escherichia coli* 10(27.7%), *Pseudomonas aeruginosa* 8(22.2%), *Klebsiella spp.* 6(16.6%), *Streptococcus pyogene*s 4(11.1%) , *Streptococcus agaltactiae* (0.0), *Staphylococcus aureus* 3(8.3%) and *Staphylococcus saprophyticus* 2(5.5%) from male patients. Statistical analysis by using the Chi – square revealed highly significant differences of *Escherichia coli* infection than other bacterial species (table 2).

Table (2): Bacterial species isolated from urine culture samples

Types of isolates	Female pts.		Male pts.	
	No.	%	No.	%
Escherichia coli	32*	35.9	10*	27.7
Pseudomonas aeruginosa	18	20.2	8	22.2
Klebsiella spp.	14	15.7	6	16.6
Streptococcus pyogenes	8	8.9	4	11.1
Streptococcus agaltactiae	6	6.7	0	0.0
Staphylococcus aureus	4	4.4	3	8.3
Staphylococcus saprophyticus	3	3.3	2	5.5
Total	**85**	**95.1**	**35**	**91.4**

** Escherichia coli showed high number of isolation from urine culture (p< 0.01)*

Table (3) showed the age distribution of patients from (20-89) years old and the high incidence of infection was at age (20-29) years old. Statistical analysis revealed a highly significant differences between age groups ($P < 0.01$).
Table (4) illustrated the relationship between diabetic patients and *bacteriuria* patients with age group. It showed that the high incidence of *bacteriuria* patients were 6(28.5%) at age (50-59) years old in females and 3(30.0%) at age (60-69) in male patients .Chi – square test revealed a highly significant difference between *Escherichia coli* and diabetic patients with age groups.

Table (3): The distribution of age groups for selected patients

Age groups	Female pts.		Male pts.	
	No.	%	No.	%
20-29	19*	21.3	12*	33.3
30-39	18	20.2	8	22.2
40-49	17	19.1	6	16.6
50-59	15	16.8	4	11.1
60-69	9	10.1	3	8.3
70-79	8	8.9	2	5.5
80-89	3	3.3	1	2.7
Total	**89**	**99.7**	**36**	**99.7**

**high incidence of bacteriuria infection at age (20-29) years old in patients (p< 0.01)*

Table (4): The relationship between diabetic patients and age groups

Age groups	Female pts.		Male pts.	
	No.	%	No.	%
20-29	2	9.5	0	0.0
30-39	2	9.5	1	10.0
40-49	2	9.5	1	10.0
50-59	6*	28.5	2	20.0
60-69	5	23.8	3*	30.0
70-79	3	14.2	2	20.0
80-89	1	4.7	1	10.0
Total	**21**	**99.7**	**10**	**100.0**

**The incidence of diabetic patients at age (50-59) years old in female and (60-69) years old in male patients (p >0.01)*

Table (5) demonstrated the relationship between *bacteriuria* patients and antibiotic uses and it reveled that the high incidences of infection were 10(22.2%) at age (60-69) years old and 7(36.8%) years old at same age group. Chi – square test revealed no significant difference between *Escherichia coli* and antibiotic uses.

Table (5): The relationship between antibiotic uses and age groups

Age groups	Female pts.		Male pts.	
	No.	%	No.	%
20-29	2	4.4	1	5.2
30-39	4	8.8	2	10.5
40-49	6	13.3	3	15.7
50-59	8	17.7	3	15.7
60-69	10*	22.2	7*	36.8
70-79	8	17.7	2	10.5
80-89	7	15.5	1	5.2
Total	**45**	**99.6**	**19**	**99.6**

**The incidence of antibiotic uses and bacteriuria infections at age (60-69) years old in female and male patients (p >0.05)*

Enzyme-linked immunosorbent assay (ELISA) is used for determination of *E.coli* antibodies in serum patients. The antibodies react with antigen in solid phase. The microplate is coated with *E.coli* antigen and this study depended on the optical density of the samples measured spectrophotometrically .The absorbance of the cut – off value equaled 0.24. Any sample that shows an absorbency value equal to or higher than the cut – off value was considered positive for IgG antibodies to *E.coli*. Samples with ratio samples absorbency / cut – off value <0 .24 were considered negative for *E.coli* IgG. The calculation of cut – off value was described by (21) . The total number of sera from *bacteriuria* patients were 42, which reveled 32(76.1%) from females and 10(23.8)%) from male patients .All those 42 gave positive reaction for specific IgG in serum against *E. coli*.

DISCUSSION

Asymptomatic bacteriuria is diagnosed if there is a large overgrowth of bacteria in the urine culture (20). Asymptomatic bacteriuria (ABU) is common. The frequency varies among different populations, depending on factors such as age, sex, and underlying disorders (eg, diabetes mellitus or spinal cord injury (21).

A study in hospitalized patients identified obesity and iron deficiency anemia as independent risk factors for ABU (22). Another study was conducted to examine the microbiology of ABU, where it revealed that *Escherichia coli* is the most common organism and the most likely to occur in healthy persons. A variety of organisms may be found, however, including *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *Enterococcus* species, and group B *Streptococcus*. In men, *Enterococcus* species and gram-negative bacilli are common. Catheterized nursing home residents may have polymicrobial ABU and studied the laboratory criteria for the diagnosis of ABU in a midstream clean-catch urine specimen. The current study is in agreement with (6). A study conducted by (23) demonstrated that the frequency of ABU in healthy young men was essentially zero. Thus, screening for ABU in this population is not recommended, but the frequency of ABU in older adults was as follows: from age (50-69) years old was (2.8-8.6%) in women and (0.6-1.5%) in men ,while at age (65-80) years old was (5.8-16%) in women and (1.5-15.3%) in men and at age older than 80 years old was (18-43%) in women and (5.4-21%) in men (24). The current study showed that the frequency of age distribution was from (20-85) years old and revealed that the frequency of ABU patients were at age (50-59) years old (16.8%) in women and (11.1%) in men, while at age (60-69) years old were (10.1%) in women and (8.3%) in men, at age (70-79) years old were (8.9%) in women and (5.5%) in men. Several factors appear to account for the increasing frequency of ABU with advancing age, including the following: obstructive uropathy (eg, urinary stones, prostatic hypertrophy, uterine prolapse, or cystocele) ,decreased bactericidal activity in prostatic secretions, perineal soiling with fecal matter in women with dementia ,neuromuscular

disease ,increased instrumentation of the urinary tract, urinary catheters, reduced Tamm-Horsfall protein secretion in urine, and increased uropathogens in the postmenopausal vagina and introitus (23). It was illustrated that asymptomatic *bacteriuria* (ABU) is more common in patients with diabetes mellitus, as well as in men, women, children, and adolescents with diabetes mellitus than in patients without diabetes (24). Diabetic patients with ABU are more likely to have *albuminuria* and symptomatic UTIs. The frequency of ABU in patients with diabetes mellitus is 7.9-17.7% in females and 1.5-2.2% in males. In the current study it was recorded that the frequency of ABU patients with diabetes mellitus was (28.5%) in women at age (50-59) years old and (30.0%) in men at age (60-69) years old. The increased frequency is probably to autonomic neuropathy of the bladder. There is no indication of adverse outcomes in women. Glucose control is not impaired. Screening is not recommended, and treatment with antibiotics is not beneficial. A randomized, controlled trial found that treatment of asymptomatic *bacteriuria* in women with diabetes did not appear to reduce complications. In conclusion, diabetes itself should not be an indication for screening or treatment of ABU(25). Antibiotic treatment may also be valuable for children aged 5-6 years and before invasive genitourinary procedures (26). However, the consensus is that catheterization has no clinical significance and that antibiotic prescription is not indicated in elderly ABU patients; in healthy school girls and young women; in diabetic women; and in patients who have indwelling catheters or undergo intermittent urinary catheterization. A study by (27) suggested the need for greater focus on optimizing the use of antibiotics in patients with enterococcal *bacteriuria*; over treatment of ABU is common, especially among patients with pyuria. Antibiotic treatment does not reduce the frequency of symptomatic UTI or improve survival; instead, it leads to an increased incidence of adverse antibiotic effects and reinfection with antibiotic-resistant organisms (6). Short-term bladder catheterization is associated with a 2-7% frequency of asymptomatic *bacteriuria* (ABU) for each day that the catheter is in place. The frequency is higher in women than in men. However, screening for ABU is not indicated unless the patient has other risk factors for UTI. Antibiotic treatment is possibly beneficial in women with persistent ABU 48 hrs after catheter removal. In general, the most effective strategy for reducing the incidence of catheter-related ABU is to reduce catheter use (28). ABU is a universal finding in patients with indwelling catheters that have been in place for longer than 30 days. These patients are at risk for acute pyelonephritis, urosepsis, catheter obstruction, renal stones, vesicoureteral reflux, renal failure, and (eventually) bladder cancer. Unfortunately, treatment of ABU in these patients does not decrease the incidence of fever and usually leads to the development of resistant bacterial strains. In asymptomatic patients with indwelling urethral catheters, cloudy or foul-smelling urine is not an indication for urinalysis, culture, or antimicrobial treatment (6). The current study is in agreement with (29).

ELISA system is as sensitive for the detection of *Escherichia coli* due to difference in number of cut- off value between control positive and test groups were 0.06 in control positive and 0.24 in test groups .This difference was due to previous exposure to *bacteriuria* or due to infection with other organisms that carry similar epitopes resulting cross reactive infections. In the current study, ELISA was sensitive test detecting all cases of *Escherichia coli* infections. *Escherichia coli* was the most common causative agent of urinary tract infection (UTI), and diagnosing this infection usually relies on bacteriologic methods.

Nevertheless, screening methods can be useful for a rapid presumptive diagnosis even though some of these screening methods have low sensitivity or are expensive. To investigate a possible new alternative approach, an antigen-based enzyme linked immunosotbent assay (ELISA) was standardized for screening for this bacterial infection (30). In urinary tract infections, screening tests can provide a more rapid presumptive diagnosis than the conventional bacteriological methods. ELISA for *E. coli* antigen detection has some interesting features. For example, results are available within 4 hrs. of urine collection diagnostic features of the ELISA are promising because of the test's high sensitivity and other diagnostic parameters (5). This study concluded that these techniques have high sensitivity and will be suitable than other routine laboratory.

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Isolation and characterization of antibiotics produced from Jordanian soil microorganisms

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ABSTRACT

Fifty soil samples were collected from clay of National Forest and wet mud from King Talal Dam bank in Jordan. Fifteen bacterial isolates were recovered on two culture media, SCM and J-agar for cultivation of *Streptomyces* and *Bacillus* species respectively. Bacterial Colonies were then picked up and transferred for further purification on suitable media. All isolates were characterized morphologically and biochemically. Six isolates were identified as Streptomyces and the other nine isolates were identified as Bacillus species. Three methods were used to determine the ability of isolates for production of antibiotics, namely: crowded plate technique, Agar streaking method and cup plate technique. Out of 15 isolates, only 9 isolates showed antimicrobial activity. All tested bacterial species inhibit *Staphylococcus aureus* (ATCC2923) except *Streptomycetes* strain NF 140, and *Escherichia coli* (ATCC25922) except *Bacillus* NF 131 and *Bacillus* KTD 136 as jugged by described methods. Butanol extracts obtained from seven isolates showed significant antimicrobial activity against indictor strains. Samples fractionated using thin layer chromatography (TLC) on silica gel plate and developed with solvent system to characterize the bioactive components. Two to three components were developed on TLC, only one component shows antimicrobial activities and indicated that these isolates may produce more than one secondary metabolites with different Rf values. According to Rf patterns behavior, one of current components have similar Rf behavior of chloramphenicol, others were similar to Ampicillin and erythromycin as confirm by chemical analysis which reveals that the component contain amino group.
This study concluded that the Jordanian soil harbors microorganisms not explored before have the ability to produce antibiotics.

Keywords: King Talal Dam, thin layer chromatography (TLC), crowded plate technique, Agar streaking method, cup plate technique

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الملخص باللغة العربية

تم جمع خمسين عينة من تربة الغابات الوطنية ومن الطين الرطب من ضفة سد الملك طلال بالأردن، وتم الحصول على خمس عشرة عزلة بكتيرية نمت على وسطين زرعيين هما أس سي أم و جي– اجار للتنمية، وتم التقاط مستعمرة واحدة نامية من مجموع المستعمرات لغرض تكاثرها وتوصيفها شكلا وكيموحيويا. وصفت ست عزلات على أنها (Streptomyces)، بينما وصفت العزل التسعة الباقية بأنها (Bacillus). استخدمت ثلاث طرق للكشف عن قابلية هذه العزلات لإنتاج المضادات الحيوية وهي: طريقة الطبق المزدحم، طريقة التخطيط على سطح الاجار و طريقة الابار على الاكار.
من مجموع خمسة عشر عزلة أظهرت تسع عزلات قابليتها على تثبيط نمو الجراثيم المذكورة سابقا كما يلي:
Staphylococcus aureus (ATCC2923) *Escherichia coli* (ATCC25922)
كل العزلات ثبطت نمو المكورات العنقودية، ما عدا *Streptomycetes* strain NF 140 ، ومن ناحية أخرى فإن العزلتين (*Bacillus* KTD 136، *Bacillus* NF 131) لم تستطع تثبيط *E.coli*.
تم اختيار سبع عزلات لدراسة موسعة وذلك بعد استعمال البيوتونال لاستخلاص المواد الفعالة التي تنتجها هذه العزلات وتنقيتها على صفائح السليكا جل باستخدام محلول مطور يساعد في حركة الحزم وتفريقها إلى حزم ، حيث أشارت نتائج التنقية إلى وجود حزمتين أو ثلاث ، ولكن حزمة واحدة فقط كان لها القدرة على التثبيط العالي للبكتيريا التي استخدمت بالفحص، وكانت مشابهة لحركة حزمة الكلورمفنيكول القياسية حتى باستخدام الصبغات.
ينتج من هذه الدراسة بأن هناك جراثيم موجودة بالتربة الأردنية لم يتم دراستها سابقا لها القدرة على إنتاج المضادات البكتيرية.

INTRODUCTION

The researchers around the world are still looking for new natural products that help in fighting diseases caused by several bacterial species. The attitude for finding new antibiotics remains the most desirable objective (1-4). Soil regards as a treasure for unknown microorganisms, since bacteria, *algae*, protozoa, yeasts, molds, and microscopic worms are routinely found in this environment. Soils may contain 10^9 to 10^{10} microorganisms per gram (dry weight), which may represent more than a million bacterial species (5).

Antibiotics are produced by many microorganisms in various ecological conditions. Producers of antibiotics can be found in the bank of rivers and lakes, decaying plants and animal remains, but majority of microorganisms that produce antibiotics inhabits soil (6-9).

Streptomyces and Bacillus have been well known for the production of secondary metabolite. The genus *Streptomyces* is represented in nature by the largest number of species and varieties, producing the majority of known antibiotics among the family Actinomycetaceae. *Streptomyces* are well known sources of antibiotics and other important novel metabolites, including antifungal agents, antitumor agents (6-8).

Several species of the genus Bacillus produce peptide antibiotics which are synthesized either through a ribosomal or non-ribosomal mechanism. The family's distinguishing feature is the production of endospores, which are highly refractile resting structures formed within the bacterial cells (10). A close relationship between sporulation and the production of secondary metabolites in microorganisms has been demonstrated by biochemical and genetic analysis of some organisms. In bacilli, the polypeptide antibiotics produced have been found to affect by spore formation directly or indirectly (11,12).

Tamehiro *et al* found a novel phospholipids antibiotic (named bacilysocin) which accumulates within (or associates with) the cells of *Bacillus subtilis* 168 and determined the structure by nuclear magnetic resonance and mass spectrometry analyses (13). Bacilysocin demonstrated antimicrobial activity, especially against certain fungi. Production of bacilysocin commenced immediately after growth ceased and before the formation of heat-resistant spores.

This study focused on the isolation and characterization of antibiotics such as substances produced from microorganisms inhabits in Jordanian soil that were not explored before.

MATERIALS AND METHODS

Sites and samples collections:

Samples were collected from two regions: National forest (NF) located at Queen Alia International Airport road and King Talal Dam (KTD) bank, located at the north of Amman city. Top soil samples to a depth of 10 cm were obtained from 25 separate sites. Samples were stored individually in separate plastic containers, refrigerated and processed for soil microbiology within 72 hrs. (8).

Isolation of streptomycetes and bacillus species:

Isolation of Streptomyces's species was preformed according to previous studies (6-8, 14); Starch Casein media (SCM) were used. A Starch Casein (SCM) agar plates (g/l): starch 10, casein 0.3, Sodium Nitrates (NaNO3) 2, Potassium phosphate (K2HPO4) 2, Sodium Chloride (CaCO3) 0.02, ferrous sulphate (FeSO4.7H2O) 0.01, Agar powder 20, (pH adjusted at 7.2) supplemented with cyclohexamide (50ug/ml) and filter-sterilized rifampicin (0.5ug/ml) were added. Procedures for isolation Bacillus species were performed on plates of J-agar (tryptone 5g/l, yeast extract 15g/l, K_2HPO_4 3g/l, glucose 2g/l, agar20g/l, pH7.4). Stock cultures can be maintained in the laboratory on soil extract agar or on special sporulation media (15).

Samples treatment:

The soil samples were dried separately at 37C^o for 1 hr in incubator. Then they were cooled to room temperature. One gm of each soil sample was added to a conical flask containing 100 ml of sterile water. All flasks were shaken for 30 minutes in orbital shaker incubator at 27 C^o. These flasks were considered as stock cultures (8). A series of culture tubes containing 9 ml of sterile water were taken. From the stock culture, 1 ml suspension was transferred aseptically to the 1st tube (10^{-1}), mixed well. From the 1st tube, 1 ml of suspension was transferred into 2nd tube (10^{-2}), mixed well. Similarly, dilutions up to 10^{-5} were made (serial dilution technique). 0.1 ml of suspension from each culture tube was spread on suitable media. Bacterial colonies were then picked up and transferred to for further purification (12).

Morphological and biochemical characterizations:

Morphological characterization of *Streptomyces* isolates were done according to the ISP recommendations (16). *Bacillus* species were characterized according to Bergey's Manual of Determinative Bacteriology (17). The biochemical test included: starch hydrolysis, casein digest and nitrate reduction, in addition to Indole production, Methyl red, Voges-Proskaure, oxidase, catalase, production of gas from glucose.

Assay for antimicrobial activity:

Test organisms: Three bacterial species were used to determine the antimicrobial activities, namely: *Staphylococcus auerus(S.aureus)* ATCC2923 , *Escherichia coli (E.coli)* ATCC25922 and *Micrococcus luteus* (ATCC 9341).

Three methods were used to determine the ability of isolates for production of antibiotics (6,8,18):
1. Crowded plate technique (Preliminary screening): Plates that contains mixed culture of microorganisms were folded by *Micrococcus luteus* and left to dry and then incubated at 37C^{o} for 3 days. Zone of inhibition around each colony were reported.
2. Agar Streak Method: The microbial sensitivity of the soil isolates were analyzed by 'Agar streak method'. Each of the isolate was streaked as a straight line on NA medium and incubated at 37C^{o} . After 3 days, *Staphylococcus aureus* and *E.coli* were streaked at right angle, but not touching to the streak and incubated at 37C^{o} for 24 hrs in case of bacteria and 27C^{o} for 48 hrs in case of fungi. If the organism is sensitive against the antibiotic produced by test isolate, then it will not grow near the isolate.
3. Agar block technique: Agar block from growing microorganism were cut and put over the nutrient agar plate which has been inoculated with: *S.aureus* and *E.coli.* Antibiotic production by a colony was defined as the inhibition of embedded bacterial growth by a ≥ 3 mm ring around the soil bacterial isolate.

Extraction of the antibiotic substance:

Fermentation :
Seven isolates were representative of 4 isolates of *Bacillus species* tagged as number KTD119, KTD120,KTD 133 and NF131 and 3 isolates of *Streptomyces species* tagged as KTD123, NF 140 and NF141 were utilized for the study after confirmation of their activity against *Staphylococcus aureus* and *Escherichia coli*. The test isolates were grown in 250ml flasks containing 50 ml of liquid medium composed of: 0.8 g NaCl, 1 g NH4Cl, 0.1 g KCl, 0.1 g KH2PO4, 0.2 MGSO4.7H2O, 0.04 g CaCl2.2H2O, 2 g glucose, and 3 g yeast extract dissolved in 1 liter DW, pH was adjusted at 7.3.
The flasks were inoculated with 1 ml of soil bacteria of the selected strain and incubated at 28 ^{o}C for 120 hours with shaking at 105 t/min. After growth, the cell free culture supernatant of each flask was extracted twice with equal volume of n-butanol (19, 20).The n-butanol layer was separated from the aqueous phase and concentrated on a rotary vacuum evaporator.

Extraction :
N-butanol extracts containing the bioactive substance: Hundred ml of n-butanol extracts containing the bioactive components were concentrated in using Rotary Evaporator machine (Heidolph) under reduced pressure at 60^{o}C to prevent destruction of the active component of extract allowing complete evaporation of n-butanol extracted solution was done (19, 20). The butanol extracts containing bioactive component of each seven sample fractionated using thin layer chromatography (TLC) on silica gel plate and developed with solvent system (20).

TLC solvent system for tested samples and certain antibiotics:
Each extract from designated isolate (KTD 119,120,123,133, NF 131,140, 141) and references were resolved on impregnated 10 X 15 silica gel layer using ethanol: acetic acid: water (50:30:30, v/v/v) as the mobile phase. Certain references antibiotics were used: Ampicilline (Merck, Germany), chlorophenicol, tetracycline and amino glycopeptide antibiotics (Sigma, USA).

TLC separation and *Rf* Value:
A small spot of solution containing the active samples that have antimicrobial activity applied to a TLC plate about 1.5 centimeters from the bottom edge plate and immerse in suitable solvent mentioned before. The solvent is allowed to completely evaporate off. Then it was air-dried. TLC plate exposed to UV light, and silica gel was fluorescing, while any organic molecule which absorbs UV light will appear as a dark blue spot (6). Spot(s) was/ were lightly traced with a pencil while visible; Circled gently with a dull pencil to permit initial method for visualization because when the UV light is removed, the spots disappear (20, 21).
Bands were scraped from the plates with a spatula under UV light, extracted with methanol and filtered through Whatman No. 5 paper. Each band was bioassayed using *S. aureus* and *E.coli*, the active bands were purified again on TLC using the same solvent system and visualized under UV light (22). The *Rf* for each band was measured. Each isolated band was also dissolved in methanol, and its UV absorption spectrum was measured to determine the maximum of the band. Compares between *Rf* value of our samples and *Rf* of references antibiotics that tested on (20,22).

TLC staining: Two stains were used to visualization and identification of components. Iodine vapor methods used for the visualization of organic compounds. Iodine was used for observation purpose because it has a high affinity for both unsaturated and aromatic compounds. Ninhydrain stain solution (1.5g ninhydrin powder dissolved in100 mlof *n*-butanol flask and then adds 3.0 ml acetic acid shaked well until complete dissolving) was used to observes the amino group of components. Ninhydrine powder is light sensitive substance, so operation done in dark away from light lamp. Ninhydrine solution placed in suitable spray bottle, and TLC spared (22).

RESULTS

Isolation of microorganisms from Jordanian soil:
Out of 50 soil samples collected from National Forest and King Talal Dam, 15 isolates were recovered (table 1). These isolates were distributed as 5 isolates from National Forest (NF), and 10 isolates from King Talal Dam Bank soil (KTD). The five isolates grow well on starch Casein agar medium (SCM) supplemented with antibacterial and antifungal agent, shows characteristic colonies, large creamy in color. Smear from colony shows the bacteria was Gram positive rods and filamentous with aerial coiled mycelia spores arranged in chain as observed by light microscope at 1000 magnification. The 10 isolates obtained from KTD shows two kinds of colonies: six isolates grow well on J-agar medium with large white-chalky colonies, smear from colony shows Gram positive Rod bacteria, while the other 4 isolates grow on SCM with characteristic large creamy elevated colonies (table 1).

Table (1): Number of isolates from different locations of Jordanian soil explored in this study

Soil description	No. of samples	No. of positive	Growth requirement	Growth morphology	Colonies color and diameter (mm)
Wet clay/KTD	25	8	J- agar	Gram positive Rods with spore forming	White-chalky large colonies
Dry soil/NF	25	1	J- agar	Gram positive chain rods	White-chalky large colonies
		6	SCM	Gram positive filamentous rods	Large creamy elevated colonies
Total	50	15			

Isolation and characterization of streptomycetes:
Six isolates out of fifty soil samples taken from National Forest (NF) were grown on SCM and show large creamy colonies with gram positive filamentous chain. The spore morphology of these strains produced aerial coiled mycelia and the spores arranged in chains. Spore chain arrangements were observed by microscope at 1000X showed that of all the isolates bear spore chains of two or more and non-motile in nature. These micro morphological and spore colors and mycelia properties strongly suggested that strains NF 128,129, 140,141,171 and 173 belonged to the genus *Streptomyces***.**

Isolation and characterization of bacillus species:
Nine isolates were grown on J-agar and shown white chalky large colonies with gram positive chain rods. The nomenclatures of these isolates were KTD 119, 120, 126, 133, 136, 143,123, 167 and NF 131. All isolates were negative to indol and not fermented glucose, also all isolates have the ability to produce oxidase and catalase with variable ability to reduce nitrate, hydrolysis of starch and digestion of casein. The morphological and biochemical characterization suggested that these isolates belong to bacillus species (table 2).

Table (2): Biochemical characterization of soil isolates from NF and KTD

Isolates code	Indole production	MR/VP	oxidase	catalase	Nitrate reduction	Starch hydrolysis	Casein digest	Glucose fermentation
KTD119	-	+ /-	+	+	+	+	+	-
KTD120	-	+/-	+	+	-	+	+/-	-
KTD 126	-	+/-	+	+	+	+	+/-	-
NF128	-	+/-	+	+	+	+	+	-
NF129	-	+ /	+	+	+	+	+	-
NF131	-	+ /	+	+	+	+	+	-
KTD 133	-	+ /	+	+	+	+	+/-	-
KTD136	-	+ /	+	+	+	+	+/-	-
NF140	-	+ /	+	+	+	+	+	-
NF141	-	+ /	+	+	+	+	+	-
KTD 143	-	+ /	+	+	+	+	+	-
NF 171	-	+ /	+	+	+	+	+	-
KTD 123	-	+ /	+	+	+	+	+/-	-
KTD 167	-	+ /	+	+	+	+	+	-
NF 173	-	+ /	+	+	+	+	+	-

Antimicrobial activity of Streptomycetes and Bacilli secondary metabolites:

Primary screening for antimicrobial activity: Primary screening of three sub cultured *Streptomycetes* species and four sub cultured *Bacillus* species were shown in table (3). All tested bacterial species inhibit *Staphylococcus aureus* (ATCC2923) except *Streptomycetes* NF 140 and *Escherichia coli* (ATCC25922) except *Bacillus* NF 131 and *Bacillus* KTD 136 as jugged by described methods (table 3).

Table (3): Antimicrobial activity of Streptomycetes and Bacilli

Isolates code	*S.aureus*	*E.coli*
Bacillus NF 131	+	-
Streptomycetes NF 140	-	+
Streptomycetes NF141	+	+
Streptomycetes NF128	+	+
Bacillus KTD 120	+	+
Bacillus KTD 119	+	+
Bacillus KTD 133	+	+
Bacillus KTD 136	+	-
Bacillus KTD 143	+	+

(+) Antimicrobial activity (-) No antimicrobial activity

Seven samples that show antimicrobial activity were selected and prepared for extraction and fermentation, after n-butanol extraction, antibacterial susceptibility testing was done by using same method.

Butanol extracts obtained from seven isolates showed significant antimicrobial activity against *E.coli* and *S.auerus* (table 4). Butanol extracts of all isolates shows more activity during secondary screening compared with primary screening (table 3). Unfortunate; isolate NF 140 shows excellent activity against *E.coli* (Zone of inhibition-34 mm),but not shows any activity against S.aureus and superior then primary screening.

Table (4): Primary screening of crude isolates against *E.coli* and *S.aureus* (Zone of inhibitions determined in (mm))

Tested samples	Diameter on *E.coli* lawn (mm)	Diameter on *S.aureus* lawn (mm)
KTD 119	12 mm	16 mm
KTD 120	14 mm	15.5 mm
KTD 123	12 mm	24 mm
KTD 133	14.5 mm	22 mm
NF 131	--	20 mm
NF 140	12 mm	--
NF 141	14 mm	11 mm

TLC Peak and *Rf* value of samples in compare of references: Figure (1) and table (5) revealed the purification and identification of components produced by isolates obtained from Jordanian soil.

Samples (KTD 119,120,123,133), (NF 131,140,141) were tested on thin layer chromatography with known antibiotics as references: Ampicillin, Chloramphenicol, Gentamicin, Neomycin and Tetracycline tested in the same circumstance. Samples were developed in proper solvent medium indicated the presence of three components for sample (KDT 120) and (KTD 123) A, B and C, and two components for samples (KTD 119), (KTD133), (NF 131) and (NF140) A and B, only one band was shown by sample (NF141).

(The band A representative for the faster moving bands were the others behind that, B= the middle spot and C= the third lowest spot). References antibiotics were developed in the same solvent system and revealed only one component. According to standard Rf value of known antibiotics. Component "B" of sample No.2 (KTD 120) shows similar Rf value with chloramphenicol (0.87), while component "B" of sample No.4 (KTD 133) shows similar Rf value with ampicillin (0.78). Component "B" of sample No.7 (NF 140) similar to Rf value of erythromycin (0.76). Other components show high value of Rf and not have the similar behavior of known antibiotics tested (Fig. 1 and table 5).

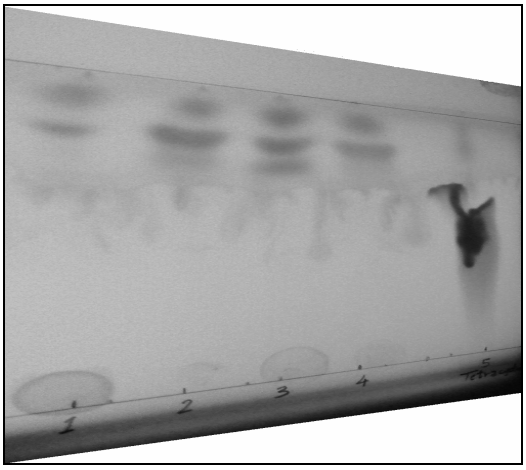

Figure (1): TLC of running butanol extracts from isolates visualized by UV light described by table (5).

Table (5): *Rf* value of components running on TLC

Tested Samples	Length in (cm)	*Rf* in (cm)
No. 1 = KTD 119	A=9.3 - B=8.2	A=0.93 - B=0.82
No. 2 = KTD 120	A=9.6 - B=8.7 - C= 8.2	A=0.96 - B=0.87 - C=0.82
No. 3 = KTD 123	A=9.8 - B=9.0 - C=8.1	A=0.98 - B=0.90 - C=0.81
No. 4 = KTD 133	A=10.2 - B=8.9	A=0.89 - B=0.78
No. 6 = NF 131	A= 9.4 - B= 8.7	A=0.94 - B=0.87
No. 7 = NF 140	A=10.4 - B=8.6	A=0.91 - B=0.76
No. 8 = NF 141	B=10.6	B=0.89
References Tested	**Length in (cm)**	***Rf* in (cm)**
No. 1 Ampicillin	8.9	0.78
No. 2 Chloramphenicole	9.9	0.87
No. 3 Gentamicin	2.1	0.18
No. 4 Neomycin	7.6	0.67
No. T Tetracyline	7.1	0.62
No. E Erythromycin	8.5	0.76

Secondary screening:
Bands from each sample developed on TLC were visualized by iodine vapor and circled them lightly with a pencil. The samples were not colored and need to be visualized with a UV lamp. UV lamp holed over the plate and circled illuminated spots. Determined bands were scraped from the TLC plates with a spatula. Eluted solution containing purified antibiotics were rescreened against *S. aureus and E.coli* and determined the zone of inhibition (table 6).

Table (6): Antibacterial activity of isolates against test organisms during secondary screening

Tested samples	Diameter at *E.coli* in (mm)	Diameter at *S.aureus* in (mm)
KTD 119	22 mm	26 mm
KTD 120	18 mm	19.5 mm
KTD 123	29 mm	24 mm
KTD 133	27 mm	17 mm
NF 131	----	28 mm
NF 140	34 mm	----
NF 141	32 mm	19 mm

Ninhydrin colorimetric method:
Ninhydrin, which is originally yellow, reacts with amino acid and turns deep purple. Band B shows purple color, while bands A and C were colorless (figure 2). Ninhydrin was reacting with a free alpha-amino group, NH_2-C-COOH, that means all purified antibiotics in this study were contain free alpha-amino group. the decarboxylation reaction will proceed for a free amino acid, it will not happen for other group. Thus, theoretically only amino acids will lead to the color development.

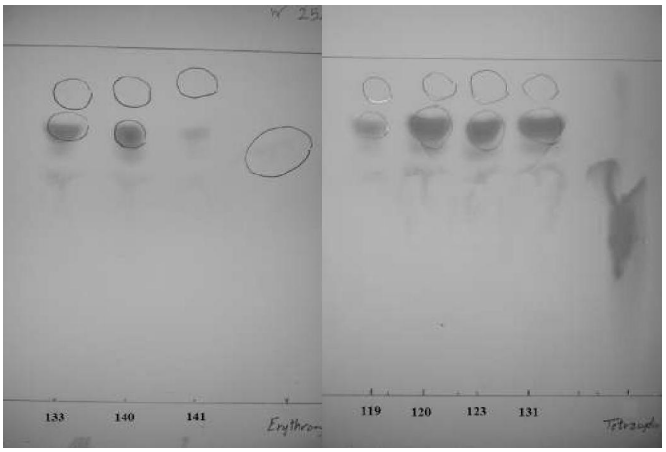

Figure (2):TLC of butanol extracts components spared by s Ninhydrin solution

DISCUSSION AND CONCLUSION

Fifteen isolates were recovered out of fifty soil samples collected from various location sites in Jordan. Jordan possesses a unique diversity of natural native soil, which has a microbial population that is not well understood. These diversities include dry sand from forests and wet clay from Dam bank. These sites either are explored or non-explored before. Our target was not to isolate microorganisms, but to search for few unexplored microorganisms that have the screen ability to produce antimicrobial agents that may help in discovering of new antimicrobial agents. These isolates were interested because it's isolated from soil have not been supplemented with either humus or sterilizer would be expected to be fast-draining and nutrient-poor with only a few dominant microbial species. Such locations yield bacteria secreting narrow-spectrum antibiotics directed against the few microbes able to survive these hostile environments such as NF 131 and NF 140 in this study. One gram of soil contains a huge number

of microorganisms, some of them of our interest, other were not. To reduce the number of non-wanted microorganisms, dilution method and selective media were used in this study (8); the plates were observed intermittently during incubation. After 72 hrs, pure culture of *Micrococcus luteus* overlay over growing colonies. Colonies which shows clear zones of inhibition around each colony were selected and purified for further studies. Mixed colonies might be picked up, since some of selected colonies did not showed antimicrobial activity, that's true especially when there are crowded colonies. Even though this method was found very useful then other two methods used in this study in screening for the isolates obtained.

King Talal Dam soil was selected because it is not explore before. The Dam is located northern Amman city and supply as reservoir of water. The bank of the Dam was composed of wet-clay. The wet clay environment is considered excellent place for harbor bacillus and fungal microorganisms (11, 23, 24,). More than third of the sample collected from the bank wet clay of King Talal Dam gave positive results when cultured on J-agar. Nine isolates were characterized as Bacillus species depend upon morphological and biochemical character, these isolates were characterized according Bergey's Determinative bacteriology (17) (tables 1,2 and 3).

In contrast to that soil nature of the second site was involved the National Forest (Queen Alia International Air port road, south Amman). The National Forest soil is characterized by dryness and not supplemented with either humus or sterilizer. Such location harbors bacteria secreting narrow spectrum antibiotics (NF131 and 140) directed against *S.aureus* and *E.coli* respectively. Only one isolate out of twenty five samples collected from National Forest soil was identified as Bacillus, while the other six, were identical morphologically and biochemically and tentatively identified as *Streptomyces* species (tables 1, 2).

All the potential isolates grew well on SCA agar media showing characteristics typical of *Streptomyces*. Streptomyces have been reported to grow well on Starch casein agar by earlier workers in this field (5-8,18,24). So SCA medium supplemented with antibacterial and antifungal agent was used for isolating *Streptomyces* strains (18).

Our results also confirm the previous studies in Jordan when they studied the Streptomyces flora of 75 soil samples, collected from different locations in Jordan (25) , were screened for their potential activities as sources of antibiotics antibiotic against resistant bacteria. All of the isolates were tested for their ability to produce inhibitory substances. The test microorganisms included Gram positive bacteria and Gram negative bacteria. We demonstrated that both narrow- and broad-spectrum antibiotic-producing bacterial species may be recovered from Dam bank (wet soil) and dry soil ecosystem. We have also showed that the antibiotics produced effect the two pathogenic bacteria; *S.aureus* and *E.coli*, Few were restrict to one type of test organism inhibit *E.coli* or *S.aureus*, while the other inhibited both of them, that may be due to hospitable ecosystem for *E.coli, Bacillus* and *Streptomycetes* at the same site together with adequate soil nitrogen and carbon levels, but were unable to effectively identify a set of environment factors selecting for narrow-spectrum antibiotic activity (5).

This study confirmed that the isolated bacteria from two location of Jordanian soils able to produce a wide variety of antibiotics with antibacterial activity and appeared promising.

In comparison between primary and secondary screening, results shows that the secondary screening were quite satisfactory as the extracted samples exhibited bigger zones of inhibition than the crude ones and at the same time did not produce any zone for the collected media supernatant to confirm the complete extraction of the antibiotic lead compound into organic solvent. It seems that logical, because re-extracted sample more concentrated than crude ones. The Rf patterns of antibiotics studied in this thesis can classified into four groups: Ampicillin, chloramphenicol, Erythromycin and non- group according to Rf value.

All antibiotics extracted from microorganisms isolated from Jordanian gave positive reaction to ninhydrine spray, as shown by purple color on the spots of TLC fractions due to the reaction of ninhydrine with amino acid. The presence of amino group in these isolates indicates that it's antibiotic like structure, the most standard and known antibiotics contain amino group in their structure. Dobrecky *et al.* monitored the behaviors of 16 antibiotics, examined by TLC sparing with Ninhydrin and detect color reaction , results in this study were similar to the findings by others (26, 27).

Seven isolates showed activity against bacteria in which all of them from Jordanian soil had succeeded in inhibition of bacterial growth.

We conclude that the bioactive substances produced by microorganisms isolated from Jordanian soil were of interest, since these compounds had antimicrobial activities.

Thus, the antibiotics described in this study had narrow and broad spectrums, which indicating that our compounds came from different sources and had diversity of action, since there is other compounds showed in TLC did not have antibacterial activities, but may have other biological functions. The Jordanian soil become a good and new source of antibiotics.

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The effect of ocimum basilicum ethanolic extract on some physiological aspects and histopathological changes in alloxanized male rats

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ABSTRACT

The present study was conducted to examine the effect of orally administered of ethanolic extract of *Ocimum basilicum* on blood serum glucose, insulin, hemoglobin A1c, hematology parameters (Hb, PCV, and RBC count) as well as its histological effect on the pancreas, liver and kidney when inducing diabetic type I in rats by alloxan. Twenty –four male rats were divided into three groups randomly, group I : (Negative control) received distilled water. Diabetes was induced in the second and third groups by alloxan injection intraperitoneal, group II : (Positive control) received distilled water. While, group III: (treated) were orally administered 200 mg/kg B.W/day of *Ocimum basilicum* extract for 6 weeks orally. The results showed that *Ocimum basilicum* extract administration significantly ($P<0.05$) decrease in the serum glucose and HbA1 concentrations in concordance with a significant ($P<0.05$) increase insulin level. While, there were significant changes ($P<0.05$)was observed on the Hb, PCV and RBC count. The histopathological changes were observed after administration of *Ocimum basilicum* extract showed a significant islet (beta cells) restoration and improvement histopathologocal changes in liver and kidney. From the results, it can be concluded that extract of *Ocimum basilicum* possess hypoglycemia effect as evidence by amelioration of pancreatic function as well as improving histopathologocal changes of pancreas, liver and kidney in diabetic animals.

Keywords: *Ocimum basilicum*, Physiological parameters, Histopathological, Male rats

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الملخص باللغة العربية

أجريت هذه الدراسة لتقييم تأثير الخلاصة الكحولية للريحان على بعض الجوانب الفسلجية والنسيجية في ذكور الجرذان المصابة بداء السكري من النوع الأول المستحث بالألوكزان الذي تم حقنه بالبريتون بجرعة 100ملغم/ كلغم. استخدمت في هذه الدراسة 24 من ذكور الجرذان وقسمت عشوائيا إلى ثلاث مجموعات، في كل مجموعة ثمانية من الجرذان، فأما المجموعة الأولى فهي مجموعة السيطرة السالبة التي أعطيت 1 مل ماء مقطر عن طريق الفم ، والمجموعة الثانية مجموعة السيطرة الموجبة مصابة بداء السكري المستحث بالألوكزان أعطيت 1 مل ماء مقطر عن طريق الفم، أما المجموعة الثالثة فكانت مجموعة معاملة مصابة بداء السكري المستحث بالألوكزان أعطيت الخلاصة الكحولية للريحان بجرعة 200 ملغم/ كلغم مذابة في 1 مل من الماء المقطر عن طريق الفم ولمدة ستة أسابيع.

أظهرت النتائج انخفاضا معنويا في كل من مستوى السكر في الدم والسكر التراكمي، بينما كان هناك ارتفاع معنوي في مستوى الأنسولين، عدد كريات الدم الحمراء، وتركيز الهيموجوبين وحجم خلايا الدم المرصوصة في مجموعة المعاملة التي أعطيت الخلاصة الكحولية للريحان، نسيجيا، فقد أظهر الفحص النسيجي في مجموعة المعاملة إصلاح تأثير الألوكزان في البنكرياس، الكبد والكلية مقارنة مع مجموعة السيطرة الموجبة (المصابة بداء السكري).

نستنتج من ذلك أن للخلاصة الكحولية للريحان تأثيرا خافضا لسكر الدم والسكر التراكمي وفي تحسين وظيفة البنكرياس وإصلاح التغيرات النسيجية في البنكرياس والكبد والكلية.

INTRODUCTION

Diabetes mellitus is a syndrome characterized by chronic hyperglycaemia and disturbances of carbohydrate, fat and protein metabolism associated with absolute or relative deficiency in insulin secretion and/or action (1). Chronic elevation of blood glucose eventually leads to long-term complications of diabetes, that leads to various tissue and organs damage that considered major causes of morbidity and mortality in human populations (2). Diabetes is commonly accompanied by other cardiovascular risk factors such as dyslipidemia, hypertension, prothrombic factors and microvascular problems involving eyes, kidney and peripheral nerves (3). However, increased free radical generation and oxidative stress are hypothesized to play an important role in the pathogenesis of diabetes and its late complications (4). The oral hypogly-caemic agents currently used in clinical practice have characteristic profiles of serious side effects such as cholestatic jaundice, aplastic and haemolytic anemias, generalized hypersensitivity reactions, liver failure and diarrhea (5) The side effects of insulin therapy which include insulin allergy, resistance and other late complications like morphological changes in kidneys and severe vascular complications (6). Today, it is estimated that about 80% of the world population relies on botanical preparations as medicine to meet their health needs (7). Several hypoglycaemic plants are potential in ameliorating lipid metabolism abnormalities of diabetes mellitus (8-10). Traditional herbal medicines are generally considered to be safer than synthetic drugs, its widely prescribed today despite the fact that their biologically active compounds are unknown, due to its minimal adverse effects, low costs, economical, effective, and their easy availability as well as to facilitate natural product drug discovery (11,12). *Ocimum_basilicum* (OB) is a plant belonging to Lamiacea family , which is widely cultivated in Asia as a nourishing food and herbal medicine. The *Ocimum basilicum*is considered as one of the most important source of medicine and drugs due to the presence of various phytochemical active compounds like alkaloids, saponins, tannins, phenols, flavonoids, isoflavonoids, proteins, steroids, terpenoids, cardiac glycosides, amino acids, sesquiterpenes, minerals, gums, mucilage, glucas and anthraquinone (13,14). Furthermore, *Ocimum basilicum* had been shown to possess diverse pharmacological properties which may be attributed to its usefulness in folk medicine to treat a wide range of diseases such as diabetes, cardiovascular diseases an antispasmodic, aromatic, digestive, carminative, stomachic and tonic agent. Many studies have established that basil leaves extracts have potent antioxidant, anti-aging, anticancer, antiviral and antimicrobial properties (15- 17). *Ocimum basilicum* has also been used externally for the topical treatment of acne, insect stings, snake bites, and skin infections (18, 19).

Aims of the study:

The present study was aimed at investigate the effects of *Ocimum basilicum* extract on blood glucose level, serum insulin hormone, hemoglobin A1c (HbA1c), Hb, PCV, RBC count and histological profile in pancreas, liver and kidney in animals induced diabetic type I.

MATERIALS AND METHODS

Plant preparation:
The fresh leaves of *Ocimum basilicum* were bought from the local market in Basra city/Iraq. The fresh leaves were collected, washed with distilled water and then dried under the shade at room temperature for six days. The dried leaves were cut into small pieces and ground into fine powder by using electric mill for 3 minutes. 50 gms of the powder were put in the round bottle flask, 200 ml of ethanol (70%) were added to flask and extracted for 12 hrs. at 70 °C. The extract was cooled and filtered with Whatman No. 1 filter paper. The filtrate was dried at room temperature and dryness powders were kept in tight closed container and stored at 4°C until use in the experimental procedure.

Experimental animals (rats):
The experiment was performed on twenty- four healthy male rats (*Rattus norvegicus*) weighing between (250 ± 25) gm and aged (12) weeks. Rats were kept for adaptation period of two weeks at the animal house of College of Veterinary Medicine / University of Basra. The animals were housed as four rats to each cage under optimum conditions (12 hrs. light/ dark cycle) and temperature of 25 ±2°C. These conditions were maintained throughout the duration of the experiment. The animals were fed with standard diet (pellet) and provided with water *ad libitum*.

Experimental design

Induction of diabetes: Diabetes was induced in overnight fasting rats by a single intraperitoneal injection of alloxan monohydrate (Sigma Ltd, USA) at dose 100 mg / kg body weight (20). Each 100 mg of alloxan was dissolved in 1ml of normal saline. Immediately after alloxan injection water replaced by 5% glucose solution for 24 hrs. in order to overcome sudden hypoglycemia (21). Diabetes was conformed 72 hrs. after induction, the rats were fasted for 12 hrs. and blood was taken from tail artery of the rats (22). The animals showing blood glucose level estimated by GOD-POD enzymetic colorimetric method (23).The animals were stabilized for a week and rats with blood glucose level more than 200 mg/dl were considered diabetic and selected for the study. Normal and diabetic rats

was randomly assigned to three groups (n = 8 in each group) as follows: Group I: (Negative control) the rats were received distilled water (1 ml). Group II: (Positive control) diabetic rats were received distilled water (1ml). Group III: (Diabetic treated) the rats were received *Ocimum basilicum* ethanolic extract (200 mg/ kg B.W) dissolved with distilled water (1ml). All treatment were continued for 6 weeks were administered by gastric intubation orally as single dose daily. After 6 weeks overnight fasting, rats of all groups were anaesthetized using ether solution inhalation. Blood samples were immediately collected from the heart and placed in plain tubes to clot at room temperature. The serum separated by centrifuge at 3000 rpm for 10 minutes. The serum used for glucose and insulin determined was done by using special enzymatic kits. Other blood samples were collected into tubes with anticoagulant (EDTA) which were used for hematological parameters. Immediately after blood collection, animals were sacrificed, Pancreas, liver and kidney carefully excised, washed with normal saline remove any red blood cells (erythrocytes) and clots.

Biochemical assay: The blood glucose concentration was measured by the glucose oxidase Method (23), the insulin level was determined using Elisa kit (24). While, the glycosylated hemoglobin (HbA1c) was determined using a hemoglobinA1c assay kit (Randox Lab., Ltd., UK) according to (25).

Haematological assay: Haematological values were measured by following standard methods at end of the experimental period. The RBC count was obtained by hematocytometer (Neubaure improved chamber) and using Hayme's solution as a diluting fluid and a special pipette for dilution (26,27). The microhematocrit method is used to calculate the percentage of PCV by the use of heparinized capillary tubes which contain heparin, the hematocrit value was obtained by service device (Hematocrit reader) (28). Hb concentrations estimated by Sahli apparatus (29).

Histological preparation: Small specimens of pancreas, Liver and Kidney from all groups were fixed in 10% neutral buffered formalin, dehydrated in ascending concentrations of ethyl alcohol (70-100%). Fixed specimens were embedded in paraffin wax and sections of 5µm thickness were cut. Slides were stained with Heamatoxylin and Eosin (H and E) for histological examination (30).

Statistical analysis: Data are presented as means ± SE. Statistical analysis was performed using one way analysis of variance (ANOVA). The values were considered to be significantly different when the P value was less than 0.05 compared to the respective control group.

RESULTS

The results showed a significant (P< 0.05) decreased in serum glucose concentration and hemoglobin A1c. This reduction was proportional with a significant (P<0.05) increased in insulin level in diabetic rats treated with *Ocimum basilicum* extract when compared to the diabetic group and control group (table 1).

Table(1): Blood glucose, hemoglobin A1c (HbA1c) and serum insulin level from control group, diabetic group and diabetic group treated with *Ocimum basilicum* extract

Groups	Blood glucose mg/dl	HbA1c %	Insulin µ Iu/ml
Negative control	79.37±2.33 C	4.43 ± 0.09 B	7.23±0.12 A
Positive diabetic	234.87 ±4.99 A	9.35 ± 0.13 A	3.58±0.05 C
Treated	96.72±2.79 B	5.15 ± 0.29 C	6.48 ±0.14 B

The different letters mean significant differences at (p < 0.05) level as compared with control group. Values are expressed as mean ± SE

The results indicated that a significantly (P< 0.05) increase in hemoglobin concentration, PCV and RBC count in diabetic group treated with *Ocimum basilicum* extract when compared to the diabetic rats and control group (table 2).

Table (2): Hemoglobin concentration (Hb), PCV and RBC count from control group, diabetic group and diabetic group treated with *Ocimum basilicum* extract

Groups	Hb g/100ml	PCV %	RBC count $x10^6$cell/ mm^3
Negative control	12.54±0.08 A	44 ± 0.49 A	4.84±0.11 A
Positive diabetic	8.25±0.17 C	29.55±0.44 C	2.92±0.07 C
Treated	11.77 ±0.26 B	42.47 ±0.50 B	3.95 ±0.14 B

The different letters mean significant differences at (p < 0.05) level as compared with control group. Values are expressed as mean ± SE

Histological findings:
The Pathological changes after administration of alloxan (100mg/kg B.W) which revealed in the Pancreas show vacuolation of islet (figure 1). The liver show diffuse vacuolation in hepatocytes and congestion in central vein (figure 2). While, in the kidney was seen atrophy of glumerulus and cortical areas of vacuolated and dilated tubules (figure 3). These changes were compared with negative control (figures 4-6). While, after administration of *Ocimum*

basilicum extract (200mg/kg B.W) it was observed that pancreas included restoration of the islet within normal limites (figure 7)). The liver showed hepatocytes and central vein within normal limites (figure 8). Whereas, kidney showed cortical areas tubules and glomerulus within normal limites (figure 9). These changes were compared with diabetic group (Positive control).

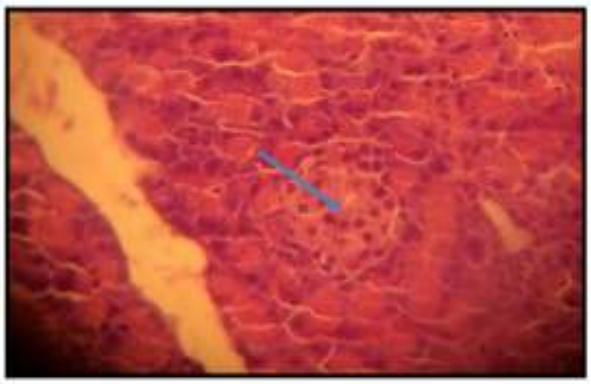

Figure (1):Pancreas section from control rats showing normal islet → H&EX100

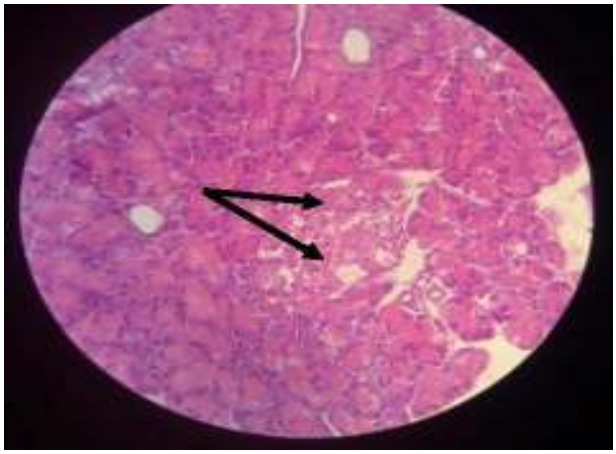

Figure (2): Pancreas section from diabetic rats, administered alloxan 100mg/kg of single dose IP showing vacuolation of islet (→) H&EX100

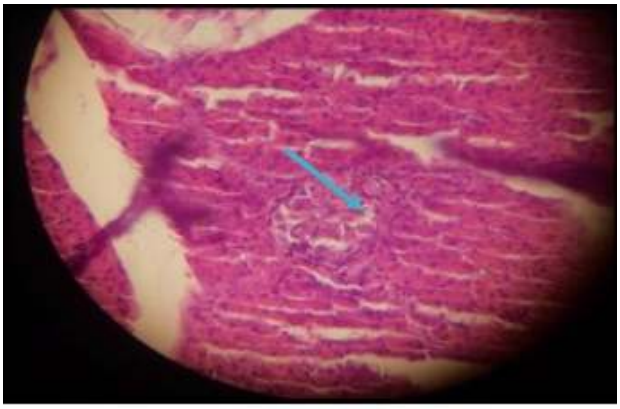

Figure (3):Pancreas section from diabetic rats treated with *Ocimum basilicum* extract 200 mg/kg for 6 weeks, demonstrates islet within normal limites (→) H&EX100

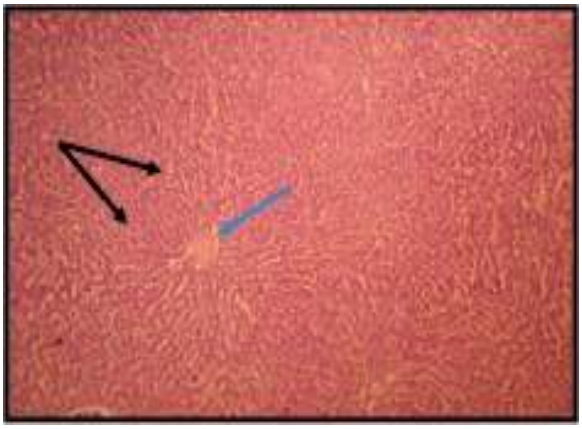

Figure (4): Liver section showing normal heypatocytes (→) central vein (→) in control rats H&EX100

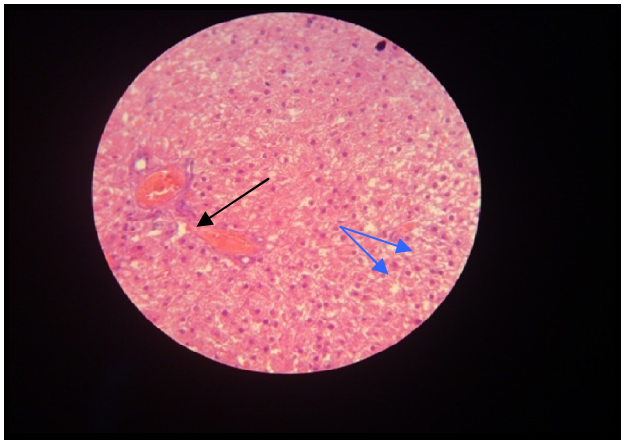

Figure (5): Liver section from diabetic rats , administered alloxan 100mg/kg single dose IP, demonstrates diffuse vacuolation of the hepatocytes (→) and congestion of the central vein (→) H&EX100

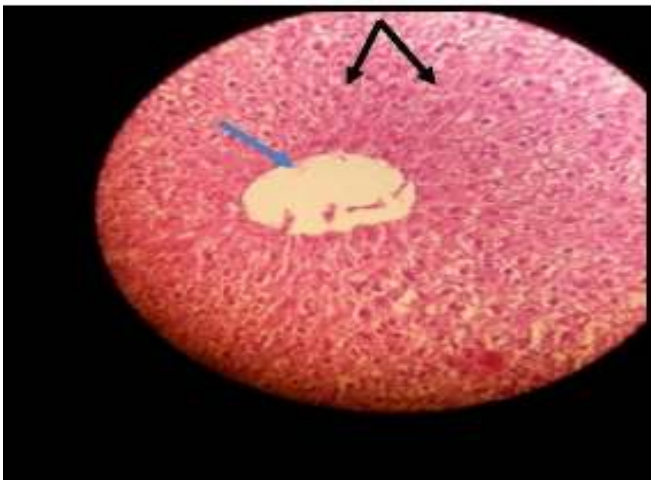

Figure (6): Liver section from diabetic rats treated with Ocimum basilicum extract 200mg/kg for 6 weeks, demonstrates hepatocytes (⟶) and central vein (⟶) within normal limits H&EX100

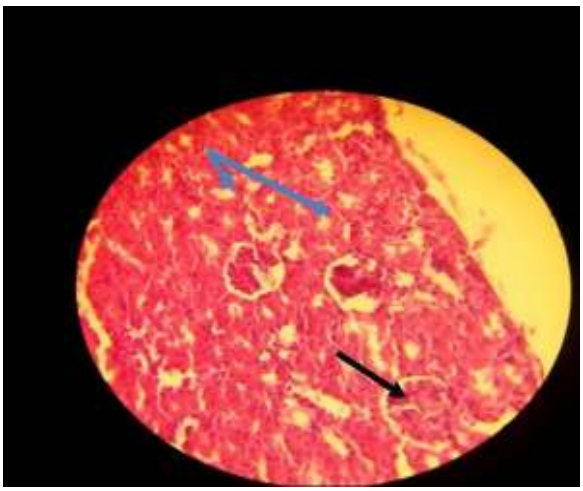

Figure (9): Kidney section from diabetic rats treated with Ocimum basilicum extract 200mg/kg for 6 weeks, demonstrates glomerulus (⟶) and cortical areas tubules within normal limits (⟶) H&EX100

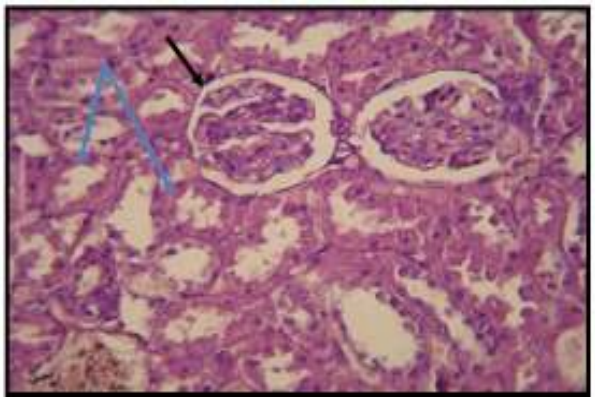

Figure (7): Kidney section showing normal glomerulus (⟶) and cortical areas tubules (⟶) in control rats H&EX400

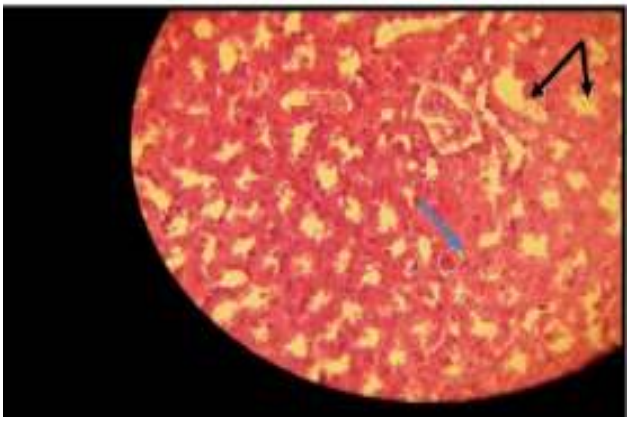

Figure (8): Kidney section from diabetic rats, administered alloxan 100mg/kg single dose IP, demonstrates atrophy of glomerulus (⟶) and cortical areas of vacuolated and dilated tubules (⟶) H&EX100

DISCUSSION

Diabetes mellitus is poised to become one of the largest global health problems in the 21st century because of its influences on multiple organ systems leading to serious complications therefore efforts remain necessary to discover new hypoglycaemic agents from plants (31). It is widely accepted that medicines of herbal origin play an essential role in treating diverse diseases since they are enriched of bioactive photochemical ingredients that might offer effective safe and potency as a therapeutic herb (32).

In the present study, alloxan was used as a diabetogen. It induces diabetes by destroying β-cells of the pancreas partially, through production of reactive oxygen species (33). In contrast in untreated diabetic rats, blood glucose levels increased due to the insulinopenia and the consequent insulin resistance (34). The oral administration of *Ocimum basilicum* extract resulted in a significant reduction in serum glucose level in diabetic rats treated. This indicated an enhanced glucose utilization triggered by insulin production from the beta cells. The profound medical effects of this herb may be attributed to its pharmaceutical potentiality due to presence of the active phyto-compounds like flavonoids, triterpenoids, alkaloids, saponins, tannins and polyphenols contents (13) These compounds are known bioactive antidiabetic principle (35). These findings are in agreement to pervious researches carried out on different ocimum species extracts (36- 38) reported that *Ocimum* species extracts has the ability to attenuate of hyperglycemia and ameliorate diabetic complications via suppressing blood sugar levels and increasing liver glycogen storage. On the other hand, Aqueous *Ocimum basilicum* extract may act via inhibition of hepatic glucose production and/or

renal glucose reabsorption, improving insulin action or stimulation of glucose utilization by the peripheral tissues (39).However, insulin level was found decreased in alloxan-induced diabetic rats. In general, several studies have demonstrated that alloxan has a β-cell cytotoxic , which significantly induced diabetes by damaging the a β-cell that causes reduction in insulin release (40, 41).There is a significant increase in serum insulin level was observed when alloxan diabetic animals were treated with *Ocimum basilicum* extract. These results have proved that the extract of *Ocimum basilicum* has a potent significant hypoglycemic effect comparable to that of effect by stimulating insulin secretion from β cells of pancreatic islets, the effects of this herb may be attributed to its flavonoids, *Ocimum basilicum*are rich source of flavonoids which have been shown to possess various biological properties related to antioxidant mechanisms (42). So it can be concluded that the extract has the potential to enhance the glucose-dependent insulin release from the pancreatic beta cells and thereby decrease the blood glucose level in alloxan-induced diabetic rats also improving insulin action (43). Moreover, further studies revealed protective effect of *Ocimum basilicum* extract on pancreatic beta cells in diminishing hyperglycemia-related oxidative stress. Indeed, it was reported that oxidative stress may have significant effect in the Glucose Transport Protein (GLUT) or at insulin receptor increasing serum glucose levels and scavengers of oxidative stress may have an effect in reducing serum glucose level in diabetes due to its strong antioxidant (44, 45). The rate of formation of HbAIC has been observed to be proportional to blood glucose level (46). The HbAIC is considered a reliable index in glycaemic control (47). In the diabetic group, HbAIC level increased significantly suggesting glycosylation of Hb in the presence of hyperglycaemia, glycosylated Hb shows reduced affinity to oxygen a process that aid free radical release (48). In extract treated, marked decrease in HbAIC concentration was observed when compared to that of diabetic animals indicating decrease in blood glucose level and recovery to Hb. A number of medicinal plants have been reported to reduce HbAIC formation due to its strong antioxidant (49). From our results, a significant decrease in hemoglobin concentration, PCV and RBC count in diabetic rats as compared with normal control indicates that the anemia occurring in DM is due to the increased non-enzymatic glycosylation of RBC membrane proteins, which correlates with hyperglycemia. On the other hand, oxidation of these glycosylated membrane proteins and hyperglyceamia in DM cause an increase in the production of lipid peroxides, which in turn cause the hemolysis of RBCs (50). However, the administration *Ocimum basilicum* extract caused increase in the hemoglobin concentration, PCV and RBC count in diabetic treated rats this may be due to the decreased level of blood glucose and/or due to lowered lipid peroxide level in RBC membrane leading to a decreased susceptibility of RBC to hemolysis. (51, 52). This in agreement with the (53, 54) demonstrated that administration of *Ocimum basilicum* in low and high dose by SRBC titre method where a good increasing values were observed in RBC, haemoglobin count and antibody in Wister albino rat this may be attributed to the presence high amount of phenolic compounds which have redical scavenging activity. Similar results were obtained by (55), who found that administration of aqueous extracts of *Ocimum basilicum* caused an increasing RBC count in *Clarias batracus.* Damage of pancreas, renal and liver tissues observed in the present study may be resulted from the increase in lipid peroxidation and decrease of antioxidant enzymes in the pancreas, kidney and liver following exposure to alloxan induced diabetes, administration of *Ocimum basilicum* extract improved the histological changes in the pancreas could be attributed to its major flavonoides components which are known to regenerate the residual beta cells after damaging effect by the diabetogenic agent (56) . This results revealed protective effect of *Ocimum basilicum* extract on pancreatic beta cell due to antidiabetic action and antioxidant properties (57, 58). Recovery of renal and hepatic tissues with treatment of the extract could be explained by the regenerative capability of the extract renal tubules and hepatocytes. The results seem to be in accordance with findings of other authors(59-62) showed that *Ocimum basilium* leaf extract suppressed histopathological alterations in liver and kidney of rats and restored creatinine, urea as well as liver function enzymes to its normal values. Similar findings were shown by (63-65) concluded that improvement liver and kidney morphology and function associated with administration of *Ocimum* species extract this explain their hepato-renal protective effect on its damage seen in diabetic rats. Similar finding were shown by (66, 67) demonstrated that dietary treatment of *Ocimum sanctum* normalized a high level of serum creatinine in diabetic rats, indicating its protective effect on renal glomerular filtration ability.

CONCLUSION

It was concluded that *Ocimum basilicum* extract possess hypoglycemia effect as evidence by amelioration of pancreatic function as well as improving of pancreas, liver and kidney structures in diabetic animals.

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Study the effect of oil and genestin extract of soybean seeds on oxidative stress and semen fluid characteristic in hyperthyrodism male rabbits induced by L-thyroxin

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ABSTRACT

Genestine extract and oil of soybean seeds are exhibited numerous interesting pharmacologic activities, very potent antioxidant and improve semen quality. Our study is to investigate the therapeutic effect of genestin extract and oil of soybean seeds against levothyroxin sodium–induced hyperthyroidism in male rabbits. The rabbit was used as a model to study the effects of hyperthyroidism induced with supraphysiologic doses of L-thyroxin sodium (L-T_4). Endocrine aspects of the thyroid in the pituitary-thyroid-gonadal axis have been studied extensively, but few controlled studies have been conducted on sperm output in males with hyperthyroidism. Thirty two male rabbits were divided randomly into four groups. Group 1: Rabbits received orally administration of normal saline (3ml) for 25 days (as served control group). Group 2: Rabbits received orally administration of L-thyroxin sodium at dose 50µg/kg B.W. /day dissolve in (3ml) normal saline for 10 days for induced hyperthyroidism. Group 3: Rabbits received orally administration of L-thyroxin sodium at dose 50µg/kg B.W. /day dissolve in (3ml) normal saline for 10 days for induced hyperthyroidism and treated with oil of soybean seeds(1ml /Kg B.W.) for15 days. Group 4: Rabbits received orally administration of L-thyroxin sodium at dose 50µg/kg B.W. /day dissolve in (3ml) normal saline for 10 days for induced hyperthyroidism and treated with genestine extract of soybean seeds(0.5g/Kg B.W.) for15 days. The experimental results revealed that hyperthyroid rabbits had significant decrease ($P<0.05$)body weight and body weight gain, testosterone concentration, TSH, zinc and total protein levels, sperm count, sperm motile while significant increase ($P<0.05$) in serum level of T3,T4 GPx, SOD, ALP, ACP, lipid profile, glucose concentration, urea, dead and abnormalities of sperm. Histological sections showed that the changes of thyroid, liver, testes and spermatogenesis was moderately depressed in hyperthyroid rabbits. Genestin extract and oil of soybean seeds treatment suppresses the hyperthyroidism-induced oxidative damage. These results suggest that experiment is accompanied with increased oxidative aggressions. A therapeutic effect of genestin extract and oil of soybean seeds on oxidative stress and semen fluid characteristic in hyperthyroidism male rabbits induced by excessive administration of thyroid hormones were detected and for the first time antithyroid activity were observed.

Keywords: Genestine, soybean seeds, L-thyroxin sodium (L-T_4).

الملخص باللغة العربية

صممت هذه الدراسة لتقصي التأثير العلاجي لزيت ومستخلص الجنستين لبذور فول الصويا على حالة الإجهاد التأكسدي وصفات السائل المنوي في ذكور الأرانب المصابة بفرط الدرقية المستحث بالثيروكسين، وقد أجريت الدراسة في البيت الحيواني لكلية الطب البيطري في جامعة البصرة على عينة مكونة من 32 من ذكور الأرانب المحلية، قسمت بصورة عشوائية إلى أربع مجموعات، الأولى هي مجموعة الأرانب السيطرة التي عوملت بالمحلول الفسيولوجي (3مل) لمدة 25 يوما، والمجموعة الثانية هي الأرانب التي عوملت بالثيروكسين بجرعة 50 مايكرو غرام /كغم لمدة 10 أيام لاستحداث فرط الدرقية ، أما المجموعة الثالثة فشملت الأرانب التي عوملت بالثايروكسين بجرعة 50 مايكرو غرام /كغم لمدة 10 أيام + (1 مل / كغم) من زيت بذور فول الصويا لمدة 15 يوما، بينما شملت المجموعة الأخيرة الأرانب المعاملة بالثيروكسين بجرعة 50 مايكرو غرام /كغم لمدة 10 أيام+ (0.5غم/كغم من مستخلص الجنسين لبذور فول الصويا) لمدة 15 يوما. بعد انتهاء التجربة تم قياس وزن الجسم ومعدل الزيادة الوزنية لجميع الحيوانات ، وتم سحب عينات الدم وجمع السائل المنوي من جميع الحيوانات.

بينت النتائج حدوث انخفاض معنوي في وزن الجسم ومعدل الزيادة الوزنية، وانخفاض معنوي في مستويات هرموني TSH , Testosterone, ومستوى Total protein, والزنك ، وكذلك انخفاض العدد الكلي للنطف وعدد النطف الحية وقلة فعاليتها وحركتها للأرانب المصابة بفرط الدرقية مقارنة مع مجموعة السيطرة والمجاميع المعالجة بالزيت ومستخلص الجنستين، بينما بينت النتائج زيادة معنوية في مستويات هرموني T4,T3، وأنزيمات GPx , SOD,ACP,ALP , Lipid profil ومستوى السكر واليوريا، وزيادة في أعداد النطف الميتة والمشوهة، وكذلك بينت النتائج حصول تغيرات نسجية بالنسبة للغدة الدرقية والكبد والخصى في مجموعة الارانب المعاملة بالثيروكسين فقط.

إن تناول 1 مل من زيت فول الصويا أو 0.5غم /كيلوغرام من وزن الجسم لمستخلص الجنستين أدى إلى انخفاض مستويات هرموني T3, T4 بالإضافة إلى تأثيره الواضح على تقليل مستويات هرموني T3, T4 إلى إرجاع مستويات كل من GPx ، SOD، الزنك وبقية المعايير الأخرى إلى مستوياتها الطبيعية مقارنة بمجموعة السيطرة، وكذلك العدد الكلي للنطف وعدد النطف الحية وفعاليتها وحركتها، وبهذا ينتج أن لزيت ومستخلص الجنستين دورا علاجيا لحالة الإجهاد التأكسدي والتغيرات المصاحبة لها لصفات السائل المنوي الحاصلة بفعل تناول الثيروكسين، وتعتبر هذه التجربة هي الأولى من نوعها في ملاحظة دور المستخلص في كبح نشاط فرط الدرقية.

INTRODUCTION

Endocrine system is the second key regulator of organ system functions after nervous system in animal body. Hormones are actual messengers in endocrine signaling.

Thyroid is a part of the hypothalamus-pituitary-thyroid axis (HPT axis). Thyroid-stimulating hormone (TSH) is secreted by the anterior pituitary. Thyrotropin-releasing hormone (TRH) from the hypothalamus binds to its receptors at the pituitary to control release of TSH. TSH binds to the TSH receptor on thyroid epithelial cells to signal thyroid gland secrete triiodothyronine T_3 and thyroxin T_4.

Thyroid gland holds a critical place in controlling brain and somatic development in infants and metabolic activities in adults. Upon stimulation by thyroid stimulating hormone (TSH), thyroid gland secretes thyroid hormones: T_3 and T_4. Although thyroid hormones have a central role in controlling basal metabolic rate, growth, as well as the development and differentiation of many cells in the body (1), their effect on spermatogenesis is not fully understood. Until very recent thyroid was thought not to affect spermatogenesis; however, research is now actively being pursued to understand the primary effects of thyroid hormones on spermatogenesis.

Spermatogenesis is generally divided into three distinct stages: (i) mitosis of spermatgonia (ii) meiosis to make haploid germ cells (iii) maturation of spermatids to spermatozoa (2). Disturbance at any step could affect the process of spermatogenesis and the spermatozoa may become defective (2).

Spermatogonia give rise to mature spermatozoa under hormonal control of the gonadotropins such as luteinizing hormone (LH) and follicle stimulating hormone (FSH). Recent identification of thyroid hormone receptors (TRs) directly on the testis and finding that thyroid hormone affects the growth and development of the male testes has accelerated research in this field (1,3). Specifically, TRs are located on the serotoxin cells in the seminiferous tubules, and it is believed that T3 binds directly to these receptors (3). Sertoli cells are first somatic cells to differentiate in the testis and they support and nurture sperm during spermatogenesis (4). TR on sertoli cells can mediate possible role, if any, of thyroid hormones in sperm production (2). More specifically, a particular interest has grown concerning the effects of thyroid disease such as hyperthyroidism on spermatogenesis and overall male fertility.

Thyroid gland activity may be affected by natural compound such as phytoestrogen. It operates directly on thyroid tissue through estrogen receptor and causes hypothyroidism. The phytoestrogens such as isoflavonoid from *glycine max* (soybean) possibly effecting on the secretion of thyroid hormones. *In vivo* it has been shown that isoflavonoid possesses goitrogenic activity and causes inhibition the activity of thyroid peroxidase (5) and *in vitro* (6). Actually thyroid peroxidase is essential to normal thyroid function as it catalyze the reactions required for thyroid hormones synthesis.

The aim of the study was to explore the relationship between thyroid hormone level and semen quality in population that included male recruited from an infertility clinic to detect associations between thyroid hormones levels and semen quality. It aimed also to study the therapeutic effect of genestin extract and oil of soybean seeds on oxidative stress and semen fluid characteristic in hyperthyroidism male rabbits.

MATERIALS AND METHODS

Experimental animals:

In the present study, a total of thirty two adult male local rabbits were obtained from the local market. Rabbits initially weighing 1600-1800 g and seven-month-old were used. Animals were acclimated to holding facilities for two weeks prior to the experiment. The rabbits were housed in groups and kept in room under controlled temperature (24°C), humidity (30-70 %) and light (12: 12 hrs / light: dark). All animals were provided balanced diet throughout the experimental period. This formed of proteins, fibers, wheat, clover, minerals and many vitamins. Animals were given food and water *ad libitum*.

Preparation of oil and genestin extract of soybean seeds:

50 gm of dried seeds powder were defatted with (500 ml) n-hexane for 16 hours by soxhlete. The combined n-hexane extract was concentrated below 50°C under reduced pressure in a rotary evaporator to get 10 gm of yellow oily mass. This mass was dried at room temperature and further (40 gm) was refluxed in (500ml) methanol (80%) in water with 3% hydrochloric acid. The sample was refluxed with solvent for one hour then filtered by Buchner funnel and filter paper (Watt man No.185). The filtrate was extracted with an equal volume of chloroform to remove pigments. The alcoholic layer was extracted with an equal volume of ethyl acetate, then treated with 2% of hydrochloric acid. The ethyl acetate layer was concentrated by rotary evaporator at 45˚C and dried at room temperature (7,8). The resultant extract (3gm) was yellowish and dry material, the percentage was (7.5% w/w).The extract was kept in dark glass container at 4°C.

Experimental design:

Thirty two adult male rabbits (8 in each groups) were divided into four groups and treated with oil and genestin extract of soybean seeds oral administration for 15 successive days as follows:

Group (1): Rabbits received orally administration of normal saline (3ml) for 25 days (as served control group).

Group (2): Rabbits received orally administration of L-thyroxin sodium at dose 50µg/kg B.W. /day dissolve in (3ml) normal saline for 10 days for induced hyperthyroidism.
Group (3): Rabbits received orally administration of L-thyroxin sodium at dose 50µg/kg B.W. /day dissolve in (3ml) normal saline for 10 days for induced hyperthyroidism and treated with oil of soybean seeds(1ml /Kg B.W.) for15 days.
Group (4): Rabbits received orally administration of L-thyroxin sodium at dose 50µg/kg B.W. /day dissolve in (3ml) normal saline for 10 days for induced hyperthyroidism and treated with genestin extract of soybean seeds (0.5g/Kg B.W.) for 15 days.

Body weight and body weight gain measurement: The animals were weighed before and after the end of the experiment.

Sampling:
Blood samples: At the end of each experimental period, blood samples were collected, from fasted male rabbits (control and treat animals), from the heart by heparinized capillary tubes in plain tubes, and allowed to be clotted at room temperature and put in centrifuge at 5000rm to obtain serum for hormonal assay and biochemical analysis such as (Lipid profile, total protein, glucose, GPx, SOD, Urea, ACP, ALP and zinc).

Hormonal Assay: Serum samples and plasma semen were assayed for TSH, T4, T3, testosterone, using the enzyme-linked immunosorbent assay (ELISA) technique using the Fortress kit.

Semen Collection: The testes were removed along with the epididymides. The caudal epididymides were separated from the testis, blotted with filter papers and lacerated to collect the semen. The semen collected dilution with normal saline and input in tubes and centrifuge for obtain semen plasma and studied physical and biochemical properties of semen such as semen volume and color.

Semen analysis:
Progressive sperm motility: This was done immediately after the semen collection. Semen was squeezed from the caudal epididymis onto a pre-warmed microscope slide (37°C) and two drops of warm 2.9% sodium citrate was added, the slide was then covered with a warm cover slip and examined under the microscope using 400Xmagnification. Ten fields of the microscope were randomly selected and the sperm motility of 10 sperms was assessed on each field. Therefore, the motility of 100 sperms was assessed randomly. Sperms were labeled as motile, sluggish, or immotile. The percentage of motile sperms was defined as the number of motile sperms divided by the total number of counted sperms (i.e. 100) (9).

Sperm viability (Live/dead ratio): This was done by adding two drops of warm Eosin/Nigrosin stain to the semen on a pre-warmed slide, a uniform smear was then made and dried with air; the stained slide was immediately examined under the microscope using 400X magnification. The live sperm cells were unstained while the dead sperm cells absorbed the stain. The stained and unstained sperm were counted and the percentage was calculated by the following equations (10):

$$\text{Live sperm \%} = \frac{\text{Live sperm}}{\text{Total sperm count}} \times 100$$

$$\text{Dead sperm \%} = \frac{\text{Dead sperm}}{\text{Total sperm count}} \times 100$$

Sperm maturation by aniline-blue: Nuclear maturation was evaluated by aniline-blue stain, according to (11). Sperm nuclei that stained with blue color were considered to be immature. But nuclear mature sperm was not stained with aniline-blue. The percentage of immature sperm was calculated from the observation of one hundred sperm preparation from each group.

Sperm morphology: A drop of Negrosin-Eosin stain was added to the sperm suspension and kept for 5 min. at 37°C. After that a drop of sperm suspension was placed on a clean slide and spread gently to make a thin film. The film was air dried and then observed under a microscope for changes in sperm morphology, according to the method of (10). The criteria chosen for head abnormality was; amorphous, pin and shortbread. For tail, the abnormalities recorded were; coiled flagellum, bent flagellum, bent flagellum tip. The result are the percentage overall abnormal form**.**

Sperm count: This was done by removing the caudal epididymis from the right testis and blotted with filter paper. The caudal epididymis was immersed in 5ml formal-saline in a graduated test-tube and the volume of fluid displaced was taken as the volume of the epididymis. The caudal epididymis and the 5ml formal-salline were then poured into a mortar and homogenized into a suspension from which the sperm count was carried out using the improved Neubauer haemocytometer under the microscope (10).

Histology examination:
After removing the thyroid, liver and testes, they were immediately fixed in Bouin's fluid for 12 hrs and the Bouin's fixative was washed from the samples with 70% alcohol. The tissues were then cut in slabs of about 0.5cm transversely and the tissues were dehydrated by passing through different

grades of alcohol: 70% alcohol for 2 hrs, 95% alcohol for 2 hours, 100% alcohol for 2 hrs, 100% alcohol for 2 hrs and finally 100% alcohol for 2 hrs. The tissues were then cleared to remove the alcohol; the clearing was done for 6 hrs using xylene. The tissues were then filtrated in molten Paraffin wax for 2 hrs in an oven at 57°C, thereafter the tissues were embedded. Serial sections were cut using rotary microtone at 5 microns (5µm). The satisfactory ribbons were picked up from a water bath (50°C - 55°C) with microscope slides that had been coated on one side with egg albumin as an adhesive and the slides were dried in an oven. Each section was deparaffinized in xylene for 1minute before immersed in absolute alcohol for 1 minute and later in descending grades of alcohol for about 30seconds each to hydrate it. The slides were then rinsed in water and immersed in alcoholic solution of hematoxylin for about 18 minutes. The slides were rinsed in water, then differentiated in 1% acid alcohol and then put inside a running tap water to blue and then counterstained in alcoholic eosin for 30 seconds and rinsed in water for a few seconds, before being immersed in 70%, 90% and twice in absolute alcohol for 30 seconds each to dehydrate the preparations. The preparations were cleared of alcohol by dipping them in \xylene for 1 minute. Each slide was then cleaned, blotted and mounted with DPX and cover slip, and examined under the microscope. Photomicrographs were taken at40X, 100X and 400Xmagnifications (12).

Statistical analysis:
The data were analyzed by SPSS software using one way variance analysis ANOVA, Version16. In all tests, a P-value of <0.05 was considered statistically significant (13).

RESULTS

Effect of oil and genestin extract of soybean seeds on TSH, T4 and T3 levels in serum hyperthyroidism male rabbits:
Results in table (1) observed that the effect of oil and genestin extract of soybean seeds on TSH,T4 and T3in serum hyperthyroidism male rabbits. The results were showed significant (P<0.05) increase of T_3 and T_4 in serum hyperthyroidism male rabbits compared with control group and other groups while the result was revealed significant (P<0.05) decrease TSH in serum hyperthyroidism of male.

Table (1): Effect of oil and genestin extract of soybean seeds on TSH, T_4 and T_3 levels in serum hyperthyroidism male rabbits. (Mean ± SD N=8)

Parameters / Groups	TSH (µIU/ml)	T4 (µg/dl)	T3 (ng/ml)
Control (Normal Saline) 0.9% NaCl	2.09 ± 0.012A	10.69± 0.14B	1.24 ± 0.04B
L-Thyroxin sodium (50µg/kg)	0.57 ± 0.054B	17.83 ± 1.96A	1.87 ± 0.057A
L-Thyroxin+Oil of Soybean Seeds (1ml/Kg)	1.69 ± 0.049A	12.42 ± 0.81B	1.38 ± 0.069B
L-Thyroxin +Genestin Extract of Soybean Seeds (0.5 g/kg)	1.85 ± 0.057A	11.73 ±0.16B	1.19 ± 0.021B

N=number of animals, A,B,C= differences between groups, P≤0.05 vs. control.

Effect of oil and genestin extract of soybean seeds on body weight and body weight gain in hyperthyroidism male rabbits:
Results in table (2) observed that the effect of oil and genestin extract of soybean seeds on body weight and body weight gain in hyperthyroidism male rabbits. The results were showed significant (p<0.05) decrease body weight and body weight gain in hyperthyroidism male rabbits compared with control group and another groups while that showed significant (p<0.05) increase body weight and body weight gain in hyperthyroidism male rabbits treated with Oils and genestin extract of soybean seeds compared with hyperthyroidism male rabbits group and non–significant (p<0.05) increase compared with control group.

Table (2): Effect of oil and genestin extract of soybean seeds on weight and body weight gain in hyperthyroidism male rabbits. (Mean ± SD N=8).

Parameters / Groups	Initial Body weight (G)	Final Body weight (G)	Body weight gain (G)
Control (Normal Saline) 0.9% NaCl	1900±20.57 A	2000±60.83 A	100 ± 9.36 A
L-Thyroxin sodium (50µg/kg)	1850±47.39A	1500±30.54 B	-350 ± 7.68 B
L-Thyroxin + Oil of Soybean Seeds (1ml/Kg)	1855±43.62 A	1950±27.10 A	95 ± 10.42 A
L-Thyroxin + Genestin Extract of Soybean Seeds (0.5 g/kg)	1870±56.82 A	1960±44.26 A	90 ± 18.51 A

N=number of animals, A,B,C= differences between groups, P≤0.05 vs. control

Effect of oil and genestin extract of soybean seeds on biochemical analysis in serum hyperthyroidism male rabbits:

Results in table (3) observed that the effect of oil and genestin extract of soybean seeds on biochemical analysis in hyperthyroidism male rabbits. The results were showed significant ($P<0.05$) increase of glucose level, ALP, ACP, urea levels while the results of GPx, SOD, total protein and zinc levels revealed significant ($P< 0.05$) decrease in serum hyperthyroidism male rabbits. Also the results were showed significant ($P<0.05$) decrease of glucose concentration, ALP, ACP and urea in serum hyperthyroidism male rabbits treated with oil and genestin extract of soybean seeds and non-significant ($P>0.05$) increase zinc concentration in serum hyperthyroidism male rabbits treated with oil and genestin extract of soybean seeds compared with hyperthyroidism male rabbits and non-significant changes compared with control group.

Effect of oil and genestin extract of soybean seeds on on Lipid profile in serum hyperthyroidism male rabbits:

Results in table (4) observed that the effect of oil and genestin extract of soybean seeds on lipid profile in hyperthyroidism male rabbits. The results were showed significant ($P<0.05$) increase of total cholesterol, triglyceride, LDL and VLDL in serum hyperthyroidism male rabbits while revealed significant ($P< 0.05$) decrease HDL in serum hyperthyroidism male rabbits compared with control group and other groups.

Table (3): Effect of oil and genestin extract of soybean seeds on on biochemical in analysis serum hyperthyroidism male rabbits. (Mean ± SD, N=8)

Parameters / Groups	Total protein mg/dl	Glucose mg/dl	GPx (mmol/L)	SOD U/dL	ALP U/L	ACP U/L	Urea mg/dl	Zinc mg/dl
Control (Normal Saline) 0.9% NaCl	67.26 ± 8.34 A	112.39 ± 15.43 B	16.25 ± 0.86 A	91.3 ± 1.29 A	26.7± 2.5 B	29.3± 7.11 B	30.62± 7.16 B	1.21± 0.012 A
L-Thyroxin sodium (50µg/kg)	42.58 ± 9.37 B	287.95 ± 17.29 A	7.68 ± 0.15 B	56. 36± 0.13 B	57.83± 5.78A	68.31± 2.89A	52.62± 14.38A	0.86± 0.023B
L-Thyroxin+Oil of Soybean Seeds (1ml/Kg)	62.37 ± 13.26 A	146.98 ± 10.07B	15.46 ± 0.37 A	89.45± 0.28 A	27.66± 3.69 B	31.47± 3.86 B	32.02± 6.24 B	1.28 ± 0.015 A
L-Thyroxin +Genestin Extract of Soybean Seeds(0.5 g/kg)	66.3 ± 11.58 A	127.83 ± 22.10 B	17.05± 0.19A	90.29± 0.15 A	25.91± 1.2 B	27.4± 3.18B	36.23± 3.69 B	1.23 ± 0.027 A

N=number of animals, A,B,C= differences between groups, P≤0.05 vs. control

Table (4):Effect of oil and genestin extract of soybean seeds on lipid profile in serum hyperthyroidism male rabbits (Mean ± SD, N=8)

Parameters / Groups	Total Cholesterol mg/dl	Triglyceride mg/dl	HDL mg/dl	LDL mg/dl	DL mg/dl
Control (Normal Saline) 0.9% NaCl	198.70 ± 28.01 B	115.60 ±24.32 A	89.42 ±56.29 A	58.7±2.5 B	45.6 ±7.11 B
L-Thyroxin sodium (50µg/kg)	338.96 ±70.8 A	198.32 ±32.19 A	67.28 ± 43.27 B	89.1±6.3 A	67.2±8.21 A
L-Thyroxin+Oil of Soybean Seeds (1ml/Kg)	219.37±52.90 B	120±17. 32 B	93.16 ± 24.97 A	64.74± 3.1 B	52.92±11.45 B
L-Thyroxin +Genestin Extract of Soybean Seeds(0.5 g/kg)	150.19±27.86 C	95.20±15.84 B	100.96±5 8.21 A	59.35 ± 7.24 B	48.39±15.57 B

N=number of animals, A,B,C= differences between groups, P≤0.05 vs. control

Effect of oil and genestin extract of soybean seeds on on Physical properties of semen analysis in hyperthyroidism male rabbits:

Results in table (5) observed that the effect of oil and genestin extract of soybean seeds on physical properties of semen analysis in hyperthyroidism male rabbits. The results were showed significant ($p<0.05$) decrease in semen volume, sperm motility, sperm concentration, total sperm cell/ ejaculate, live-dead sperm and significant ($p<0.05$) increase in sperm abnormalities in hyperthyroidism male rabbits compared with control and another groups while The results were showed significant ($p<0.05$) increase in semen volume, sperm motility, sperm concentration, total sperm cell/ ejaculate, live-dead sperm and significant ($p<0.05$) decrease in sperm abnormalities in male rabbits treated with L-T4+Genestin extract of soybean seeds compared with control and another groups but its non-significant ($p<0.05$) in semen volume, sperm motility, sperm concentration, total sperm cell/ ejaculate, live-dead sperm in male rabbits treated with L-T4+ oil of soybean seeds compared with control.

Effect of oil and genestin extract of soybean seeds on testosterone level in serum and semen hyperthyroidism male rabbits:

Results in table (6) observed that the effect of oil and genestin extract of soybean seeds on testosterone in serum and semen hyperthyroidism male rabbits. The results were showed significant ($P<0.05$) decrease of testosterone level in serum and semen hyperthyroidism male rabbits compared with control group and other groups while the results were showed non- significant ($P <0.05$) changes of testosterone level in serum and semen male rabbits treated with oil and genestin extract of soybean seeds compared with control.

Table (5): Effect of oil and genestin extract of soybean seeds on physical properties of semen analysis in hyperthyroidism male rabbits (Mean ± SD, N=8)

Parameters / Groups	Control (Normal Saline) 0.9% NaCl	L-Thyroxin sodium (50µg/kg)	L-Thyroxin + Oil of Soybean Seeds (1ml/Kg)	L-Thyroxin + Genestin Extract of Soybean Seeds (0.5 g/kg)
Semen volume(ml)	0.70 ± 0.06B	0.45±0.02C	0.75±0.01B	0.80±0.03A
Semen colour	Creamy	Creamy	Creamy	Creamy
Mass activities	++	+	+++	++++
Sperm motility%	75.14 ±9.42B	45.20 ±6.93C	78.32 ±9.17B	88.70 ±15.24A
Sperm concentration ($\times10^6$/ml)	6.32 ± 0.12B	3.75±0.19C	6.91±0.53AB	7.74±0.67A
Total sperm cell / ejaculate($\times10^6$/ml)	4.74±0.36B	1.98 ± 0.14C	5.35 ± 0.13B	6.79 ± 0.21A
Live-dead sperm ratio	70:30 ± 5.98B	35:65 ± 6.17C	82:18 ± 3.04A	87:13 ± 2.27A
Sperm abnormalities	17.56 ±2.5B	24.67 ± 1.57A	12.67 ± 1.57AB	9.67 ± 1.57C

N=number of animals, A,B,C= differences between groups, P≤0.05 vs. control

Table (6): Effect of oil and genestin extract of soybean seeds on testosterone level in serum and semen male rabbits (Mean ± SD, N=8)

Parameters / Groups	Testosterone in serum ng/ml	Testesterone in semen ng/ml
Control (Normal Saline) 0.9% NaCl	1.47±0.036	1.69±0.015
L-Thyroxin sodium (50µg/kg)	0.47±0.018	0.58±0.012
L-Thyroxin+Oil of Soybean Seeds (1ml/Kg)	1.23±0.011	1.52±0.014
L-Thyroxin +Genestin Extract of Soybean Seeds (0.5 g/kg)	1.44±0.016	1.61±0.013

N=number of animals, A,B,C= differences between groups, P≤0.05 vs. control

Effect of oil and genestin extract of soybean seeds on biochemical analysis in plasma semen male rabbits:

Results in table (7) revealed that the effect of oil and genestin extract of soybean seeds on biochemical analysis in plasma semen hyperthyroidism male rabbits on total protein, GPx, SOD, ALP,C ACP and zinc concentrations. The results revealed significant($P<0.05$) decrease in total protein and zinc levels in hyperthyroidism semen rabbits while the results revealed significant ($P<0.05$) increase in GPx, DOS, ACP and ALP concentrations in hyperthyroidism semen rabbits compared with control and other groups.

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Table (7): Effect of oil and genestin extract of soybean seed on biochemical analysis in plasma semen male rabbits (Mean ± SD , N=8)

Parameters / Groups	Total protein mg/dl	GPx (mmol/L)	SOD U/dL	ALP U/L	ACP U/L	Zinc mg/dl
Control (Normal Saline) 0.9% NaCl	62.19 ± 7.04A	19.57 ± 0.43A	85.1 ± 9.14A	54.7 ± 8.23B	49.3 ± 7.11B	1.98 ± 0.015A
L-Thyroxin sodium (50µg/kg)	40.34 ± 3.01B	5.25 ± 0.02C	51.27± 4.06B	78.7 ± 12.3A	76.1 ± 10.01A	0.97 ± 0.016B
L-Thyroxin + Oil of Soybean Seeds (1ml/Kg)	58.41 ± 9.16A	14.23 ± 0.49B	79.23± 11.14A	57.1 ± 5.7B	51.2 ± 4.32B	1.91 ± 0.012A
L-Thyroxin + Genestin Extract of Soybean Seeds(0.5 g/kg)	57.25 ± 11.09A	15.12± 0.05B	83.16± 15.04A	52.1 ± 3.89B	48.2 ± 9.26B	2.10 ± 0.001A

N=number of animals, A,B,C= differences between groups, P≤0.05 vs. control

Sperm examination:

Sperms of rabbits (control). Showing almost of sperms normal, live and decrease number of mature sperm and present large number of sperms dead and large number of different types of abnormalities sperm coiled tail, double–Tail, Only Head when stained with eosin and negrosin but sperms of rabbits treated with L-Thyroxin sodium. Showing all sperm abnormal, increase number of dead sperm and immature sperm and decreased in number of live sperm when stained with aniline-blue stain and showing high number of different types of abnormalities sperms 1-Coiled tail 2-Only Head in sperms of rabbis treated with L-Thyroxin sodium while sperms of rabbits treated with oil and genestin extract of soybean seeds. Showing all sperm normal, increase number of live sperm and mature sperm and decreased in number of dead sperm when stained with aniline-blue stain and showing low number of different types of abnormalities sperms 1-Coiled tail 2-Only Head in sperms of rabbis treated with oil and genestin extract of soybean seeds (figures 1-8).

Histological changes:

Thyroid gland: Thyroid gland of control male rabbit. Showing normal architecture, thyroid follicles, filled with colloid lined by cuboidal thyrocytes parafollicular cells while thyroid gland of rabbit treated with L-T_4. Showing hyperatrophied follicular cells and depletion of parafollicular cells, almost microfollicles hyperplasia, some follicle present a variety in size of thyroid follicles but thyroid gland of male rabbit treated with L-T4+Oil of soybean. Showing colloid-rich uniform thyroid follicles are lined by a layer of cuboidal epithelial cells (thyrocyte) and parafollicular cells can be distinguished, also thyroid gland of male rabbit treated with L-T_4+Genestin extract of soybean. Showing colloid-rich uniform thyroid follicles are lined by a layer of cuboidal epithelial cells (thyrocyte) and parafollicular cells can be distinguished (figures 9-12).

Liver: Section of Liver of control male rabbit. Showing normal hepatocyte, normal portal vein, sinusoid while liver of rabbit treated with L-T_4. Showing irregular arrangement of hepatocyte, enlarged spaces of sinusoid ,occasional foci of inflammatory cells, minimal diffuse vacuolation of hepatocytes but Section of Liver of male rabbit treated with L-T_4 + Oil of soybean seeds. Showing normal hepatocyte normal central hepatic vein, sinusoid also Section of Liver of male rabbit treated with L-T_4 + Genestin extract of soybean seeds. Showing normal hepatocyte normal central hepatic vein, siunsiod (figures 13-16).

Testes: Section of testis of rabbits (control). Showing mild vacuolation of spermatognia while Section of testis of rabbits treated with L-T_4. Showing vacuolation and widening of inter

seminiferous tubules, arrested of spermatogenesis, decrease of interstitial leydig cells but Section of testis of rabbits treated with L-T_4+ Oil of soybean seeds. Showing normal seminiferous tubules and spermatogenesis, interstitial leydig cells also Section of testis of rabbits treated with L-T_4+ Genestin extract of soybean seeds showing: normal seminiferous tubules and spermatogenesis, Interstitial leydig cells (figures 17-20).

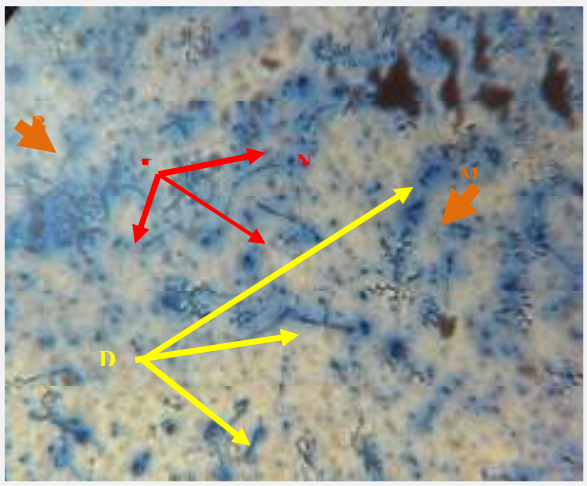

Figure (1): Sperms of rabbits (control). Showing almost of sperms normal (N), live (L) and mature (M) and present some of sperms dead (D)and abnormalities (B). Stained with aniline-blue 400X.

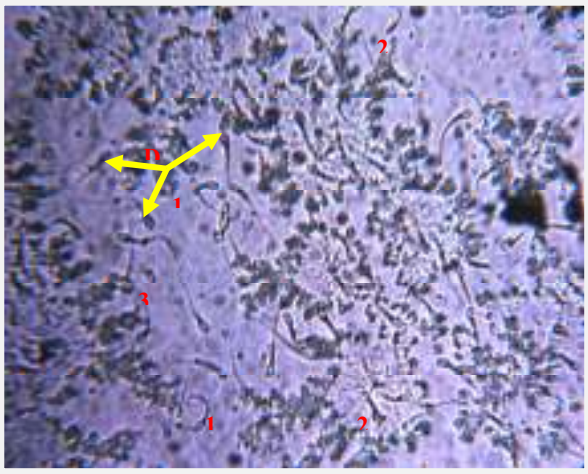

Figure (2): Sperms of male rabbis treated with L-T4. Showing large number dead sperms(D) and different types of abnormalities sperm1-Coiled tail 2-Only–Tail 3-Only Head. Stained with aniline-blue ,400X.

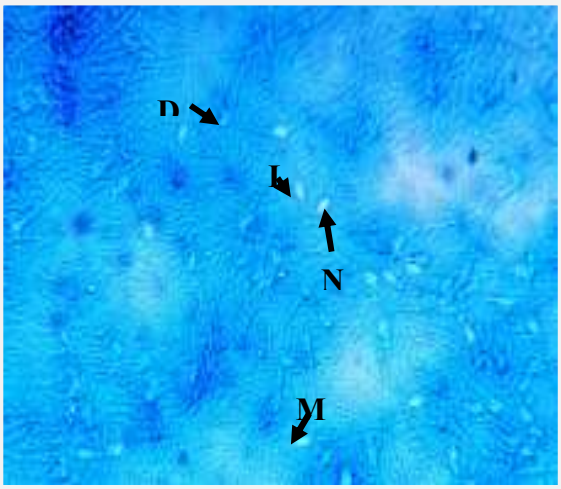

Figure (3): Sperms of male rabbits traeted with L-T4+Oil of Soybean. Showing almost of sperms normal (N), live (L) and mature (M) and present some of sperms dead (D). Stained with aniline-blue 400X.

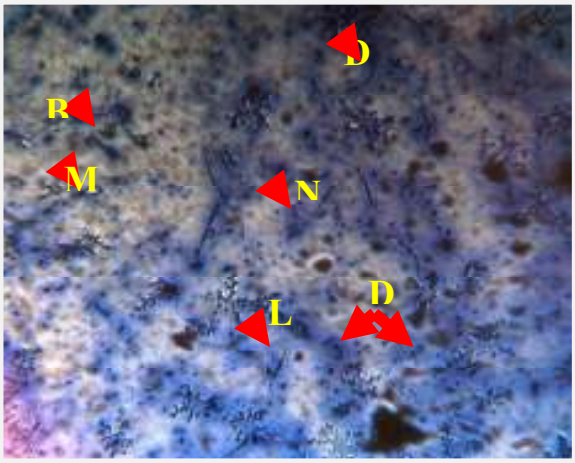

Figure (4): Sperms of male rabbits traeted with L-T4+Genestin extract of Soybean. Showing almost of sperms normal (N), live (L), mature (M) and present some of sperms dead (D) and abnormalies(B). Stained with aniline-blue 400X.

Figure (5): Sperms of rabbits (control). Showing almost of sperms normal, live and mature and present some of sperms dead and abnormalities. Stained with aniline-blue, 1000X.

Figure (6): Sperms of male rabbis treated with L-T4. Showing large number dead sperms and different types of abnormalities sperm, Coiled tail, Only–Tail, Only Head. Stained with aniline-blue, 1000X.

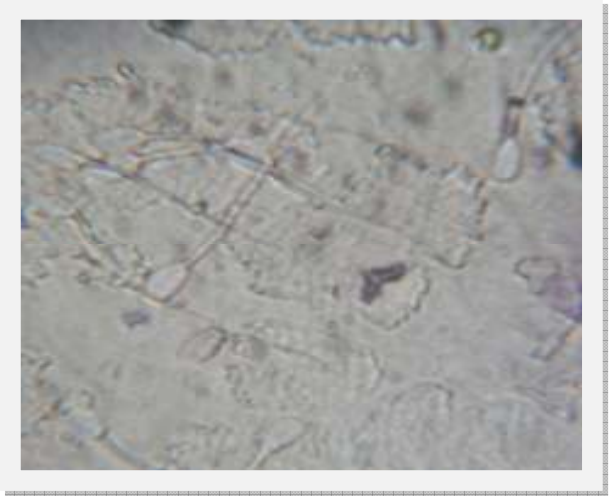

Figure (7): Sperms of male rabbits traeted with L-T4+Oil of Soybean. Showing almost of sperms normal, live, mature. Stained with aniline-blue, 1000X.

Figure (8): Sperms of male rabbits traeted with L-T4+ Genestin extract of Soybean. Showing almost of sperms normal, live and mature and present some of sperms dead. Stained with aniline-blue,1000X.

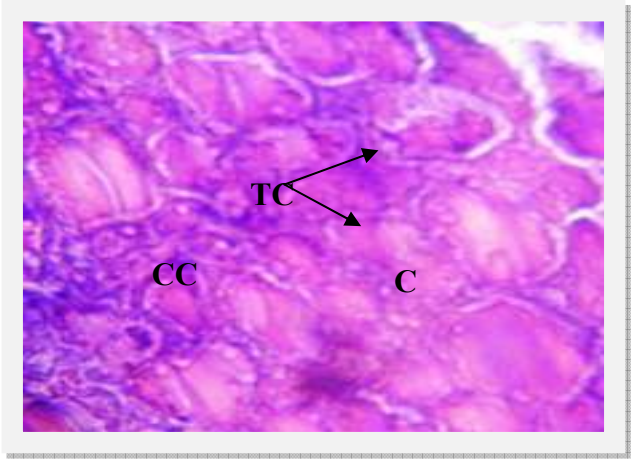

Figure (9): Thyroid gland of control male rabbits. Showing normal architecture, thyroid follicles (tf), filled with colloid (C) lined by cuboidal thyrocytes (TC) (arrow), parafollicular cells (CC) stain (H&E) 400X.

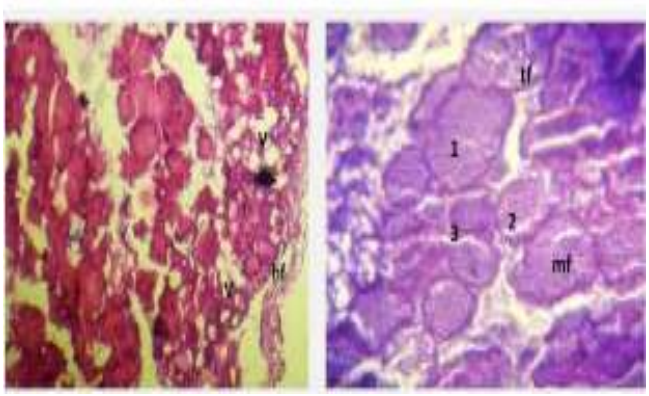

Figure (10): Thyroid gland of rabbit treated with L-T4. Showing hyperatrophied follicular cells (hf) and depletion of parafollicular cells, almost microfollicles hyperplasia (mf), some follicle present a varity in size of thyroid follicles (tf) (1, 2, 3), stain (H&E) 400X.

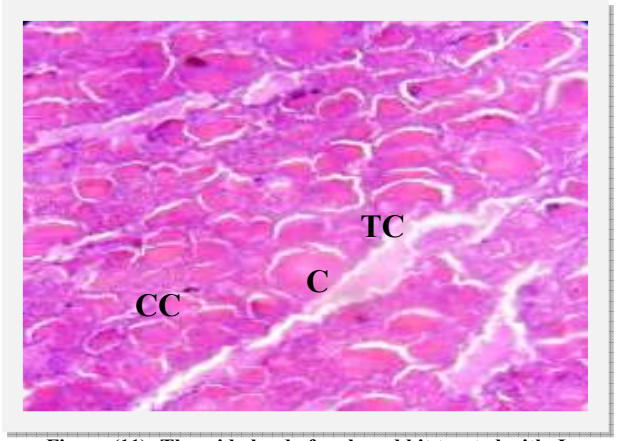

Figure (11): Thyroid gland of male rabbit treated with L-T4+Oil of soybean. Showing colloid-rich(C) uniform thyroid follicles (tf) are lined by a layer of cuboidal epithelial cells (thyrocyte) (TC) and parafollicular cells (CC) can be distinguished, stain(H&E) 400X.

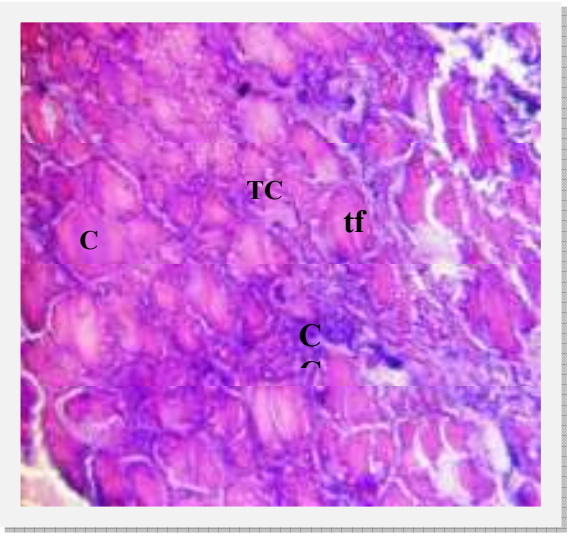

Figure (12): Thyroid gland of male rabbit treated with L-T4+Genestin extract of soybean. Showing colloid-rich(C) uniform thyroid follicles (tf) are lined by a layer of cuboidal epithelial cells (thyrocyte) (TC) and parafollicular cells (CC) can be distinguished, stain(H&E) 400X.

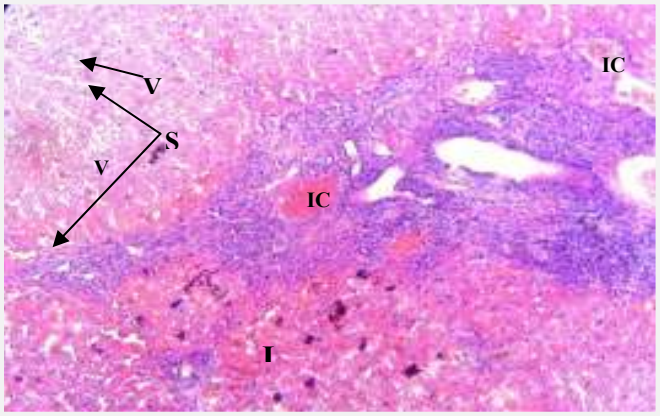

Figure (14): Liver of rabbit treated with L-T4. Showing irregular arrangement of hepatocyte, enlarged spaces of sinusoid (S), necrosis of hepatocyte, fibrosis and occasional foci of inflammatory cells (IC),minimal diffuse vacuolation of hepatocytes(V), stain (H&E) 400X.

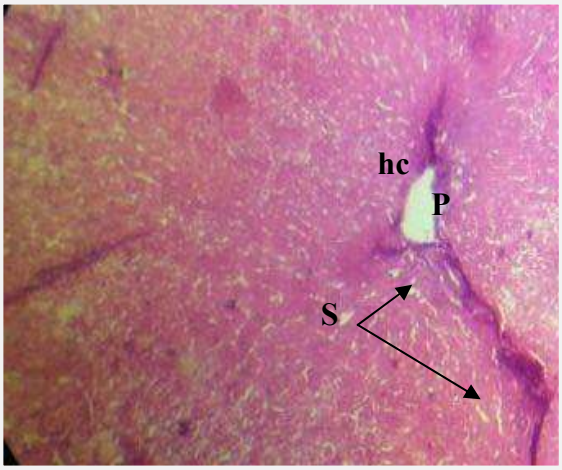

Figure (13): Section of Liver of control male rabbit. Showing normal hepatocyte (hc) normal portal vein (PV), siunsiod (S), stain (H&E) 400X.

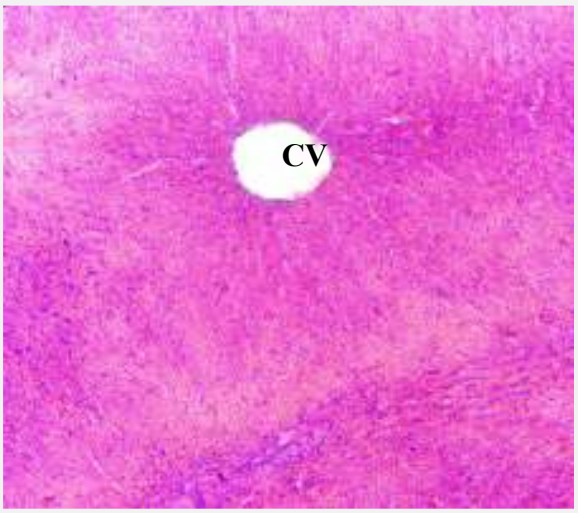

Figure (15): Section of Liver of male rabbit treated with L-T4 + Oil of soybean seeds. Showing normal hepatocyte (hc) normal central hepatic vein (CV), siunsiod (S), stain (H&E) 400X.

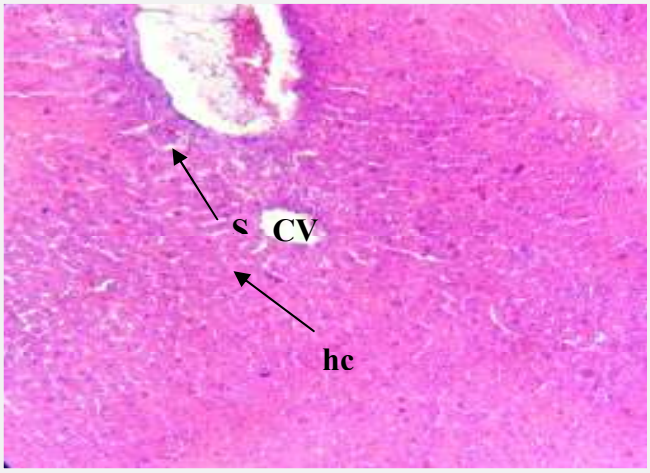

Figure (16): Section of Liver of male rabbit treated with L-T4 + Genestin extract of soybean seeds. Showing normal hepatocyte (hc) normal central hepatic vein (CV), sinusoid (S), stain (H&E) 400X.

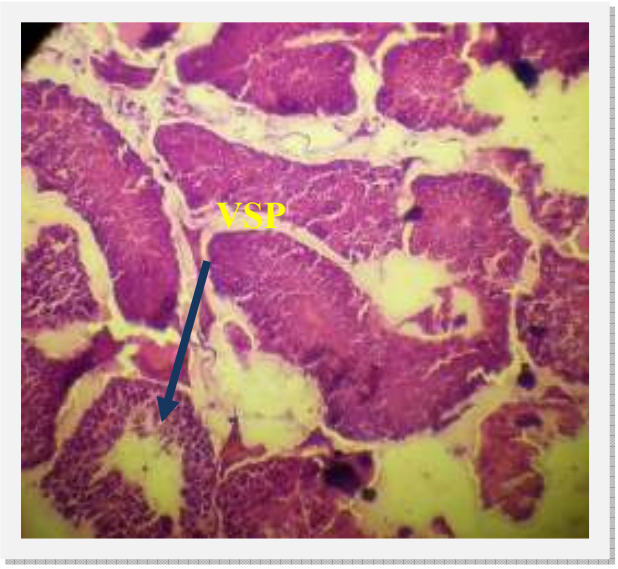

Figure (17): Section of testis of rabbits (control). Showing mild vacuolation of spermatgnia(VSP), (H&E,stain)100X.

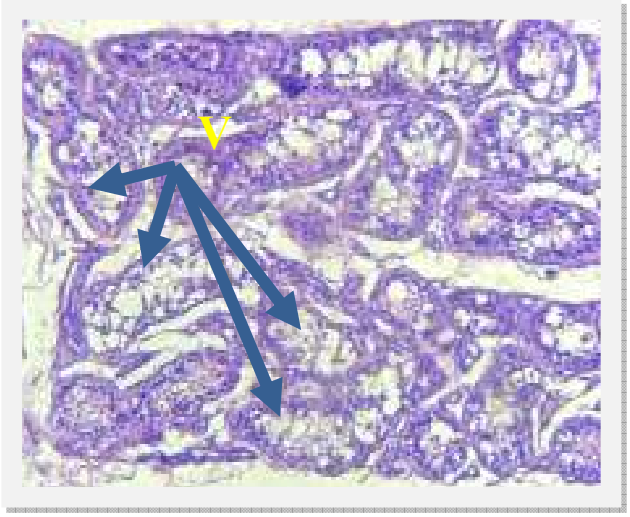

Figure (18): Section of testis of rabbits treated with L-T4. Showing vacuolation and widening of inter seminiferous tubules, arrested of spermatogenesis(Sp), decrease of interstitial leydig cells (Lc). (H&E,stain)100X.

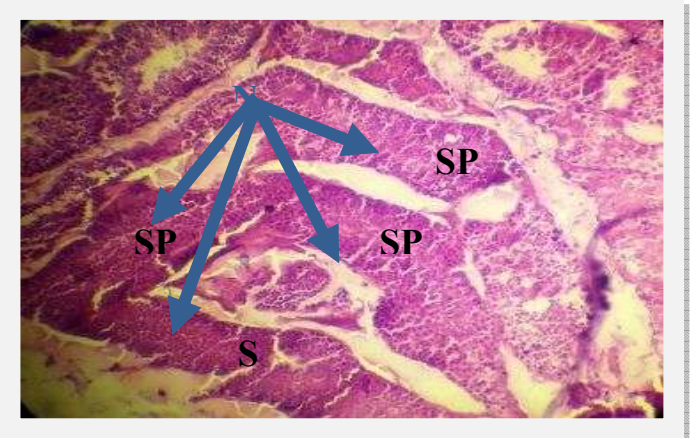

Figure (19): Section of testis of rabbits treated with L-T4+ Oil of soybean seeds. Showing normal seminiferous tubules (N) and spermatogenesis(SP), interstitial leydig cells (Lc). (H&E stain, 100X).

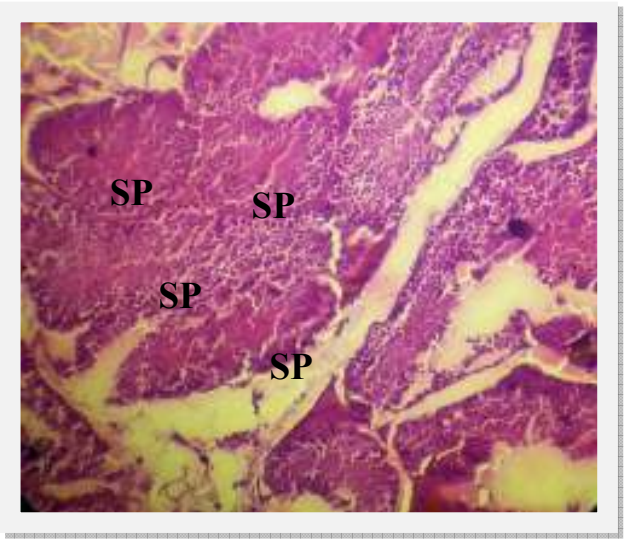

Figure (20): Section of testis of rabbits treated with L-T4+ Genestin extract of soybean seeds. Showing) normal seminiferous tubules (N) and spermatogenesis(SP), Interstitial leydig cells (Lc). (H&E stain)100X.

DISCUSSION

In the present study, increased serum T_3 and T_4 levels and decreased in TSH levels were observed in the hyperthyroid animals induced by thyroxine. In this respect, the results of our study appear to be consistent with the findings of others [14-17]. Also, the TSH level was significantly lower in the hyperthyroid group compared to the control group and the histological changes of thyroid glands indicate this result. The mechanisms behind the oil and genestin extract of soybean seeds-induced reduction in thyroid hormone are not clear. Possibilities include oil and genestin extract of soybean seeds induced modulation in deiodination system, which affects deiodinase activity through its antioxidant properties. Based on the results obtained, it can be concluded that the hyperthyroid group, which received oil and genestin extract of soybean seeds, shows a significantly different decrease of plasma T_3 and T_4 levels and significantly different increase of TSH levels. Pharmacological antioxidants may have an effect on the peripheral conversion of thyroid hormones by way of deiodination and/or mechanism of cell membrane defence, the integrity of which may have an effect on the activity of deiodinases (21).

The present study revealed a decrease in body weight and body weight gain in hyperthyroidism male rabbits compared control. This result agrees with (22) the likelihood of weight loss occurring is related to the severity of the overactive thyroid. Thus, if the thyroid is extremely overactive, the individual's BMR increases which leads to increased caloric requirements to maintain that weight. If the person does not increase the calories consumed to match the excess calories burned, then weight loss will ensue. As indicated earlier, the

factors that control our appetite, metabolism, and activity are very complex and thyroid hormone is only one factor in this complex system. Nevertheless, on average the more severe the hyperthyroidism, the greater the weight loss observed. Weight loss is also observed in other conditions where thyroid hormones are elevated, such as in the toxic phase of thyroiditis and if one is on too high a dose of thyroid hormone pills. A study showed that the rats treated with LT4-were lost body weight and attributed that to catabolic effect of L-T4 (23). Another study that was conducted by (24) also noticed that the L-T4 induce reductions in bone mineral mass and reduce of growth because increase metabolic catabolism while significant effect administration of oil and genestin extract of soybean seeds increase in body weight and body weight gain due to increases metabolic processes in body (25).

The present study revealed that the hyperthyroidism affect on biochemical parameters such as increase in levels of glucose, cholesterol, triglyceride, low density lipoprotein (LDL-), ALP, ACP, and decrease (HDL-) high density lipoprotein, GPx and SOD (Table2-3). The extracts exhibited significant reduction of serum cholesterol level in hyperthyroidism male rabbits. The abnormal high concentration of serum lipids in the hyperthyroid rabbits is mainly due to increase in the mobilization of free fatty acids from the peripheral fat depots (26). Maintenance of serum cholesterol profile indicates that genestin and oil of soybean may exert their role in maintenance (27). Genestin and oil of soybean treatment decreased the elevated glucose concentration significantly ($P<0.05$) in treated hyperthyroid rabbits; however, their glucose concentrations were still significantly higher ($P<0.05$) than those of the control group. A reduction in the serum glucose levels of the groups treated with oil and genestin extract of soybean were observed in this work because of genestin and oil of soybean lead to stimulate insulin secretion when insulin is increased it leads to decrease glucose concentration. This result is in agreement with (28), who found that the high isoflavone soy diet increases insulin secretion. This result is attributed often to estrogen receptor agonism by isoflavones thus used to regulate glucose absorption and elevation in diabetes (28). Isoflavone compounds found in soybean, especially genistein may help to stay lean by causing us to produce fewer and smaller fat cells (28).

Previous studies have suggested that hyperthyroidism increased free radical production and lipid peroxidation levels (18-20). Hyperthyroidism accelerates ROS generation and produces changes in the antioxidant systems of various tissues (2-4). The cellular GSH plays an important role as biological antioxidant defence systems, which act as protective mechanisms against oxidative damage, therefore, the decreased level of GSH may be due to overproduction of free radicals and increased lipid peroxidation in hyperthyroidism (16). In our study, serum GSH levels were decreased in hyperthyroid animals as compared to control animals, possibly secondary to increased ROS generation.

Oxidative stress has been identified as one of the very important factors that affect fertility status. Sperm, like any other aerobic cells, are constantly facing the “oxygen-paradox”. Oxygen is essential to sustain life as physiological levels of ROS are necessary to maintain normal cell function and all that is true for sperm as well. However, excessive production of ROS (oxidative stress) is well known to be detrimental to sperm by adversely affecting the quality of sperm DNA. The main function of thyroid hormone within physiological ranges is to regulate and enhance metabolic reaction and oxygen consumption of different cells of the body. ROS which are the by-products of tissue metabolism are normally treated by physiological antioxidants. The results in this investigation clearly indicate that thyroid hormones play a fundamental role in reproductive function. The results obtained here showed that both sperm viability, sperm count decrease and testosterone hormone, while the abnormalities and dead sperm increase and histological changes of testis indicate this result. It can envision a number of ways that could lead to decrease sperm viability. It was found that testosterone hormone decrease alters the androgen dependent maturation of spermatozoa and causes various changes in the lipid composition in epididymides (29, 30). The other is a reduction in FSH secretion which decreases acrosine activity (31, 32).

However, researchers (33) proposed that sperm morphology as well as motility may be affected by thyroid hormones. Those authors have shown that hyperthyroidism was capable to cause histological and endocrinological epididymides dysfunction by affecting epididymal epithelium which participated in disposal of cytoplasmic droplets detached from spermatozoa, leading to epididymal sperms with high percentage of these droplets. Several possibilities may be considered with respect to where and how thyroid hormones act to promote sperm count decrease, previous work showed that depressed serum FSH level may be the cause of delayed Sertoli cell maturation in hyperthyroid rats (34). As a result, low protein content, low levels of enzyme activity and decrease androgen binding protein (ABP) production are consistent with impaired gametogenic development (35). It was also demonstrated that FSH is a mitogenic factor during the Sertoli cell proliferative phase (36), therefore, it is a reasonable to speculate that FSH reduction may cause a decline in sertoli cell number. The contribution of T to spermatogenesis has been investigated by several authors (37,38). It is generally accepted that this hormone might be affected either by expression ARs in sertoli cells or by enhanced binding sperms to Sertoli cells which prevent their premature detachment from the epithelium (38). Furthermore, it should be taken into

account that this hormone might be expected to inhibit spermatogenesis by regulating the meiotic stage of this process (39,40). Because of, the fact that Sertoli cells provide support to the germ cells during spermatogenesis (41), hyperthyroidism may reduce sperm count due to its effect on these cells, so that one report showed that thyroid hormones stimulate sertoli cells to uptake glucose and to secrete substances such as lactate which is essential for germ cells survival and growth factors such as Insulin-like growth factor-1 (IGF-1) stimulating DNA synthesis in mitotic germ cells (42).

The role of thyroid in regulating oxidative stress in male reproductive organs is recently being explored. Previous reports showed that both hyper- and hypothyroidism are associated with increased oxidative stress in semen (43). In testis there are two highly energy consuming physiological processes; spermatogenesis and steroidogenesis. In addition, testis is rich in polyunsaturated fatty acids (PUFA) which are liable to peroxidation by pro-oxidant agents. Testis on the other hand has enzymatic and non-enzymatic antioxidant defense systems with limited potentials (44). In cases of thyrotoxicosis, part of sustained injury to various body tissues is attributed to oxidative damage (45). Choudhury *et al.* conducted a study on rat testis after inducing hyperthyroidism and discovered that although there is positive regulatory effect on antioxidant enzyme catalase, there is negative effect on level of testicular glutathione peroxidase. In addition Choudhury *et al*. found decreased concentration of reduced glutathione GSH, 'the important antioxidant molecule in Sertoli and spermatogenic cells' (46). Malgorzata *et al.* found that excess T_3 and T_4 induce DNA damage in male sperm (47).

Also the hyperthyroidism lead to zinc deficiency and affected on reproductive system. The finding of low sperm count, decreased motility, and increased percentage of abnormal forms agreed with Valle *et al* (17), who found that zinc deficiency causes atrophy of the seminiferous tubules, failure of spermatogenesis and decreased testosterone secretion in rats. Zinc deficiency impairs the responsiveness of Leydig cell to gonadotropins and may cause primary hypogonadism in humans as well as in experimental animals (18).

The most important male hormone produced by the testis is testosterone (a steroid that stimulates the development of sex characteristics). The essential mineral zinc, is important in prostate gland function and growth of the reproductive organs. Moderate to severe zinc deficiency produces regression of the testes, mild deficiency leads to low sperm count (12). Male infertility is influenced by zinc in several different ways, low zinc levels have a negative effect on serum testosterone concentration and semen volume (13). Our findings of low serum testosterone in zinc deficient subjects was in agreement with (14), who found that zinc deficient animals develop impairment of testicular growth, low serum testosterone and elevated FSH and LH. A clinical study demonstrated that adult males experimentally deprived of zinc showed that the Leydig cell synthesis of testosterone was disturbed (15).

Zinc is a natural aromatize enzyme inhibitor. Aromatize enzymes cause the body to block the pituitary gland from releasing lutein and follicle stimulation of hormones which stimulate the production of testosterone, aromatize enzyme converts testosterone into estrogen and result in lower amounts of available testosterone (1).

Zinc is not only vital in the production of testosterone, it also works to maintain healthy semen volume and has been implicated in testicular development and sperm.

Maturation (1). Zinc in seminal plasma stabilizes the cell membrane and nuclear chromatin of spermatozoa (2) and protects the testis against the degenerative changes (3). It may play a regulatory role in the process of capacitating and across some reaction (4). It contributes to the stable attachment of sperm head to tail and its removal induces head-tail detachment (5).

The present study referred that the effect of oil and genestin extract of soybean seeds on hormones shows the decreased levels in T3, T4, and increase TSH and testosterone in serum and plasma semen. This changes in levels of hormones could be attributed to oil and genestin extract of soybean seeds genestinis polyphenols that showed antioxidant and free radical scavengers (5,6). And may be genestin play a regulatory role in sex hormones. Because genestin exhibit a wide range of biological effects, including antioxidant and enzyme-modulating action and anti-allergic, antiatherosclertic, antithrombotic, antiviral, anticarcinogenic, antispasmodic, and diuretic effect. Previous reports showed that both hyper- and hypothyrodisim are associated with increased oxidative stress (43). In cases of thyrotoxicosis, part of sustained injury to various body tissues is attributed to oxidative damage therefore can be inhibited effect of excess of oxidative stress by treatment of genestin extract.

Recently, there is great evidence that genestin prevents oxidative injury by modulating the expression of antioxidant enzyme systems (30)

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Effects of ivermectin on lipid profiles, antioxidant enzymes and proteins with the beneficial effects of vitamin C in rabbits

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ABSTRACT

Ivermectin is a semisynthetic, anthelmintic drug which is derived from the avermectins, a class of highly active broad-spectrum, anti-parasitic agents isolated from the fermentation products of *Streptomyces avermitilis*in soil. This study was trying to investigate the effect of repeated doses of ivermectin alone or with the combination of vitamin C on lipid profiles, antioxidant enzymes, and total protein, albumin and globulin of female rabbits. For this purpose,48 mature female rabbits were divided into 8 groups in equal number (6/group). The first group was administered 0.9% Nacl, which act as control, the second, third, and fourth groups were given(0.5mg/Kg,1mg/Kg,2mg/Kg B.W Ivermectin) respectively, while the fifth group was administered 50mg/Kg B.W Vitamin C only, whereas the sixth, seventh, and eighth groups were administered 50mg/Kg vitamin C in addition to ivermectin (0.5mg, 1mg, &2mg/Kg B.W)respectively. The ivermectin was given subcutaneous route weekly, while vitamin C was administered by oral route daily for 8 weeks.

The results showed significant decrease $P<0.05$ in triglyceride level in 6^{th} and 8^{th} groups, as well as, the HDL revealed significant increase in 2^{nd} and 6^{th} groups, while the total cholesterol, and LDL did not altered significantly. The catalase activity demonstrated significant increased ($P<0.05$) in 2^{nd} group which administered therapeutic dose of ivermectin, while the superoxide dismutase clarified significant decrease ($P< 0.05$) in 5^{th} , 6^{th} and 7^{th} groups, and glutathione peroxidase showed significant decrease ($P<0.05$) in 5^{th} group which administered vitamin C only. There were no significant differences in total protein, albumin, and globulin in all treated groups as compared with control group. It can be concluded that Ivermectin has no effect on lipid profiles and proteins, but it causes oxidative stress. Vitamin C is considered as ameliorative agent and can have a protective effect in rabbits.

***Keywords*:** Ivermectin, Vitamin C, Lipid profiles, Antioxidant enzymes, protein, Rabbit.

الملخص باللغة العربية

الإيفرمكتين هو دواء شبه صناعي مشتق من صنف الإفرمكتين، يستعمل كمضاد ديدان ويعمل بفعالية عالية كمضاد طفيلي واسع الطيف تم عزله من نواتج التخمر للبكتيريا السبحية في التربة *Streptomycesavermilitis*. حاولت هذه الدراسة التعرف إلى تأثير الجرع المتكررة من الإيفرمكتين منفردا أو مع إضافة فيتامين سي على محتويات الدهون، والإنزيمات المضادة للأكسدة ،والبروتين الكلي، والألبومين والجلوبيولين في إناث الأرانب. ولهذا الغرض تم تقسيم 48 أنثى أرنب بالغة في ثماني مجموعات وبعدد متساو في كل مجموعة (6)، أعطيت المجموعة الأولى 0.9% من محلول الملح الفسيولوجي واعتبرت مجموعة سيطرة، في حين أعطيت المجموعات الثانية والثالثة والرابعة الإيفرمكتين بجرعات (0.5ملغم/كغم، 1ملغم/كغم و 2ملغم / كغم من وزن الجسم) على التوالي، بينما أعطيت المجموعة الخامسة جرعة 50ملغم /كغم من وزن الجسم من فيتامين سي فقط ، في حين أعطيت المجموعات السادسة والسابعة والثامنة جرعات 50ملغم/ كغم من وزن الجسم من فيتامين سي بالإضافة إلى الإيفرمكتين بجرعات (0.5 ملغم/كغم، 1 ملغم/كغم و 2 ملغم/ كغم من وزن الجسم) على التوالي، وقد أعطي الإيفرمكتين عن طريق الحقن تحت الجلد أسبوعيا، بينما أعطي فيتامين سي عن طريق الفم يوميا لمدة ثمانية أسابيع.

وقد أظهرت النتائج انخفاضا معنويا ($P<0.05$) في مستوى الدهون الثلاثية في المجموعتين السادسة والثامنة، كما أظهرت الدهون عالية الكثافة زيادة معنوية ($P<0.05$) في المجموعتين الثانية والسادسة، بينما لم يلاحظ حدوث أي تغيرات معنوية في الكوليسترول الكلي والدهون منخفضة الكثافه، وأظهرت النتائج وجود زيادة معنوية ($P<0.05$) في فعالية إنزيم الكاتليز في المجموعة الثانية والتي حقنت بالجرعة العلاجية من الإيفرمكتين، بينما أظهر إنزيم السوبر أوكسايد دسميوتيز انخفاضا معنويا ($P<0.05$) في المجموعات الخامسة والسادسة والسابعة ، وأظهر إنزيم الكلوتاثيون بيروكسيديز انخفاضا معنويا ($P<0.05$) في المجموعة الخامسة والتي أعطيت فيتامين سي فقط . ولم يلاحظ أي فروقات معنوية في البروتين الكلي، والألبومين والجلوبيولين في كل المجموعات المعاملة، مقارنة بمجموعة السيطرة، وبهذا، يمكن الاستنتاج أن الجرع المتكررة من الإيفرمكتين لا تؤثر على مستويات الدهون والبروتينات ، لكنها تسبب إجهادا تأكسديا. ويعتبر فيتامين سي عاملا محسنا ذا تأثير وقائي على إناث الأرانب.

INTRODUCTION

Ivermectin is macrocyclic lactone, acts as broad spectrum antiparasitic drug against many internal and external parasites. Theivermectin appearance is off-white, nonhygrscopic crystalline powder with melting point about 155C, also it is very poorly soluble in water but is freely soluble in propylene glycol, polyethylene glycol and vegetable oils (1). It is considered to have high safety margin in ruminants, horses, and swine. In high doses, it may exhibit central nervous system depression as evidenced by listlessness, mydriasis, ataxia, recumbency and coma (2).Antioxidant are substances that either directly or indirectly protect cells against adverse effects of xenobiotic, drugs, carcinogens and toxic radical reaction (3).The antioxidant system are classified into two major group's enzymatic antioxidant which include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). While the non-enzymatic antioxidant contain Ascorbic acid (vitamin C), Vitamin E (α- tocopherol), Vitamin A, Glutathione (GSH), Uric acid; Bilirubin and Flavonoids (4).

Catalase is an enzyme present in the cells of plants, animals and aerobic bacteria. It regarded one of the very important enzyme in the protecting the cell from the oxidative damage which caused by reactive oxygen species ROS (5) .Glutathione peroxidase is an enzymes catalysed the oxidation of glutathione at the expense of hydro peroxide, which may be hydrogen peroxide or another species such us a lipid hydro peroxide (6). Superoxide dismutase is one of the most important intracellular enzymatic antioxidant which catalysed the destruction (dismutation) of superoxide free radical ion responsible for lipid peroxidation and peroxidative haemolysis of erythrocytes, on the other hand, the function of superoxide dismutase were resulted in the protection of the biological integrity of the cells and tissues against the harmful effects of superoxide free radicals (7).

It is well known that ascorbic acid is water soluble vitamin and actsas reducing agent. Many animals and most of plants synthesized it from glucose, also it has been implicated as a free radical scavenger (8). Another of evidences revealed that vitamin C is very important in a regulation of catabolism of cholesterol to bile acid in guinea pig and play essential role in lipid regulation (9). On the other words, vitamin C participates in cholesterol metabolism and when marginal vitamin C deficiency was occurred this lead to increase in plasma cholesterol concentration (10). Ii is very clear that the dietary supplements of vitamin C and E elevate the activities of antioxidant enzyme which include catalase, superoxide dismutase and glutathione peroxidase which in turn lead to reduce the oxidative stress and intravascular damage of internal organ (11). In fact the oxidative stress is consider as a pathophysiological process in which intracellular balance between endogenous as well as exogenous pro-oxidants and antioxidant is shifted toward pro-oxidant leaving cell unprotect from free radical attach which in turn may cause hepatotoxicity, neurotoxicity, and nephrotoxicity in human and animals (12). Some researchers clarified the S/C injection of Ivermectin in rabbits at dose of 1mg/Kg B.W caused significant decrease in total antioxidant capacity at 120 hours, whereas, the plasma nitric oxide level showed significant increase at 24 hours of treatment(13). This study aimed to investigate the effects of repeated administration of ivermectin alone and with the combination of vitamin C on lipids profiles, antioxidant enzymes and total protein, albumin and globulin in female rabbits.

MATERIALS AND METHODS

Ivermectin 10% was purchased from local market (VET Product Office, KIPRO Company, Holland) and Vitamin C (Al-Shahba Labo, Syria).

Animal husbandry:

Forty eight female rabbits (*Lepus cuniculus*), (1200-2000gm) body weight and (8-12 months) of age were brought from local market in Basra Province. The rabbits were housed (6 rabbits / cage) in a wire silk cages measuring (100 X 50 X 50 cm) under controlled animal house condition at temperature (25 ± 3 C^o) and relative humidity (50 ± 5 %) in the animal house of Veterinary Medicine College in Basra University. The rabbits were kept under observation for one month. The animals were offered a rabbit's diet, green leaves, alfalfa, and water.

Experimental design:

Forty eight female rabbits were divided into eight groups (6 rabbits in each group). Each group was treated for 8 week as follows:

Group 1 : Injected (0.9 % NaCl) which acts as a control.

Group 2: Injected (0.5 mg/kg B.W Ivermectin).

Group 3: Injected (1 mg/kg B.W Ivermectin).

Group4: Injected (2 mg/kg B.W Ivermectin).

Group 5: administered (50mg/ Kg B.W Vitamin C).

Group 6: Injected (0.5 mg/Kg B.W Ivermectin +50mg/kg B.W Vit.C).

Group 7: Injected (1mg/kg B.W Ivermectin + 50mg/Kg B.W Vit. C).

Group 8: Injected (2mg/ Kg B.W Ivermectin +50mg/kg B.W Vit. C). The Ivermectin were given subcutaneously and weekly, while vitamin C were given daily and orally.

At the end of experiment (8 Weeks), the blood samples were taken directly from the heart by using disposable syringe and put in screw tube without anticoagulant then centrifuged at 4000 rpm for 10 minutes to get serum for biochemical assay (Lipid profiles, CAT, SOD, GPx, and total protein, albumin and globulin).

Biochemical analysis:

All the biochemical kits were measured spectrophotometrically.Total cholesterol was measured according to CHOD-PAP method, triglyceride was done according to Fossati and prencipe method. Catalase was measured according to modified method by Aebi, 1984, glutathione peroxidase was done according to method of Flohe and Gunzler,1984, superoxide dismutase was based on its ability to inhibit the epinephrine oxidation to adrenochrome. Total protein was demonstrated according to Biuret method, and albumin was measured according to BCG method.

Statistical analysis:

The results were analysed by one- way ANOVA test. Least significant different test (LSD) was calculated to test the difference between means when there is significant differences. All statistical calculations were carried out by the aid of the statistical SPSS V. 22 (SPSS Inc.).

RESULTS

The results of the effect of Ivermectin alone or with the combination of vitamin C on lipid profiles (total cholesterol, triglyceride, high density lipoprotein HDL, and low density lipoprotein LDL) on female rabbits after 8 weeks of treatment are clarified on table (1).

The total cholesterol and LDL level did not show significant differences among all treated groups as compared with control group. The triglyceride level revealed significant decrease ($p<0.05$) in 6^{th} and 8^{th} groups as compared with control group, while HDL recorded significant increase ($p< 0.05$) in 2^{nd} ,and 6^{th} groups in comparison with the control group.

In table(2), the Catalase level showed significant increase ($p< 0.05$) in 2^{nd} group which administered 0.5mg/Kg B.W Ivermectin, while the superoxide dismutase (SOD) level revealed significant decrease ($p< 0.05$) in 5^{th} , 6^{th} ,and 7^{th} group in comparison with control group, As well as, There was significant decrease ($p< 0.05$) in glutathione peroxidase level in 5^{th} group which administered vitamin C only as compared with control group.

Depending on the results clarified in table (3) there were no significant differences on total protein, albumin and globulin level present in all treated groups as compared with control group.

Table (1): Effect of ivermectin alone or with the combination of vitamin C on Lipids profiles of female rabbits after 8 weeks of treatment. (Mean ± SE, N=6)

Groups	Total cholesterol mg/dl	Triglyceride mg/dl	LDL mg/dl	HDL mg/dl
1st Group Control 0.9%Nacl	91.703± 8.222	189.280±13.815 a	147.966±30.071	119.055±13.468 b
2nd Group (0.5mg/Kg IVM)	115.110± 19.865	173.581± 7.513 ac	189.782±58.997	182.589±35.169 a
3rd Group (1mg/Kg IVM)	125.530± 12.459	165.214± 7.208 ac	138.315±31.385	112.191±7.340 b
4th Group (2mg/Kg IVM)	120.740± 28.225	168.879± 8.952 ac	70.766±18.422	128.965±15.095 bc
5th Group 50mg/Kg Vit.C	106.468± 8.054	163.761± 8.462 ac	191.308±23.380	162.255±13.408 ab
6th Group (0.5mg/Kg IVM) + (50mg/Kg Vit.C)	98.468± 4.152	144.605±20.734 bc	178.524±38.660	170.712±7.597 ac
7th Group (1mg/Kg IVM) + (50mg/Kg Vit.C)	77.431± 7.867	162.516± 6.810 ac	135.099±19.138	132.315±24.959 ab
8th Group (2mg/Kg IVM) + (50mg/Kg Vit.C)	80.839± 11.403	148.339±25.999 bc	160.832±42.915	121.211±6.395 bc

**IVM=Ivermectin, Vit.C= Vitamin C*

**Different letters denote significant differences (p< 0.05) between groups*

Table (2): Effect of ivermectin alone or with the combination of vitamin C on antioxidant enzymes Catalase (CAT), Super oxide dismutase (SOD), and Glutathione peroxidase (GPx) of female rabbits after 8 weeks of treatment. (Mean ± SE, N=6).

Groups	CAT µmol /min/ml	SOD	GPx µmol/L
1st Group Control 0.9%Nacl	1.916 ± 0.251 b	0.782± 0.019 ac	0.335± 0.022 a
2nd Group (0.5mg/Kg IVM)	3.206± 0.846 a	0.827± 0.016 a	0.370± 0.062 a
3rd Group (1mg/Kg IVM)	1.546±0.189 b	0.834± 0.010 a	0.319± 0.071 a
4th Group (2mg/Kg IVM)	1.882± 0.231 b	0.747±0.020 cd	0.296± 0.080 a
5th Group 50mg/Kg Vit.C	1.867± 0.297 b	0.685± 0.005 bf	0.070± 0.018 b
6th Group (0.5mg/Kg IVM) + (50mg/Kg Vit.C)	1.489± 0.220 b	0.633± 0.018 f	0.210± 0.065 ab
7th Group (1mg/Kg IVM) + (50mg/Kg Vit.C)	1.953 ± 0.272 b	0.709± 0.035 bd	0.318± 0.071 a
8th Group (2mg/Kg IVM) + (50mg/Kg Vit.C)	2.658 ± 0.657 ab	0.801± 0.020 ac	0.293± 0.083 a

**IVM=Ivermectin, Vit.C= Vitamin*
**Different letters denote significant differences (p< 0.05) between groups*

Table (3): Effect of ivermectin alone or with the combination of vitamin C on Total protein, albumin and globulin of female rabbits after 8 weeks of treatment. (Mean ± SE N=6).

Groups	Total protein g/dl	Albumin g/dl	Globulin g/dl
1st Group Control 0.9%Nacl	12.685 ± 1.183	8.937± 0.492	4.085± 0.519
2nd Group (0.5mg/Kg IVM)	13.656± 0.779	8.090± 0.616	6.416± 0.823
3rd Group (1mg/Kg IVM)	14.400±0.703	8.842± 0.414	3.897± 1.067
4th Group (2mg/Kg IVM)	12.685± 1.830	8.485±0.600	5.171± 0.812
5th Group 50mg/Kg Vit.C	13.318± 1.333	8.054± 1.366	6.346± 1.410
6th Group (0.5mg/Kg IVM) + (50mg/Kg Vit.C)	12.684± 1.832	8.668± 0.451	5.352± 1.286
7th Group (1mg/Kg IVM) + (50mg/Kg Vit.C)	13.318 ± 1.333	7.393± 0.886	5.925± 0.881
8th Group (2mg/Kg IVM) + (50mg/Kg Vit.C)	13.574 ± 0.617	7.881± 1.042	5.693± 1.472

**IVM=Ivermectin, Vit.C= Vitamin*

DISCUSSION

The results in present study revealed that Ivermectin did not change significantly the triglyceride level in the blood, but when combined with vitamin C it caused a decrease in triglyceride. The decrease in triglyceride level was due to the administration of vitamin C. Vanapalli*, et. al* (14) demonstrated the ivermectin bioavailability had been decreased by orange juice administration in healthy volunteers, as well as, the mechanism of decreased ivermectin bioavailability is unknown but the fruit juices and constituents are potent inhibitor of certain drug transports. Similarly many researcher team showed that vitamin C was effective in decreasing lipid profile up to some extent but not up to statistically significant level (15,16).This observationis in line with (17), who suggested the supplementation of 500mg/day of vitamin C for 4weeks to patient with hypercholesterolemiacaused significant decrease in serum triglyceride. Similar finding but with aanother compound (Halothane)had found the I/P injection of vitamin C to rats caused significant decrease in triglyceride level when compared with group which dosing with halothane only (18).

On the other hand, the increased in HDL in present study in2nd and 6thgroupswhich are in agreement with other investigators (19, 20). Those researchers concluded that vitamin C prevent oxidation of LDL-cholesterol, decrease total LDL-cholesterol and triglyceride level, as well as, increased HDL-cholesterol level. Moreover, another researcher claimed that the administration of Ivermectin for 15 day was caused significant reduction in hepatic phospholipid and cholesterol contents in rats (21). Actually some authors explained the hypocholesterolaemic effect of vitamin C by prevention LDL-cholesterol from oxidative damage and it aid in degradation of cholesterol, as well as, it has been observed that vitamin C is needed by the enzyme cholesterol 7 α hydroxylase in the first step of bile acid synthesis by directing cholesterol toward bile acid synthesis and it reduce its level in serum (22).

The results in present study was clarified significant elevation in catalase level in second group (0.5mg/Kg Ivermectin), and Superoxide dismutase level showed significant decrease in 5th, 6th and 7th groups (50mg/Kg Vit. C, 0.5mg/Kg,1mg/Kg IVM + Vit.C respectively), in addition the glutathione peroxidase level proved significant decrease in group, which administered vitamin C only (5th group).

In fact, the catalase is as an enzyme that decomposes hydrogen peroxide into oxygen and water. The increased in catalase activity in current study can be explained due to excess free radicals which may produced due to ivermectin administration (23,24).This event occur in normal dose, while when using in repeated doses, the free radicals may be exceed than normal production and may caused harmful effect on the body organs, hence the activities of catalase enzyme should be increased to eliminate these free radicals. One must maintain that free radicals is define as an atom or group of atoms possessing one or more unpaired electrons (25). So the antioxidant enzymes (CAT, GPx ,SOD, Vit. C, Vit, E, B-Caroten, Coenzymes Q)will provide electron to free radicals to neutralize them.However,this results were disagreement with (26) who showed the single dose of ¼ LD50 of Abamectin (similar to ivermectin) caused significant decrease in catalase and glutathione-S-transferase activity in male albino rats.

In present study the SOD activity only was decreased in groups which received vitamin C alone or with ivermectin combination (5th, 6th and 7th). Superoxide dismutase (SOD) is considered as the first line of defence against deleterious effects of oxygen radicals in the cell by the catalysing the dismutation of superoxide radicals to hydrogen peroxide H2O2 and molecular oxygen (5) and due to Vitamin C can replace glucose in many chemical reactions because of its structure similarity to glucose and this is very useful in prevention of non-enzymatic glycosylation of proteins and it mops up free radicals formed in the body (27), so the production of SOD diminish.

This results is in agreement with(28) who illustrated the coadministration of Doramectin and another antioxidant vitamin (AD_3E) in rabbits were caused decrease in superoxide dismutase (SOD) activity in day 7 of treatment.

Generally, the glutathione peroxidase isregardedan antioxidant enzyme that reduce hydrogen peroxide and lipid peroxidation (29).Glutathione peroxidase GPxactivity in current study shows a decrease in group which administered vitamin C only. This observation is possibly related to antioxidant activities of vitamin C (30, 31).previous worker has indicated that vitamin C is able to scavenger reactive oxygen species and reactive nitrogen species which are known to reduce glutathione peroxidase activity due to interference on glutathione which is a substrate for glutathione peroxidase and they showed the administration of vitamin C to pregnant women could reduce the activity of glutathione peroxidase (32). Besides another investigators found that vitamin C exhibits a protective effect against free radical induced oxidative damage (33).

Based on the results of table (3) there were no significant differences in total protein, albumin and globulin level. This finding is in accordance with (13), who found the single S/C injection of ivermectin (0.5mg, 1mg/Kg B.W) to rabbits did not change or effect on the total protein, albumin and globulin level in each concentration. In another study, it was found that the S/C injection of 0.2mg/Kg of ivermectin to goat infested with mange in single dose caused an elevation in total protein, albumin and globulin(34). Some researchers (35) claimed the S/C injection of 0.2mg, 0.4mg/Kg B.W of ivermectin to albino rats for 8 weeks caused significant reduction in total protein and albumin. As well as,(36) observed decrease in total protein,

albumin, and globulin in goat after treatment with Ivermectin.

CONCLUSION

Ivermectin has no effect on lipid profiles and proteins, but it causes oxidative stress. Vitamin C is considered as ameliorative agent and can protect rabbits.

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قسم الدراسات والبحوث العربية

ARABIC STUDIES AND RESEARCHES SECTION

الخصائص الفسلجية للبكتيريا الخيطية المحبة للملوحة

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الملخص باللغة العربية

جمعت 75 عينة طبيعية من مصادر مختلفة (بيئات مائية ومياه أبار وترب مالحة مختلفة) خلال الفترة من 18 آب إلى 15 تشرين الثاني من عام 2013. وتم عزل 55 عزلة تابعة للأجناس Nocardiopsis و Streptomyces ، وبلغت أعلى نسبة عزل لجنس Nocardiopsis (77.1%) ، وتلاه جنس Streptomyces بنسبة (22.9%)، أنتخبت منها 35 عزلة وشخصت العزلات إلى مستوى النوع اعتمادا على 80 صفة من الصفات الشكلية ، الكيموحيوية ، الفسلجية والجزيئية باستخدام التصنيف العددي بطريقة التحليل العنقودي Cluster analysis من خلال معامل التشابه البسيط وطريقة المعدل. تم انتقاء 19 عزلة وإجراء تضخيم لمورث 16s rDNA الذي ظهر تقريبا عند الموقع 1350 زوجا قاعديا، شخصت العزلات ضمن المركز الوطني لمعلومات التقانة الحيوية National Center for Biotechnology Informaion (NCBI) وذلك باستخدام برنامج Basic Local Alignment Search Tool (BLAST) وقورنت تتابعات القواعد النيتروجينية للأنواع قيد الدراسة باستخدام برنامج Mega 5 وطريقة Clustal W والتعقد باستخدام المعدل غير الموزون Un-weighted Pair Group Method for the arithmetic Average (UPGMA) لإيجاد العلاقة التطورية بين الأنواع ، حيث تم تقسيم المخطط الشجري إلى عشرة عناقيد رئيسة J,I,H,G,F,E,D,C,B,A. وقع النوع *N. dassonvillei* ضمن العنقود A ، والنوع *N. lucentensis* ضمن العنقود B ، والنوع *N. aegyptia* ضمن العنقود C ، والنوع *N. arvandica* ضمن العنقود D ، والنوع *N. halotolerans* ضمن العنقود E ، والنوع *N. xinjiangensis* ضمن العنقود F ، والنوع *N. halophila* ضمن العنقود G ، والنوع *S. rochei* ضمن العنقود H ، والنوع *S. albiaxialis* ضمن العنقود I ، ووقع النوع *S. cacaoi* ضمن العنقود J.

تميزت أفراد جنس Nocardiopsis بكونها غير منتجة لرائحة التربة الرطبة earthy odour التي تنفرد بها أنواع جنس Streptomyces . تميزت بعض الأنواع التابعة لجنس Nocardiopsis بامتلاكها تراكيب خاصة عبارة عن مجموعات من خيوط متجمعة مع بعضها تتكون في نهاية قمة خيوط الغزل الهوائي، أو قد تظهر على جانبي خيوط الغزل وتدعى هذه المجموعات Synnemata أو قد تسمى أحيانا Coremia ، ولهذه المجموعات دور مهم في تمييز وتشخيص النوعين *N. lucentensis* و *N. halotolerance* اللذين يمتلكان هذه التراكيب الخاصة عن بقية أنواع جنس Nocardiopsis قيد الدراسة والتي تفتقد لمثل هذه التراكيب.

صنفت الأنواع قيد الدراسة اعتمادا على قدرتها على النمو بوجود تراكيز مختلفة من ملح الطعام NaCl إلى ثلاثة أصناف : المحبة لدرجات الملوحة العاليـــة extreme halophiles التـــي استطاعت النمو بتراكيز ملحية وصلت إلى أكثر من 12% NaCl كما في الأنواع *N. xinjiangensis* و *N. halophila* و *S. caccaoi* ، والمحبة لدرجات الملوحة المعتدلة moderate halophiles التي استطاعت النمو بتراكيز ملحية أقل من 12 % NaCl كما في الأنواع *N. aegyptia* و *S. rochei* ، ومتحملة للملوحة العالية halotolerans والتي اســتطاعت النمــو بوجود وغياب ملح الطعام NaCl كما في الأنواع *S. albiaxialis* و *N. lucentensis* و *N. halotolerans* و *N. arvandica* و *N. dassonvillei*.

الكلمات المفتاحية: *التصنيف العددي*، Streptomyces ، Nocardiopsis ، 16s rDNA، المحبة للملوحة

Physiological characteristics of halophilic actinomycetes

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ABSTRACT

Seventy five samples were collected from various sources (saline water and different soils environments) for the period from August 18 to November 15, 2013. Fifty-five isolation belonging to the genera Nocardiopsis and Streptomyces, the highest rate of isolation was 77.1 % which belonged to the genus Nocardiopsis, followed by 22.9% to Streptomyces, thirty-five isolates were selected and had been diagnosed to species level, depending on 80 morphology, biochemical, physiological and molecular characteristics using numerical taxonomy by using cluster analysis and simple matching coefficient with average linkage. Nineteen isolation were selected and an amplification of 16s rDNA gene which almost appeared at 1350 base pairs, the isolates were diagnosed within the National Center for Biotechnology Information (NCBI) in USA using the Basic Local Alignment Search Tool (BLAST), the nitrogen bases of the sequencing were compared using Mega 5 and the method of Clustal W and clustering using the Un-weighted Pair Group Method for the arithmetic Average (UPGMA) to find the evolutionary relationship between species, the tree has been planned split into ten main clusters A,B,C,D,E,F,G,H,I,J. The species *N. dassonvillei* occurred within the cluster A, and *N. lucentensis* within the cluster B, and *N. aegyptia* within the cluster C, and *N. arvandica* within the cluster D, and *N. halotolerans* within the cluster E, and *N. xinjiangensis* within the cluster F, and *N. halophila* within the cluster G, and *S. rochei* within the cluster H, and *S. albiaxialis* within the cluster I, and *S. cacaoi* within the cluster J. Members of the genus Nocardiopsis were distinguished of being unproductive earthy odour that are unique in members of the genus Streptomyces. Some species of the genus Nocardiopsis characterized by possessing a private structures of filaments grouped together at the end of the aerial mycelium or may appear on both sides of the mycelium called Synnemata or may sometimes called Coremia, which play an important role in the diagnosis and discrimination of *N. lucentensis* and *N. halotolerance* who possess these special types of structures from the rest of the genus Nocardiopsis under study that lack of such structure. The species under study were classified depending on their ability to grow in the presence of different concentrations of NaCl salt into three categories: Extreme halophiles that have managed growth in salt concentrations reached more than 12% NaCl as in the species *N. xinjiangensis* , *N. halophila* and *S. caccaoi,* and Moderate halophiles that have managed growth in salt concentrations less than 12% NaCl, as in the species *N. aegyptia* and *S. rochei*, and Halotolerans which was able to grow in the presence and absence of NaCl as in the species , *N. dassonvillei* ,*N. lucentensis, N. halotolerans* ,*N. arvandica* and *S. albiaxialis*

المقدمة

تصنف البكتيريا الخيطية المحبة للملوحة halophilic actinomycetes بأنها بكتيريا موجبة لصبغة جرام حرة المعيشة ، مترممة ، وهي من أكثر المجاميع البكتيرية المتواجدة في التربة (1) ، ويعد جنس Streptomyces أكثرها شيوعا في التربة ومعروف بدوره الرئيس في عملية المعدنة في التربة ، وأيضا في قدرته على إنتاج أنواع مختلفة من المضادات الحيوية (2).
إن أول أنواع البكتيريا الخيطية المحبة للملوحة العالية التي سجلت هي *Actinopolyspora halophila* والتي تميزت بقدرتها على النمو بتراكيز ملحية عالية تصل إلى 25% NaCl (3) ، وأيضا *A. iraqiensis* المحبة للملوحة المعتدلة التي عزلت من الترب المالحة في العراق وتستطيع النمو بتراكيز ملحية 10–15 % NaCl (4) ، كما تم تسجيل عزل للنوع *N. halophila* المحب للملوحة العالية الذي ينمو بتراكيز ملحية 3–25% NaCl من الترب المالحة في العراق (5).
ونظرا للأعداد الهائلة للبكتيريا الخيطية المتواجدة في مثل هذه البيئات المتطرفة ، جاءت هذه الدراسة لتحقيق الأهداف الآتية:
1. عزل وتشخيص أنواع البكتيريا الخيطية المحبة للملوحة من البيئات المختلفة.
2. تصنيف العزلات عدديا ومقارنة عدة طرق للتعنقد وإعداد جداول تشخيصية ثابتة ومقبولة للعزلات قيد الدراسة.
3. مقارنة تتابعات المورث 16s rDNA للأنواع المعزولة.

المواد وطرق العمـــــل

جمعت 70 عينة من بيئات مائية وترب مالحة لمناطق مختلفة من أنحاء القطر إضافة لعينات البحر الميت و 5 عينات من ترب زراعية للمقارنة كما هو موضح في الجدول (1).
جمعت 55 عينة تربة وجففت العينات بدرجة حرارة الغرفة وأخذ 1 غم من التربة وحضرت منها تخافيف عشرية وصولا إلى التخفيف 10^{-4} ، أخذ 1 سم^3 من التخفيف 10^{-3} وزرع بطريقة الصب على وسط Streptomyces agar الحاوي 12% ملح الطعام وحضنت في درجة حرارة 28 °م لمدة أسبوع في ظروف هوائية (6، 7).
جمعت 20 عينة مياه مالحة من مناطق مختلفة وأخذ 1 سم^3 من العينة وزرعت بطريقة الصب على وسط Streptomyces agar الحاوي على 12% ملح الطعام وحضنت في درجة حرارة 28 °م لمدة أسبوع في ظروف هوائية (6).

جدول رقم (1): عدد العينات المأخوذة ومناطق العزل

نوع العينة		عد العينات	مناطق العزل	الأجناس المعزولة
ترب مالحة	سطح التربة	23	البصرة/العمارة/بغداد/سد الموصل /دهوك/زاخو/سنجار/ زاويتة/عقرة/ جبل سنجار/ناحية القيروان/ناحية البعاج /ناحية الذلجة/جبل كورك في اربيل/الترب المجاورة لمعامل الذلوج في سنجار ونينوى.	Nocardiopsis Streptomyces
	على عمق 10 سم	23		
ترب زراعية		5	موصل/قضاء سنجار	لا يوجد
مياه آبار		20	كركوك/بغداد/البصرة/العمارة/دهوك/سنجار/بعاج/الشرقاط	Nocardiopsis
طين		4	الأردن/البحر الميت	Nocardiopsis Streptomyces

الاختبارات الشكلية : تم ملاحظة خصائص المستعمرات النامية على الوسط المستخدم في العزل الأولي ، مثل حجم المستعمرات وشكلها ولون كل من الغزل الارضي والهوائي وإنتاجها للصبغات الخارجية والرائحة وغيرها من الصفات (8، 9).
الاختبارات المجهرية : استخدمت تقنية الزرع على الشريحة الزجاجية Slide culture technique للتحري عن شكل كل من الغزل الأرضي والهوائي (10).

الاختبارات الكيموحيوية :
1. تحديد التركيز الملحي المثالي للنمو : حضرت تراكيز مختلفة من ملح الطعام بإضافة المحلول الملحي المذاب إلى وسط yeast extract agar وبالتراكيز (25، 20 ، 15 ، 12 ، 9 ، 7 ، 5 ، 3 ، 0%) وحضنت تحت نفس الظروف سابقة الذكر (11) ، وعلى ضوئه تم تحديد إضافة تركيز 5% لكل من الأوساط الزرعية المستخدمة لاحقا .
2. إنتاج إنزيم سايتوكروم أوكسديز(12) .
3. إنتاج إنزيم الكتاليز(13).
4. استهلاك الكاربوهيدرات أستخدم وسط الفينول الأحمر Phenol red agar بإضافة ملح الطعام بتركيز5% Nacl والمضاف إليه بعد التعقيم كل من السكريات الآتية والمعقمة لمدة 5 دقائق في جهاز الموصدة تحت الظروف المثالية: D- Mannose , Maltose , Sucrose , Cellulose , Xylose , Galactose , Dextrine , Mannitol , Fructose , Lactose , Glucose Sorbitol , L-Arabinose. حضنت الأطباق في درجة حرارة 28 درجة مئوية لمدة 48–72 ساعة وتم ملاحظة النتيجة من خلال تغير لون الوسط (14،15).
5. تحليل النشا (12،15).
6. تحلل الجيلاتين (12).
7. استهلاك السترات (13) .
8. تحلل الأحماض الأمينية: حضر وسط تحلل الأحماض الأمينية بإضافة 0.5 غم من وسط الأكار المغذي nutrient agar و0.5 غم سكر الكلوكوز و0.02 غم من الكاشف Bromocresol purple و0.5 غم من كل من الأحماض الأمينية: L-Serine , Glycine , DL- Methionine , Valine , L- L-Arginine Therionine , L-lanine L-Cyctine في 100 سم^{3}من الوسط

مع إضافة 5% من ملح الطعام وعقمت في 121 درجة مئوية لمدة 10 دقائق.وحضنت في درجة حرارة 28 درجة مئوية لمدة 28- 72 ساعة في ظروف هوائية وتم ملاحظة النتيجة من خلال تغير لون الوسط (16).

9. اختبار إنتاج غاز H_2S و Co_2 باستخدام وسط ثلاثي السكر و الحديد (17).

10. تأثير ملح كلوريد البوتاسيوم على النمو : حضر وسط yeast extract agar بتراكيز Kcl مختلفة (15,12,9,7,5,3%) وحضنت الأطباق في درجة حرارة 28 درجة مئوية لمدة 7 أيام (18، 19).

11. الحساسية للمضادات الحيوية : استخدمت المضادات الحيوية المجهزة من شركة Bio analysis اعتماداً على الطريقة المتبعة من قبل منظمة الصحة العالمية (20، 21).

12. النمو بوجود المثبطات : نميت العزلات على وسط yeast extract agar المضاف له 5% ملح الطعام والحاوي على تركيز 0.1 % من كل من مثبطات الفينول والصوديوم آزايد والكرستال فايوليت ، وحضنت الأطباق في درجة 28 درجة مئوية لمدة 7 أيام في ظروف هوائية (8). 13. الفعالية الضد ميكروبية للعزلات Antimicrobial activity: تم اختبار قدرة العزلات قيد الدراسة على تثبيط نمو أنواع بكتيرية موجبة وسالبة لصبغة كرام ، ونميت البكتريا الخيطية أولاً بشكل خط مستقيم منتصف الطبق الحاوي على وسط Muller Hinton agar المعقم الحاوي على 3% ملح الطعام NaCl وحضنت في درجة 28 درجة مئوية لمدة 7 أيام ثم زرعت بكتريا الاختبار على جانبي الخط وحضنت في 37 درجة مئوية لمدة 24 ساعة في ظروف هوائية (22).

14. النمو بمدى أس هيدروجيني مختلف: حضر وسط yeast extract agar بقيم PH مختلفة (4 , 6 , 7 , 9 , 12) وبتركيز ملحي 5% وحضنت الأطباق بعد زرعها في درجة حرارة 28 درجة مئوية لمدة 7 أيام (23).

التصنيف العددي: استخدم التصنيف العددي في هذه الدراسة لتفريق الأجناس المعزولة ، وتمييز الانواع التابعة لها وباتباع نظام التحليل العنقودي وبطريقة الربط باستخدام المعدل الموزون.

تتألف المصفوفة من عدد (m=80) من المتغيرات للصفات الشكلية والفسلجية والكيموحيوية والبيئية والتراكيب الجزيئية لعدد من العزلات (n=35) من الأجناس والأنواع المعزولة ، وقد نظمت المصفوفة على أساس الارقام الثنائية لتمثيل حالة وجود الصفة (+ او 1) وحالة عدم وجود الصفة (- او 0) الخاص بالعزلة الواحدة.

تم تحليل البيانات باستخدام البرنامج الاحصائي SPSS Statistical Packege for the Social Sciences (24).

الاختبارات الجزيئية:

1. عزل الدنا البكتيري بإستخدام Colony PCR المحورة (25) .

2. طريقة الترحيل الكهربائي: تم الكشف عن الدنا المجيني وناتج تفاعلات PCR بإستخدام الترحيل الكهربائي على هلام الاكاروز Agarose gel electrophoresis.

3. تفاعلات PCR للتحري عن مورث 16s rDNA: حضر المزيج الرئيس بحجم 50 مايكروليتر لجميع العينات وتم ترحيل العينات بجهاز الترحيل الكهربائي بفولتية 50 لمدة 75 دقيقة ثم صبغه بإستخدام بروميد الاثيديوم للتحري عن وجود مورث 16s rDNA عند الموقع 1350 زوج قاعدي.

4. تحديد تتابعات القواعد النتروجينية لمورث 16s r DNA: تم اجراء تحديد التتابعات الوراثية لمورث 16s r DNA لـ 19 عزلة تابعة للبكتريا الخيطية وأنجز الفحص في شركة (Microgen Laboratory / USA in both directions using an ABI 3730 XL DNA Analayser) تم التحري عن التطابق الجيني للمورث بإستخدام برنامج Basic Local Alignment Search Tool (BLAST) والمتوفر في المركز الوطني لمعلومات التقانات الحياتية National Center for Biotechnology Information (NCBI)

5. العلاقة التطورية للسلالات المشخصة: تم الحصول على العلاقة التطورية بين السلالات التابعة للبكتريا الخيطية المحبة للملوحة البالغ عددها 19 باستخدام Clustal W ضمن برنامج Mega 5.0 باستخدام طريقة UPGMA اعتماداً على طريقة Tamura and Nie لسنة (2001).

النتائـــج والمناقشـــة

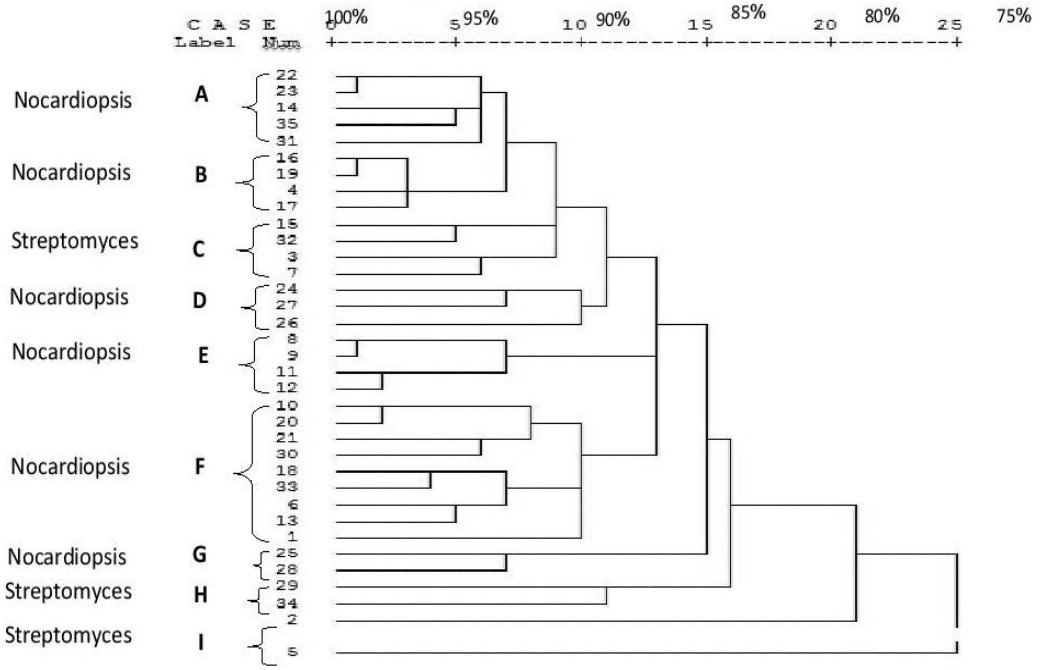
تم الحصول على 55 عزلة في العزل الأولي من البيئات المالحة في حين لم يتم الحصول على عزلات بكتيرية من الترب الزراعية ، انتخب منها 35 عزلة محبة لدرجات الملوحة العالية التي لها القدرة على النمو بتراكيز ملحية تصل الى 12% NaCl في وسط العزل الاولي Streptomyces agar وذلك اعتمادا على تقسيمات الباحثين Kushner و Kamekura حيث أن الكائنات المحبة للملوحة العالية تحتاج إلى تركيز ملحي 12 % NaCl لتنمو بصورة جيدة (26) والذي كان له دور كبير في تمييز البكتريا الخيطية المحبة للملوحة (27- 29).

التحليل العددي:

شخصت العزلات المنتخبة اعتمادا على 80 اختبار واقع ضمن صفاتها الشكلية باستخدام طريقة الزرع على الشريحة الزجاجية والكيموحيوية والفسلجية إلى جنسي Streptomyces و Nocardiopsis باستخدام ومقارنة ثلاث طرائق للتعنقد وتم الحصول على ثلاث مخططات شجرية واتضح ان افضل الطرائق هي طريقة الربط باستخدام المعدل الموزون ومن ثم طريقة الربط المنفرد للمجاور الاقرب تليها الربط المنفرد للمجاور الابعد واستبعدت بقية طرق الربط. تم الحصول على 28 عزلة تابعة لجنس Nocardiopsis إذ يعد من أكثر أجناس البكتريا الخيطية المحبة للملوحة الواسعة الانتشار التي عزلت في مثل هذه البيئات المالحة ويتبعها جنس Streptomyces بعدد 8 عزلات ، في حين لم يتم عزل الأجناس الأخرى التابعة للبكتريا الخيطية والسبب في ذلك يعود الى صعوبة عزل هذه الأجناس فضلا عن كونها أقل انتشارا مقارنة بجنسي Nocardiopsis و Streptomyces (30، 31) ، إضافة الى كون الوسط المستخدم في العزل الاولي Streptomyces agar من الأوساط الفقيرة low nutrient المفضل لنمو جنسي Streptomyces و Nocardiopsis وقد يكون غير ملائم لبعض الاجناس التي تحتاج الى متطلبات نمو خاصة (32، 33).

قسم المخطط الشجري الأولي إلى 9 عناقيد (I,H,G,F,E,D,C,B,A) كما هو موضح في الشكل (1) والجدول (2) ، تميزت أفراد جنس Nocardiopsis بكونها غير منتجة للرائحة earthy odour التي تنفرد بها انواع جنس Streptomyces ، حيث تقوم الأخيرة بإنتاج مركبات عضوية تدعى بـ Geosmin والمسؤولة عن رائحة التربة (34) .

تميزت بعض الأنواع التابعة لجنس Nocardiopsis بامتلاكها تراكيب خاصة عبارة عن مجاميع من خيوط متجمعة مع بعضها تتكون في نهاية قمة خيوط الغزل الهوائي او قد تظهر على جانبي خيوط الغزل وتدعى هذه المجاميع بـ Synnemata او قد تسمى احيانا بـ Coremia ، ولهذه المجاميع دور مهم في تمييز وتشخيص النوعين *N. lucentensis* و*N. halotolerance* ،اللذان يمتلكان هذه التراكيب الخاصة عن بقية أنواع جنس Nocardiopsis قيد الدراسة والتي تفتقد لمثل هذه التراكيب (35- 37).



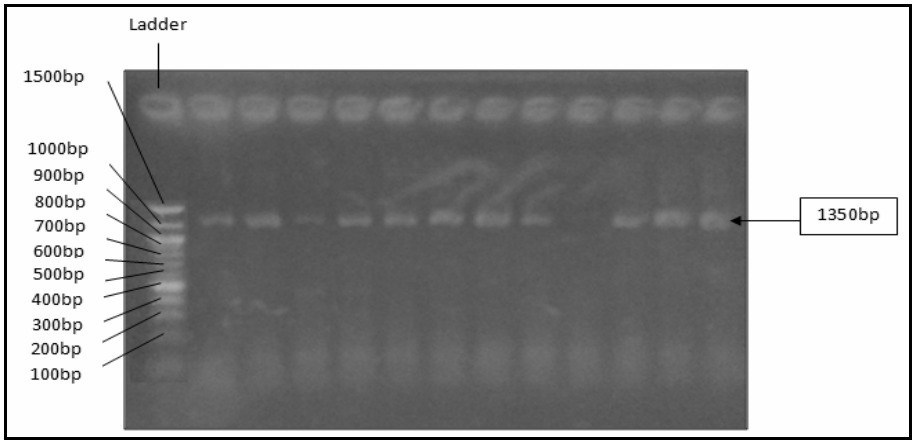
شكل رقم: (1) المخطط الهرمي الشجري للعزلات التابعة لجنسي Streptomyces و Nocardiopsis التي تم الحصول عليها بالتحليل العنقودي باستخدام طريقة المعدل الحسابي الموزون ومعامل التشابه البسيط Ssm

جدول رقم (2): النسب المئوية لتشابه العناقيد الرئيسة ومصادر عزلها حسب المخطط الشجري في الشكل رقم (1)

رمز العنقود	عدد العزلات	الأجناس المعزولة	النسبة المئوية للتشابه%	مصدر العزل
A	5	Nocardiopsis	94	مياه بئر /تربة
B	4	Nocardiopsis	97	مياه بئر /تربة
C	4	Streptomyces	91	تربة/ طين البحر الميت
D	3	Nocardiopsis	90	تربة
E	4	Nocardiopsis	93	تربة/ طين البحر الميت
F	9	Nocardiopsis	90.5	تربة
G	2	Nocardiopsis	93.5	تربة
H	2	Streptomyces	89	تربة
I	2	Streptomyces	75.5	تربة
المجوع	**35**			

تشخيص العزلات اعتمادا على تحليل تتابعات مورث 16s rDNA:

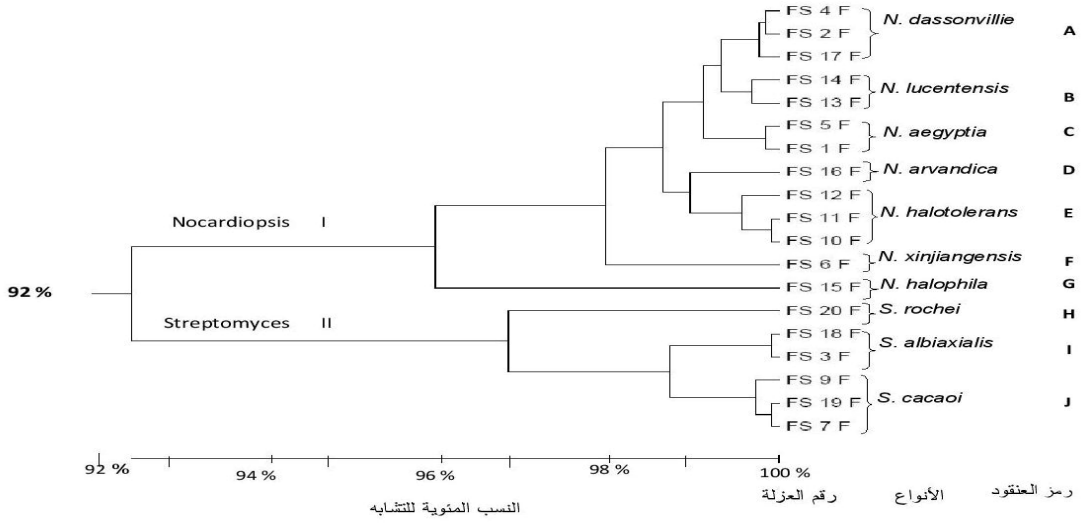
اعتمادا على الشجرة التصنيفية الأولية في الشكل السابق رقم (1)، تم انتقاء 19 عزلة ، 13 عزلة بكتيرية تابعة لجنس Nocardiopsis ، 11 منها عزلت من الترب المالحة وطين البحر الميت وعزلتين من عينات المياه المالحة ، و6 عزلات بكتيرية تابعة لجنس Streptomyces تضمنت 4 عزلات من الترب المالحة وطين البحر الميت وعزلتين من المياه المالحة لتشخيصها جزيئيا. تم اجراء تضخيم لمعظم المورث 16s rDNA الذي ظهر تقريبا عند الموقع (1350 زوجا قاعديا) باستخدام البادئ العام 27f و 1392r المجهز من شركة Promega للدنا الكروموسومي كما هو موضح في الصورة (1) ، استخدم المورث 16s rDNA المضخم كقالب لتحليل وتحديد تتابع القواعد النتروجينية وذلك لتشخيص العزلات التابعة لأجناس البكتريا الخيطية المحبة للملوحة ، تم مقارنة نتائج تتابعات القواعد النتروجينية للسلسلة الامامية والخلفية لمورث 16s rDNA للسلالات قيد الدراسة مع السلالات القياسية ضمن المركز الوطني لمعلومات التقانة الحياتية NCBI ، وذلك باستخدام برنامج BLAST، إذ اظهرت نسبة تشابه مابين (96.6–99.9) مع العزلات التابعة لجنس Streptomyces ونسبة تشابه مابين (98.5–99.8) مع العزلات التابعة لجنس Nocardiopsis.



صورة رقم (1): موقع الحزم لمورث 16s rDNA عند 1350 زوج قاعدي

قورنت تتابعات القواعد النتروجينية للانواع قيد الدراسة باستخدام برنامج Mega 5 وباستخدام طريقة Clustal W والتعنقد باستخدام المعدل غير المورون The Un-weighted Pair Group Method for the arithmetic Average(UPGMA)، لإيجاد العلاقة التطورية بين الأنواع. قسم المخطط الشجري إلى عنقودين رئيسين I و II ضم جنسي Nocardiopsis و Streptomyces على التوالي و10 عناقيد رئيسة (J,I,H,G,H,F,E,D,C,B,A) ، حيث ارتبطت الأنواع التابعة لجنس Nocardiopsis مع بعضها ضمن مستوى تشابه (98.5–99.8) % ، في حين ارتبطت الأنواع التابعة لجنس Streptomyces مع بعضها عند مستوى تشابه (96.9–99.9) % ، في حين ارتبط العنقودين الرئيسين مع بعضهما عند مستوى تشابه (92%) كما هو موضح بالمخطط الهرمي الشجري (شكل رقم 2) والجداول (3–6).

اعتمادا على تتابعات القواعد النتروجينية لمورث 16s rDNA لكلا الجنسين وباستخدام البادئ العام 27f و 1392r فقد لوحظ وجود تغاير ونقص في القواعد النتروجينية بين جنسي Streptomyces و Nocardiopsis من القاعدة 406–462 ، كذلك لوحظ وجود تغاير في القواعد النتروجينية بين الأنواع الموضحة في الشجرة التطورية (شكل رقم 2)، حيث ظهر التغاير في المنطقة بين القاعدة رقم 20–79 ، كذلك في القواعد النتروجينية من القاعدة رقم 145–265 حيث ظهر مثلا النوع *S. cacaoi*في عنقود منفصل وأيضا ظهر النوع *S. rochei* في عنقود منفصل عن الانواع التابعة لنفس الجنس وكذلك نفس الحال مع بقية الانواع ، وهذا التغاير (زيادة او نقصان) في القواعد النتروجينية بين الجنسين يدل على ان جنس Nocardiopsis بعيد وراثيا عن جنس Streptomyces وهذا ما أكده الباحثين Lane و Tamura (38,39) حيث أن كل منها يقع ضمن عائلة مختلفة اضافة الى كون مجموعة البكتريا الخيطية بصورة عامة واسعة التنوع ومتغايرة جينيا خاصة جنس Streptomyces.



شكل رقم (2): المخطط الهرمي الشجري التطوري للأنواع التابعة لجنسي Streptomyces و Nocardiopsis باستخدام طريقة UPGMA

جدول رقم (3): العناقيد التي تم الحصول عليها من الشجرة التصنيفية شكل رقم (2) والنسب المئوية لإظهار الصفات المظهرية للعزلات

رمز العنقود		A	B	C	D	E	F	G	H	I	J
الأنواع / الصفات		*Nocardiopsis dassonvillei*	*Nocardiopsis lucentensis*	*Nocardiopsis aegyptia*	*Nocardiopsis arvandica*	*Nocardiopsis halotolerans*	*Nocardiopsis xinjiangensis*	*Nocardiopsis halophila*	*Streptomyces rochei*	*Streptomyces albiaxialis*	*Streptomyces cacaoi*
عدد العزلات		3	2	2	1	3	1	1	1	2	3
نسبة الارتباط		%99.7	% 99.6	%99.8	%98.9	%99.5	%95.9	%95.9	%96.7	%99.9	%99.7
النسب المئوية للصفات / الاختبارات											
ألوان الغزل الهوائي للمستعمرات على اكار مستخلص الخميرة 5% NaCl	حليبي فاتح	0	0	100	0	0	100	100	0	0	100
	حليبي غامق	33.3	0	0	0	33.3	0	0	0	0	0
	ابيض فاتح	0	0	0	0	33.3	0	0	0	50	0
	ابيض غامق	66.7	100	0	0	33.3	0	0	0	0	0
	قهواني فاتح	0	0	0	100	0	0	0	0	50	0
	ذهبي فاتح	0	0	0	0	0	0	0	100	0	0
ألوان الغزل الارضي للمستعمرات على اكار مستخلص الخميرة 5% NaCl	اصفر فاتح	100	0	100	0	66.7	100	100	0	0	100
	اصفر غامق	0	100	0	0	33.3	0	0	100	0	0
	قهواني فاتح	0	0	0	100	0	0	0	0	100	0
حجم المستعمرات	كبيرة	0	0	0	0	0	0	0	0	0	33.3
	متوسطة	66.7	50	100	100	66.7	0	0	100	50	0
	صغيرة	33.3	50	0	0	33.3	100	100	0	50	66.7
قوام المستعمرات	هشة	100	100	100	0	66.7	0	0	100	50	66.7
	جلدية ملتصقة	0	0	0	100	33.3	100	100	0	50	33.3
انتاجها للرائحة		0	0	0	0	0	0	0	100	100	100
الصبغة الخارجية المنتجة	قهواني مصفر	0	0	0	0	0	0	0	100	0	0
	وردي مصفر	0	0	0	100	0	0	0	0	0	0
	قهواني فاتح	33.3	0	0	0	0	0	0	0	0	0
	قهواني غامق	33.3	50	0	0	0	0	0	0	50	0
	اصفر مخضر	0	0	0	0	0	0	100	0	0	0

جدول رقم (4): العناقيد التي تم الحصول عليها من الشجرة التصنيفية (شكل رقم 2) والنسب المئوية لإظهار الصفات المجهرية للعزلات

رمز العنقود			A	B	C	D	E	F	G	H	I	J
الأنواع / الصفات			*Nocardiopsis dassonvillei*	*Nocardiopsis lucentensis*	*Nocardiopsis aegyptia*	*Nocardiopsis arvandica*	*Nocardiopsis halotolerans*	*Nocardiopsis xinjiangensis*	*Nocardiopsis halophila*	*Streptomyces rochei*	*Streptomyces albiaxialis*	*Streptomyces cacaoi*
عدد العزلات			3	2	2	1	3	1	1	1	2	3
نسبة الارتباط			%99.7	% 99.6	%99.8	%98.9	%99.5	%95.9	%95.9	%96.7	%99.9	%99.7
النسب المئوية للصفات / الاختبارات												
الغزل الهوائي بتقنية الزرع على الشريحة	شكل الغزل	مستقيم	0	100	100	*	66.7	0	0	100	0	33.3
		متعرج (حلزون)	100	0	0	*	33.3	100	0	0	100	0
		حلزون مكبوس	0	0	0	*	0	0	0	0	0	66.7
		سلسلة من السبورات	0	0	0	*	0	0	100	0	0	0
	طول الغزل	طويل	100	100	100	*	100	100	100	0	0	33.3
		قصير	0	0	0	*	0	0	0	100	100	66.7
	سمك الغزل	سميك	100	0	0	*	100	100	100	100	100	100
		نحيف	0	100	100	*	0	0	0	0	0	0
	التفرع		0	0	0	*	0	0	0	100	0	33.3
	وجود السبورات		0	100	0	*	0	0	100	0	0	0

جدول رقم (5): العناقيد التي تم الحصول عليها من الشجرة التصنيفية (شكل رقم 2) والنسب المئوية لإظهار الصفات الكيموحيوية للعزلات

رمز العنقود		A	B	C	D	E	F	G	H	I	J
الأنواع / الصفات		*Nocardiopsis dassonvillei*	*Nocardiopsis lucentensis*	*Nocardiopsis aegyptia*	*Nocardiopsis arvandica*	*Nocardiopsis halotolerans*	*Nocardiopsis xinjiangensis*	*Nocardiopsis halophila*	*Streptomyces rochei*	*Streptomyces albiaxialis*	*Streptomyces cacaoi*
عدد العزلات		3	2	2	1	3	1	1	1	2	3
نسبة الارتباط		%99.7	% 99.6	%99.8	%98.9	%99.5	%95.9	%95.9	%96.7	%99.9	%99.7
النسب المئوية للصفات / الاختبارات											
النمو بتراكيز NaCl مختلفة	% 0	66.7	50	100	0	33.3	0	100	0	100	66.6
	% 3	100	100	100	100	66.7	0	100	100	100	100
	% 5	100	100	100	100	100	100	100	100	100	100
	% 7	100	100	100	100	100	100	100	100	100	100
	% 9	100	100	100	100	100	100	100	100	100	100
	% 12	100	100	0	100	100	100	100	0	100	100
	% 15	66.7	100	0	100	100	100	100	0	100	100
	% 20	0	0	0	0	0	100	100	0	50	33.3

رمز العنقود		A	B	C	D	E	F	G	H	I	J
الأنواع / الصفات		*Nocardiopsis dassonvillei*	*Nocardiopsis lucentensis*	*Nocardiopsis aegyptia*	*Nocardiopsis arvandica*	*Nocardiopsis halotolerans*	*Nocardiopsis xinjiangensis*	*Nocardiopsis halophila*	*Streptomyces rochei*	*Streptomyces albiaxialis*	*Streptomyces cacaoi*
	% 25	0	0	0	0	0	0	100	0	0	0
النمو بتراكيز KCl مختلفة	% 3	33.3	100	100	100	66.7	100	0	0	50	100
	% 5	100	100	100	100	100	100	100	0	50	100
	% 7	100	100	100	100	100	100	100	100	100	100
	% 9	100	100	100	100	100	100	100	0	100	100
	% 12	100	100	100	100	100	100	100	0	50	100
	% 15	66.7	100	50	0	33.3	100	100	0	50	66.7
النمو بوجود المثبطات 1%	الفينول	0	0	0	0	0	0	0	0	0	0
	صوديوم أزايد	0	0	0	0	0	0	0	0	0	0
	كرستال فايوليت	100	100	100	100	100	100	100	100	100	100
تخمر السكريات	مانوز	33.3	100	100	0	100	0	0	0	0	33.3
	مالتوز	33.3	100	0	0	33.3	0	100	100	0	0
	سكروز	66.7	100	100	0	100	0	0	100	0	0
	سيليلوز	0	0	0	0	0	0	0	0	0	0
	زايلوز	33.3	0	100	0	0	0	100	0	0	100
	كالاكتوز	33.3	0	100	100	100	0	0	100	0	0
	كلوكوز	100	100	0	0	100	100	0	100	100	100
	دكستروز	0	0	0	0	0	0	0	100	0	66.7
	مانيتول	33.3	100	0	0	0	0	100	100	0	100
	فركتوز	33.3	100	0	0	33.3	0	100	100	0	100
	لاكتوز	33.3	0	100	0	0	0	0	100	0	0
	سوربيتول	0	0	0	0	0	0	0	0	100	0
	ارابينوز	33.3	0	100	0	0	0	100	100	100	100
استهلاك الاحماض الامينية	L-ثريونين	33.3	0	50	0	0	0	0	0	50	33.3
	L-ارجنين	0	0	0	100	0	0	0	0	0	0
	L-النين	66.7	0	100	100	100	100	0	100	100	66.7
	كلايسين	33.3	0	0	0	0	0	0	0	0	66.7
	L-سيرين	33.3	100	50	100	33.3	100	0	0	0	66.7
	فالين	33.3	0	0	0	0	0	0	0	0	33.3
	DL-مثيونين	33.3	0	50	0	0	0	0	0	0	66.7
	L-سستين	100	50	100	0	66.7	0	100	100	50	100
إنتاج أنزيم	الاوكسيديز	33.3	50	100	100	0	100	0	100	50	33.3
	الكتاليز	100	100	100	100	100	100	100	100	100	100
إنتاج غاز	H2S	0	0	0	0	0	0	0	0	0	0
	CO2	33.3	50	100	100	0	100	0	100	0	66.6

جدول رقم (6): العناقيد التي تم الحصول عليها من الشجرة التصنيفية (شكل رقم 2) والنسب المئوية لإظهار صفات الفعالية الضد الميكروبية والحساسية للمضادات الحيوية للعزلات

رمز العنقود		A	B	C	D	E	F	G	H	I	J
الأنواع / الصفات		*Nocardiopsis dassonvillei*	*Nocardiopsis lucentenssis*	*Nocardiopsis aegyptia*	*Nocardiopsis arvandica*	*Nocardiopsis halotolerans*	*Nocardiopsis xinjiangensis*	*Nocardiopsis halophila*	*Streptomyces rochei*	*Streptomyces albiaxialis*	*Streptomyces cacaoi*
عدد العزلات		3	2	2	1	3	1	1	1	2	3
نسبة الارتباط		%99.7	% 99.6	%99.8	%98.9	%99.5	%95.9	%95.9	%96.7	%99.9	%99.7
النسب المئوية للصفات / الاختبارات											
الفعالية الضد ميكروبية	Micrococcus luteus	66.7	100	0	100	66.7	100	100	100	50	66.7
	Salmonella typhimurium	33.3	0	50	0	0	0	0	100	50	0
	Bacillus sp.	0	100	0	100	66.7	0	100	100	50	66.7
	Escherichia coli	33.3	50	0	100	66.7	0	100	100	50	33.3
	Staphylococcus aureus	0	50	0	100	66.7	0	100	100	0	66.6
	Staphylococcus epidermidis	0	0	50	100	66.7	0	0	100	0	33.3
	Pseudomonas aeruginosa	0	0	0	0	33.3	0	0	0	0	0
	Klebsiella pneumonia	0	100	0	0	33.3	0	0	100	50	33.3
الحساسية للمضادات الحيوية	Erythromycin 15 Mg/disc	66.7	50	50	100	100	0	100M	0	50 50M	33.3 66.7M
	Neomycin 30 Mg/disc	100	100	50	100	100	100	100	100	100	66.6
	Ampicillin 25 Mg/disc	0	0	0	0	0	0	0	0	0	0
	Gentamicin 30 Mg/disc	100	100M	50 50M	100M	100M	100	100	100M	100M	66.7 33.3M
	Vancomycin 10 Mg/disc	100	50	50 50M	100	100	100	100M	100	100	100
	Rifampin 5 Mg/disc	100	100	100	100	100	100	100	100	100	100
	Ciprofloxacin 10 Mg/disc	100M	100M	50 50M	100M	100M	100M	100M	0	100M	33.3 66.6M
	Streptomycin 25 Mg/disc	100M	100M	50M	100M	33.3M	100M	100M	100	50M	33.3 33.3M
	Ceftazidim 10 Mg/disc	0	0	0	0	0	0	0	0	50	0
	Penicillin G 10 Mg/disc	100	50 50M	50 50M	100M	66.7 33,3M	100	100	0	50 50M	66.7 33.3M
	Tetracyclin 10 Mg/disc	66.7M	50M	50 50M	100M	100M	100M	100M	0	100M	100M
	Chloramphinicol 10 Mg/dis	100M	100M	50 50M	100M	100M	100M	100M	0	100M	33.3 66.7M

100% : موجبة للاختبار أو حساسة للمضاد الحيوي ، 0 % : سالبة للاختبار أو مقاومة للمضاد الحيوي ، M : متوسطة الحساسية للمضاد الحيوي ،
** : نمو ضعيف للغزل الهوائي للمجموعة D*

العنقود الرئيس A: ضم أفراد تعود إلى النوع *N. dassonvillei* تميزت بكون الغزل الهوائي عبارة عن خيوط طويلة سميكة غير متفرعة أو متفرعة تفرعات قليلة جدا ويتميز بشكله المتعرج ذو النهايات المتحلزنة في بداية عملية التبوغ ، ثم تصبح الهايفات مستقيمة أو متعرجة تعرج بسيط كما موضح في الصورة رقم (2) ، لا تنمو على الأوساط الزرعية الحاوية على تركيز ملحي 20% NaCl ، التركيز الملحي المثالي للنمو 5% NaCl وتصنف بكونها متحملة لدرجات الملوحة العالية (40، 41).

العنقود الرئيس B: ضم هذا العنقود أفراد تعود إلى النوع *N. lucentensis*، يبدأ الغزل الهوائي بالنمو عندما يكون التركيز الملحي للوسط 10% NaCl فما فوق ويتميز بكونه ذو خيوط طويلة نحيفة غير متفرعة متعرجة قليلا والذي ينقسم بدوره إلى سبورات صغيرة الحجم متطاولة ويتميز الغزل الهوائي بتكوينه لتراكيب تعرف بـــ synnemata كما هو موضح في الصورة رقم (3) ، تنمو بتراكيز ملحية 3-15 % NaCl والتركيز الملحي المثالي للنمو 7 % NaCl وتصنف بكونها متحملة لدرجات الملوحة العالية (42).

العنقود الرئيس C: ضم افراد تعود للنوع *N. aegyptia*، يتميز الغزل الهوائي بكونه ذو خيوط طويلة مستقيمة نوعا ما نحيفة غير متفرعة كما في الصورة رقم (4) ، التركيز الملحي المثالي للنمو 5% NaCl ولا تستطيع النمو بتراكيز ملحية تتجاوز 10 % NaCl وتصنف بكونها محبة لدرجات الملوحة المعتدلة (43).

العنقود الرئيس D: ضم عزلة تعود إلى النوع *N. arvandica* ، مستعمراتها متوسطة الحجم ، جلدية القوام تظهر بلون قهوائي فاتح ، محللة للجيلاتين ، تنمو بتراكيز ملحية 3−15 % NaCl ، التركيز الملحي المثالي للنمو 12 % NaCl ، ولا تنمو بتراكيز ملحية تتجاوز 20 % NaCl وتصنف بكونها متحملة لدرجات الملوحة العالية ، لا تستطيع النمو بتركيز 15 % KCl وتعتبر صفة تشخيصية في تميز أفراد هذا النوع عن بقية أنواع جنس Nocardiopsis (44، 45).

العنقود الرئيس E: ضم أفراد تعود إلى النوع *N. halotolerans*، يمتاز الغزل الهوائي بكونه ذو خيوط طويلة سميكة بشكل متعرج zigzag قبل عملية تكوين السبورات الخارجية ، مكونة للـــ Synnemata كما هو موضح في الصورة رقم (5) ، تنمو بتراكيز ملحية من 0−15 % NaCl في اوساط قاعدية ، وتنمو بصورة مثالية على وسط مستخلص الخميرة بتركيز ملحي 10 % NaCl وعند قيمة pH 8.5 ، لكنها لا تستطيع النمو عند الـــ pH الحامضي 4 وبتركيز ملحي 20 % NaCl وتصنف بكونها متحملة لدرجات الملوحة العالية (37، 46).

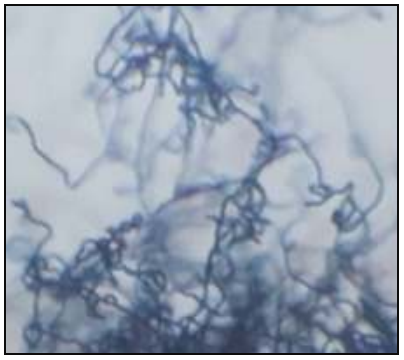
العنقود الرئيس F: ضم عزلة واحدة تعود للنوع *N. xinjiangensis*، موجبة لإختبار الكتاليز والاوكسديز ، لا تستطيع النمو بتركيز ملحي 3 % NaCl ولكنها تستطيع النمو بتركيز ملحي 20 % NaCl وتصنف بكونها محبة لدرجات الملوحة العالية (47، 48).

العنقود الرئيس G: ضم عزلة واحدة تعود للنوع *N. halophila*، الغزل الهوائي متعرج ويتميز بكونه عبارة عن سلسلة من السبورات البيضوية كما في الصورة رقم (6)، تستطيع النمو بتراكيز ملحية عالية تصل إلى 25 % NaCl لذلك صنفت بكونها محبة لدرجات الملوحة العالية (49).

العنقود الرئيس H: ضم عزلة واحدة تعود للنوع *S. rochei*، يمتاز الغزل الهوائي بكونه ضعيف ذو خيوط مستقيمة قصيرة سميكة ومتفرعة كما في الصورة رقم (7) ، تستطيع النمو عند pH 6−12 ، الـــ pH المثالي للنمو 9 ، وتستطيع النمو بتراكيز ملحية 3−10 % NaCl والتركيز المثالي للنمو 7 % NaCl وصنفت بكونها محبة لدرجات الملوحة المعتدلة ، تستطيع النمو فقط بتركيز 7 % كلوريد البوتاسيوم (50، 51).

العنقود الرئيس I: ضم أفراد تعود للنوع *S. albiaxialis*، الغزل الهوائي حلزوني مفتوح كثير التعرج ذو خيوط قصيرة نحيفة غير متفرعة كما في الصورة رقم (8) ، تستطيع النمو بوجود تراكيز ملحية 0−15 % NaCl ، التركيز الملحي المثالي للنمو 7 % NaCl وتصنف بكونها متحملة لدرجات الملوحة العالية (52).

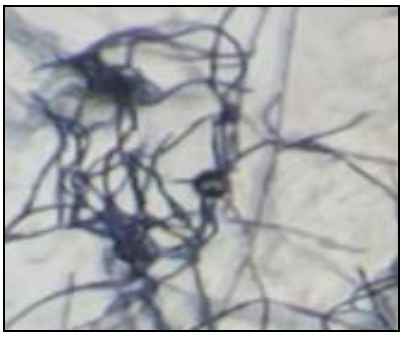
العنقود الرئيس J: ضم أفراد تعود للنوع *S. cacaoi* خيوط الغزل الهوائي عبارة عن حلزون مفتوح يظهر بشكل متعرج كما في الصورة رقم (9) ، تنمو بصورة مثالية على وسط مستخلص الخميرة بظروف قاعدية ، pH المثالي للنمو هو 9 ، وتستطيع النمو بتراكيز ملحية 3−15% NaCl ،التركيز الملحي المثالي للنمو 5 % NaCl وتصنف بكونها محبة لدرجات الملوحة العالية (53).



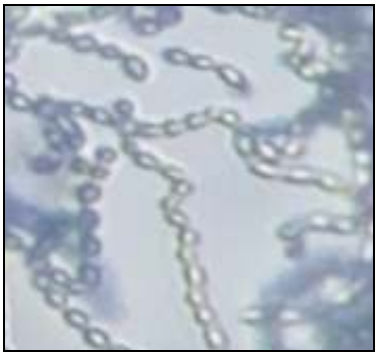
صورة رقم (2): الشكل المتعرج ذو النهايات المتحلزنة للغزل الهوائي لأفراد النوع *N. dassonvillei*

صورة رقم (3): الغزل الهوائي وتركيب الـــ Synnemata في النوع *N. lucentensis*

صورة رقم (4): الشكل المستقيم القليل التعرج للغزل الهوائي لأفراد النوع *N. aegyptia*



صورة رقم (5): الغزل الهوائي وتركيب Synnemata في النوع N. halotolerans



صورة رقم (6): السبورات البيضوية الشكل للنوع **N. halophila**

صورة رقم (7): الشكل المستقيم والمتفرع للغزل الهوائي للنوع **S. rochei**

صورة رقم (8): الشكل الحلزوني المفتوح للغزل الهوائي لأفراد النوع **S. albiaxialis**

صورة رقم (9): الشكل الحلزوني المفتوح للغزل الهوائي لأفراد النوع **S. cacaoi**

المصـــــــــادر

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تقدير الخواص الانتفاخية في طبقات التحميل السطحية باستخدام الطرائق غير المباشرة في مناطق مختـــارة من محافظة البصرة – جنوبي العراق

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الملخص باللغة العربية

تعـــد الترب المنتفخة مشكلة عالمية، توجد في أنحاء مختلفة من العالم وتسبب الكثير من المشاكل الهندسية للأسس وأمان المنشآت المقامة عليها. تهدف الدراسة الحاليـــة إلى التعرف على قابلية الانتفاخ في الترب السطحية الممتدة إلى عمق 4 متر من سطح الأرض والتي تعد طبقات التحميل للمنشآت الهندسية ذات الأسس الضحلة في مناطق مختارة من محافظة البصرة / جنوبي العراق، وتحديد الأسباب المؤدية إلى الانتفاخ في هذه الترب، والمعالجات المقترحة لتلافي الآثار السلبية للانتفاخ. ولتحقيق أهداف الدراسة، تم اختيار 10 مواقع موزعة في مناطق مختارة من المحافظة لحفر عشر مجسات اختبارية بعمق أربعة أمتار لكل منها، وتم إنجاز الفحوصات التصنيفية المتمثلة بالتحليل الحجمي للحبيبات والمحتوى المائي وحدود اتربرغ في 40 نموذجا من الترب المستخرجة من المجسات الاختبارية. استخدمت قيم حد السيولة ومعامل اللدونة في تصنيف ترب منطقة الدراسة على وفق مخطط اللدونة ، واستخدمت نسب المحتوى المائي و الطيني وقيم حد الانكماش ومعامل اللدونة والفاعلية في التربة لتقديرجهد الانتفاخ فيها بحسب تصانيف (Holtz and Gibbs,1956)، و(Lambe,1960)، و(Seed *et al.*,1962).

أظهرت النتائج أن ترب منطقة الدراسة هي من النوع الطيني الغريني والغرين الطيني. وأظهر مخطط اللدونة وجود نموذجين يمكن اعتبارهمـــا أطيان لا عضوية قليلة اللدونة و 29 نموذجا أطيانا لا عضويـــة متوسطة اللدونة و9 نماذج أطيانـــا لا عضوية عاليـــة اللدونة. تعد الترب فـــي مناطق القرنة وحي القادسية والبريهة ذات جهد انتفاخ منخفض، بينما تعد الترب في مناطق كرمة علي وشط العرب والأصمعي وأبو الخـــصيب وشط البصرة والفاو ذات جهد انتفاخ متوسط ، وتعد منطقة القبلة ذات جهد منخفض إلى متوسط.

الكلمات المفتاحية: الترب المنتفخة، ترب البصرة، الخواص التصنيفية

Estimation of Swelling Properties of Superficial Bearing Strata by using Indirect Methods at selected regions in Basrah Governorate / Southern of Iraq

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ABSTRACT

Swelling of soils is considred as a universal problem occured in different parts of the world and causes a lot of engineering problems for the foundations and stability of building. The aim of this study is to show the swelling properties in topsoil extended to 4 meters depth of surfacial bearing strata selected region in Basrah governorate southern of Iraq as a study area ,and the causes leading to swelling in these soils, and suitable treatments are selected in 10 sites distribution in selected areas and ten boreholes were drilled to take soil samples. Accomplished tests of classification test were carried out to calculate the percentage of clay, silt and sand, water content and Atrbergs limits. Liquid limit and plasticity index value were used to classify soils to plasticity chart. The water content, clay ratios, shrinkage limit, plasticity index and activity of the soil are used to estimate the swelling potential of soils according to,(Holtz and Gibbs,1956) ،(Lambe,1960) ،(Seed *et al.*,1962) classifications . Results showed that the soils of the study area are silty clay and clayey. Plasticity chart shows that two samples are classified inorganic clay of low plasticity,29 samples are inorganic clay of medium plasticity and 9 samples are inorganic clays of high plasticity .soils in Qurnah, He-Alqadisiyah, Albreha have low swelling potential, while soils at karmit Ali, Shatt al-Arab, Asma'i, Abu AL- kassib, Shatt al-Basra and Fao have intermediate swelling potential, and alqabla samples is show low – Medium swelling potential.

المقدمـــــــة

تعــد الترب المنتفخة مشكلة عالمية، توجد في أنحاء مختلفة من العالم خصوصا في المناطق الاستوائية. وتسبب الترب الانتفاخية الكثير من المشاكل الهندسية للأسس وأمان المنشآت المقامة عليها. تعرف الترب التي يزداد حجمها أو يقل عند التغير في المحتوى الرطوبي بالترب المنتفخة، وهي غالبا ما تكون بسبب المواد الطينية الموجودة في الترب الطينية، ويرجع أصل هذه الترب للبيئات الترسيبية الصحراوية أو النهرية أو بيئة المياه الضحلة أو بيئة المصبات، وهي بيئات غنية بالأيونات المختلفة التي تساعد في تكوين الترب الانتفاخية. وإذا كانت هذه الترب محصورة فإن الزيادة في الحجم بسبب الانتفاخ تؤدي إلى تكوين ضغط يدعى ضغط الانتفاخ (Swelling pressure) الذي قد يسبب أضرارا تنعكس على الأسس ومن ثم على المنشآت (1). تظهر المعادن الطينية من مجموعة السمكتايت مثل المونتمورلينايت والفرميكولايت والهالوسايت والسيبولايت قابلية عالية على الانتفاخ بسبب امتصاص الماء بين شرائح المعدن، ويمكن أن يصل انتفاخ المونتمورلينايت النقي إلى 15 مرة قدر حجمه وهو جاف مما يؤدي إلى حدوث أضرار كبيرة في المنشآت الهندسية والطرق وبطانة القنوات وغيرها (2).

يصبح وجود الترب الانتفاخية مشكلة إذا تعرضت هذه التربة لتغيرات كبيرة في المحتوى الرطوبي بسبب الأمطار والحرارة والرياح، ويجب أن تكون طبقة التربة المنتفخة ذات سمك كاف لتحدث حركة لإحداث الضرر في سطح الطبقة.

تشهد محافظة البصرة بناء العديد من المشاريع التنموية والخدمية والتعليمية، مما يتطلب دراسة الخصائص الانتفاخية للترب في طبقات التحميل السطحية ومدى تأثيرها في الأسس الضحلة للمنشآت الهندسية بغية إنشاء مشاريع آمنة من مخاطر التصدع والانهيار.

تهدف الدراسة الحاليـــة إلى التعرف على قابلية الانتفاخ في الترب السطحية الممتدة إلى عمق 4 م من سطح الأرض والتي تعد طبقات التحميل للمنشآت الهندسية ذات الأسس الضحلة في مناطق مختارة من محافظة البصرة، وبيان التغاير الأفقي والعمودي في قابلية الانتفاخ على امتداد منطقة الدراسة، وتحديد الأسباب المؤدية إلى الانتفاخ في هذه الترب، والمعالجات المقترحة لتلافي الآثار السلبية للانتفاخ.

المواد وطرق العمـــــل

موقــع ومناخ منطقة الدراســة:

تقع منطقة الدراسة ضمن الحدود الإدارية لمحافظة البصرة/ جنوبي العراق في الجزء الشمالي الغربي من الخليج العربي، وقد تم اختيار عشرة مواقع موزعة في مناطق مختارة من المحافظة، والجدول رقم (1) يبين إحداثياتها نسبة إلى خطوط الطول ودوائر العرض.

جدول رقم (1): إحداثيات مواقع النمذجة في منطقة الدراسة

رقم الموقع	اسم الموقع	خطوط الطول ° ′ ″	دوائر العرض ° ′ ″
S1	القرنة	31 1 34.5	47 25 25.4
S 2	كرمة علي	30 34 56	47 44 37.7
S 3	القادسية	30 30 2.5	44 44 10.1
S 4	شط العرب	30 31 28.1	47 50 43.1
S 5	الأصمعي	30 30 8.9	47 47 31.3
S 6	بريهة	30 30 45.1	47 50 29.4
S7	ابو الخصيب	30 29 2	47 52 17.8
S8	شط البصرة	30 28 35	47 44 39.3
S 9	القبلة	30 28 29.5	47 48 9.3
S 10	الفاو	29 58 43	48 28 26.6

تمتاز مدينة البصرة بالمناخ شبه الاستوائي الحار، الجاف صيفا والبارد الرطب شتاء، وبالتطرف الكبير في درجات الحرارة وارتفاع نسبة الإشعاع الشمسي وقلة الأمطار والرطوبة العالية مقارنة ببقية أجزاء القطر، لمجاورتها للمسطحات المائية وقربها من الخليج العربي (3). وقد بلغ معدل درجات الحرارة الصغرى للسنوات العشر الأخيرة حوالي 19.8 درجة مئوية ومعدل درجات الحرارة العظمي حوالي 34.05 درجة مئوية . تسقط معظم الأمطار في فصلي الشتاء والربيع خلال الفترة من شهر تشرين الثاني لغاية نيسان. وقد بلغ معدل تساقط الأمطار في المدينة 8.95 ملم للسنوات من 2004 – 2014 ومعدل التبخر 39.87 ملم (4)، ونتيجة للمناخ الحار وخاصة في فصل الصيف، فإن معدلات التبخر تزيد على معدلات التساقط مما يعرض الترب السطحية للجفاف طيلة أيام السنة.

تغلب على المنطقة الرياح الشمالية الغربية والجنوبية الشرقية والجنوبية الغربية (5)، كما تهب العواصف الترابية الحاوية على نسب عالية من الترب والغبار ذات الحجوم الدقيقة مصدرها إما تربا محلية أو منقولة من دول الجوار (6).

جيولوجية منطقة الدراسة:

تقع محافظة البصرة على وفق التقسيم الثلاثي للقطاعات التكتونية للعراق ضمن المنطقة غير الالتوائية (Unfolded zone) قطاع سهل الدلتا (7)، كما تقع جيومورفولوجيا ضمن وحدة المسطحات المائية التي تمتاز بوجود بيئات مائية مختلفة منها العذبة والمويلحة والبحرية متمثلة بمناطق شط العرب والأهوار وخور الزبير على التوالي. هي منطقة منبسطة التضاريس يعد شط العرب من أهم الملامح الجيومورفولوجية المؤثرة فيها إذ يقطع أراضيها من الشمال الغربي إلى الجنوب الشرقي ليصب في الخليج العربي حاملا معه تصاريف مياه دجلة والفرات وجزء من رواسب نهر الكارون. تتفرع في منطقة الدراسة انهار عديدة لها تأثير كبير في طبيعة المنطقة الجيومورفولوجية أهمها الجبيله و الرباط و الخندق والعشار والخورة والسراجي (8).

تتمثل ترب منطقة الدراسة بالترسبات التماسكية الحديثة السطحية الممتدة فوق تكوين الحمار والتي تغطي معظم أجزاء الإقليم الشرقي من محافظة البصرة ويصل سمكها إلى سبعة أمتار، وهي مزيج من الطين والغرين النهري الفيضي والمترسب من انهار دجلة والفرات والكارون وشط العرب (9). يسود في هذه الترسبات معادن الكلورايت والالايت والكاؤولينايت الطينية وكمية من السليكا (10).

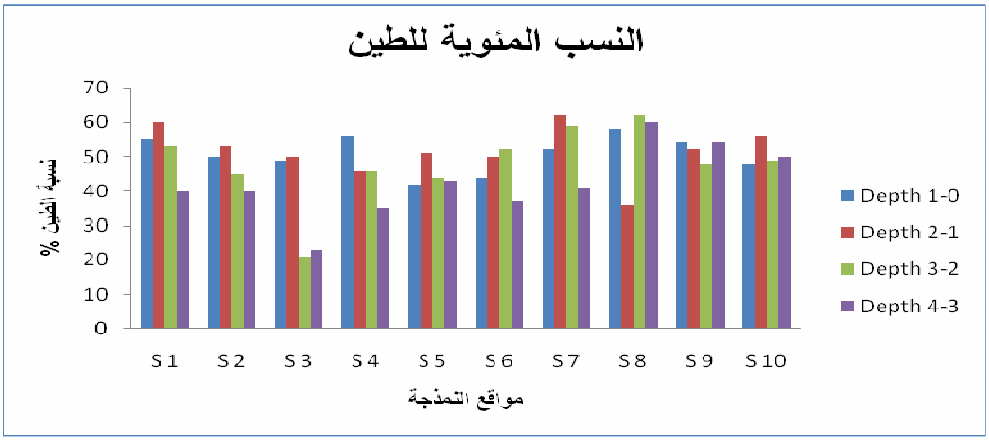
يكون مستوى المياه الجوفية قريبا من السطح ويتراوح عمقها بين 0.15 – 1.9 مترا في عموم المدينة (11)، ويؤثر قرب المياه الجوفية في الأسس الضحلة للمنشآت، وفي سعة التحميل لطبقات التربة العليا، أما المواد العضوية فنسبتها تكون غير مؤثرة وهي أقل من 3 % (12).

طريقة البحث:

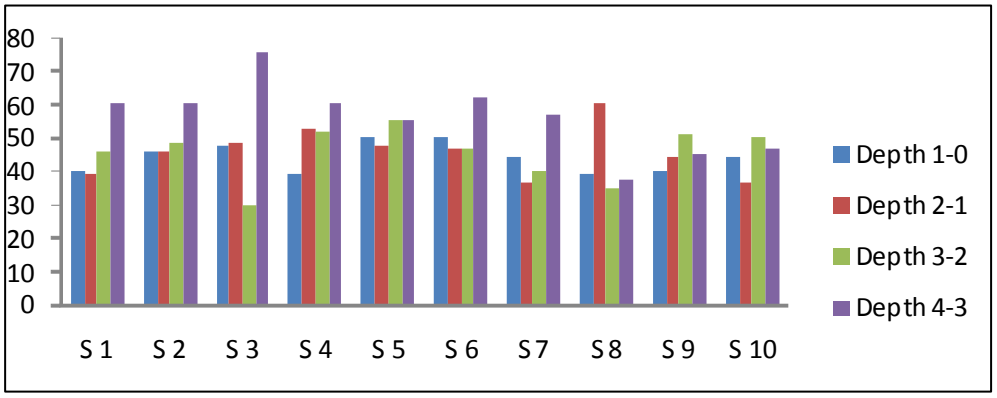
تم الاستعانة بفريق الحفر التابع لمختبر البصرة الإنشائي لحفر عشر مجسات اختبارية بعمق أربعة أمتار لكل منها على وفق المواصفة البريطانية BS 5930:1981 في عشرة مواقع مختارة موزعة في أرجاء محافظة البصرة، وتم إنجاز الفحوصات التصنيفية المتمثلة بالتحليل الحجمي للحبيبات والمحتوى المائي وحدود اتربرغ على وفق المواصفة الأمريكية ASTM D422-63 (13) في 40 نموذجا من الترب المستخرجة من المجسات الاختبارية (جدول 2، أشكال 1، 2، 3) ، واستخدمت قيم حد السيولة ومعامل اللدونة في تصنيف ترب منطقة الدراسة على وفق مخطط اللدونة في (14) وكما هو مبين في شكل رقم (4). واستخدمت نسب المحتوى المائي و الطيني وقيم حد الانكماش ومعامل اللدونة والفاعلية في التربة لتقدير جهد الانتفاخ فيها بحسب تصانيف (15– 18).

جدول رقم (2): الخواص التصنيفية لترب منطقة الدراسة

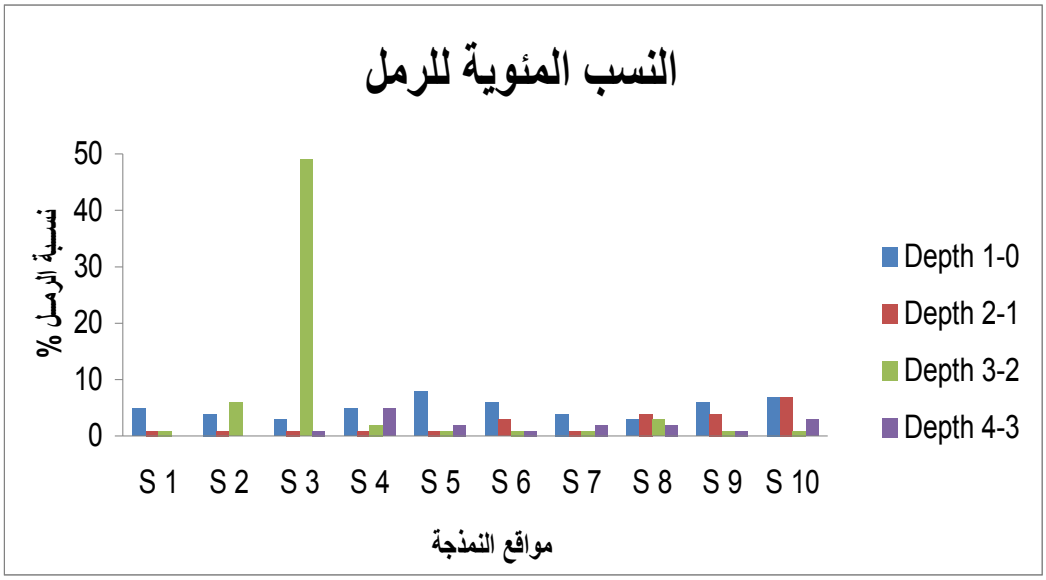
الخواص / الموقع	العمق (م)	التوزيع الحجـمي للحبيبات			المحتوى الـمائي %	حـد السيولـة %	دليـل اللدونة %	حـد الانكماش %	الفـاعلية
		طين%	غرين %	رمل %					
القرنة	1 – 0	53	40	5	20	42	19	8	0.34
	2 – 1	60	39	1	24	40	17	6.5	0.28
	3 – 2	53	46	1	24	40	16	7	0.30
	4 – 3	40	60	0	23	38	17	6.5	0.34
كرمة علي	1 – 0	50	46	4	20	57	28	16.5	0.56
	2 – 1	53	46	1	25	55	25	14	0.47
	3 – 2	45	49	6	25	52	22	9	0.48
	4 – 3	40	60	0	26	45	20	5	0.50
حي القادسية	1 – 0	49	48	3	29	43	18	12.5	0.36
	2 – 1	50	49	1	28	30	12	12.3	0.24
	3 – 2	26	30	49	29	30	12	6.3	0.57
	4 – 3	23	76	1	34	35	14	5	0.60
شط العرب	1 – 0	56	39	5	16	49	24	13.5	0.43
	2 – 1	46	53	1	18	48	23	6.42	0.50
	3 – 2	46	52	2	20	46	22	6.5	0.48
	4 – 3	35	60	5	26	44	20	15	0.57
الأصمعي	1 – 0	42	50	8	16	43	28	15.5	0.66
	2 – 1	51	48	1	16	41	20	7.5	0.39
	3 – 2	44	55	1	17	43	22	11.5	0.50
	4 – 3	43	55	2	18	43	21	10	0.49
بريهة	1 – 0	44	50	6	18	41	18	8	0.41
	2 – 1	50	47	3	15	44	19	7.5	0.38
	3 – 2	52	47	1	22	46	21	7.3	0.40
	4 – 3	37	62	1	20	41	17	10	0.45
أبو الخصيب	1 – 0	52	44	4	29	47	18	14	0.35
	2 – 1	62	37	1	31	53	25	18	0.40
	3 – 2	59	40	1	32	52	23	14.5	0.39
	4 – 3	41	57	2	31	48	22	10	0.54
شط البصرة	1 – 0	58	39	3	21	42	19	8	0.32
	2 – 1	36	60	4	22	44	20	10.2	0.55
	3 – 2	62	35	3	24	52	28	14.5	0.45
	4 – 3	60	38	2	12	54	32	13	0.53
القبلة	1 – 0	54	40	6	18	53	32	11.5	0.59
	2 – 1	52	44	4	15	49	24	9.1	0.46
	3 – 2	48	51	1	22	39	15	7.6	0.31
	4 – 3	54	45	1	20	39	18	7	0.33
الفاو	1 – 0	49	44	7	30	55	26	16.5	0.53
	2 – 1	56	37	7	28	44	19	13	0.34
	3 – 2	49	50	1	30	45	20	8.2	0.41
	4 – 3	50	47	3	32	38	16	7.9	0.32



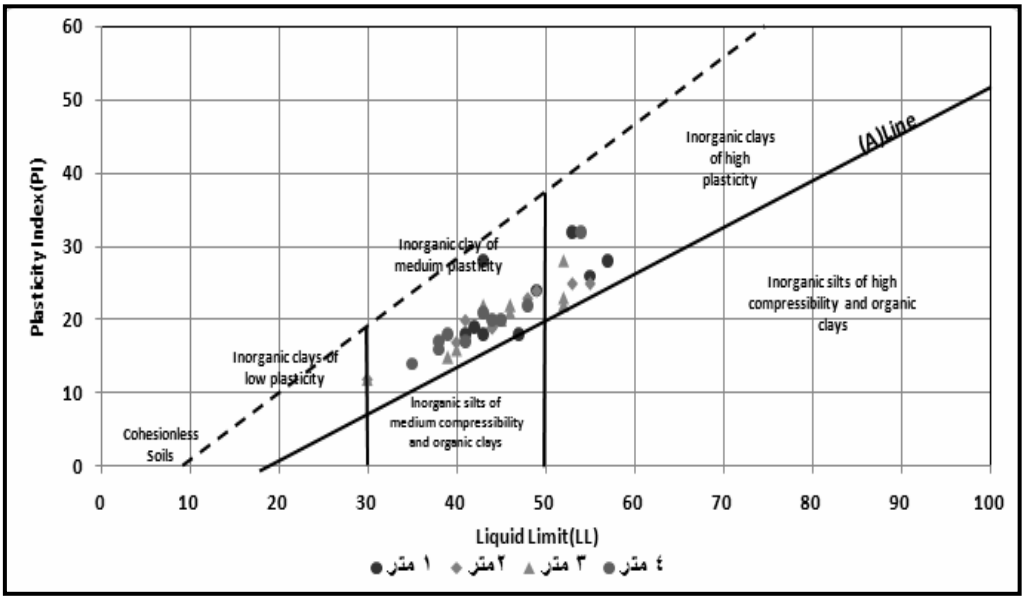
شكل رقم (1): النسب المئوية للطين في ترب منطقة الدراسة



شكل رقم (2): النسب المئوية للغرين في ترب منطقة الدراسة



شكل رقم (3): النسب المئوية للرمل في ترب مواقع الدراسة



شكل رقم (4): تصنيف نماذج ترب منطقة الدراسة حسب مخطط اللدونة في (14)

النتائـــــــــج والمناقشـــــــة

يبين الجدول رقم (2) والأشكال (1، 2، 3) نتائج التوزيع الحجمي للحبيبات في ترب منطقة الدراسة. تظهر النتائج أن نسب الطين تتراوح ما بين 23% في منطقة حي القادسية عند العمق 3- 4 متر إلى 62 % في منطقة شط البصرة عند العمق 2-3 متر وأبو الخصيب عند العمق 1-2 متر وبمعدل 50.8 %، و نسب الغرين ما بين 30% في منطقة حي القادسية عند العمق 2-3 م و 76 % في منطقة حي القادسية عند العمق 3-4 م وبمعدل 47.9 %. تتراوح نسب الرمل ما بين 0 % في منطقتي كرمة علي عند العمق 3-4 م والقرنة عند العمق 3-4 م ايضا 49% في منطقة حي القادسية عند العمق 2–3 م وبمعدل 3.9%.

يلاحظ من نتائج التوزيع الحجمي للحبيبات فيها أن ترب منطقة الدراسة هي من النوع الطين الغريني والغرين الطيني.

أظهرت نتائج فحوص حدود اتربرغ أن حد السيولة يتراوح ما بين 30% في منطقة حي القادسية بالعمقين 1-2 و 2-3 م و57% في منطقة كرمة علي بعمق 0-1 م وبمعدل 44.2 % . يتراوح دليل اللدونة ما بين 12% في منطقة حي القادسية بالعمقين 1-2 م و -3 2 م إلى 32% في منطقة شط البصرة بعمق 3-4 م ومنطقة القبلة بعمق 0-1 م وبمعدل 20.4 %.

كما أظهر مخطط اللدونة المبين في شكل رقم (4) بأن نموذجين تم تصنيفهما أطيانا لا عضوية قليلة اللدونة و 29 نموذجا أطيانا لا عضويـــــة متوسطـــة اللدونة و9 نماذج أطيانـــا لا عضويـــة عالية اللدونة.

حساب الانتفاخ في ترب منطقة الدراسة:

تم حساب جهد الانتفاخ في التربة بطرق غير مباشرة اقترحها عدد من الباحثين ومنها:

1. تصنيف الانتفاخ بحسب (15) والذي اعتمد قيم حد الانكماش في التربة لتقدير جهد الانتفاخ فيها، (جدول رقم 3).

جدول رقم (3): تصنيف الانتفاخ في التربة بحسب (15).

قيم حد الانكماش	جهد الانتفاخ
70-60	عالي جدا
60-40	عالي
40-30	متوسط
30-20	قليل

2. تصنيف الانتفاخ بحسب (16) والذي اعتمد قيم المحتوى المائي في التربة لتقدير جهد الانتفاخ فيها. (جدول رقم 4).

جدول رقم (4): تصنيف الانتفاخ بحسب (16).

المحتوى المائي	جهد الانتفاخ
<14	عالي
20-14	متوسط
>20	قليل

3. تصنيف الانتفاخ بحسب (17)، والذي اعتمـــد قـــيم المحتـــوى الطيني والفاعلية في التربة لتقدير جهد الانتفاخ فيها ، والجدول رقم (5)، يلخص النتائج لاختبار نماذج الدراسة وفقا للطـــرق الثلاثـــة الواردة في (15– 17)، حيث تظهر النتائج أن نموذجا واحدا كان عالي الانتفاخ، و 21 نموذجا متوسط الانتفاخ، فيما كان 18 نموذجا منخفض الانتفاخ.

وبشكل عام، يلاحظ من الجدول رقم (5) أن نماذج التربـــة فـــي مناطق القرنة وحي القادسية والبريهة هي ذات جهد انتفاخ واطئ، بينما تعد الترب في مناطق كرمة علي وشط العرب والأصـــمعي وأبو الخصيب وشط البصرة والفاو ذات جهد انتفاخ متوسط، وتعد منطقة القبلة ذات جهد منخفض – متوسط.

جدول رقم (5): نتائج الانتفاخ لترب منطقة الدراسة

الخواص / الموقع	العمق (م)	انتفاخ حسب تصنيف Holtez &Gibbs (15) الانكماش	الانتفاخ حسب تصنيف Lambe (16)	الانتفاخ حسب تصنيف Seed *et.al.* (17) المحتوى الطيني والفاعلية %	معدل الانتفاخ
القــرنة	1 – 0	متوسط	متوسط	متوسط	منخفض
	2 – 1	منخفض	منخفض	منخفض	
	3 – 2	منخفض	منخفض	منخفض	
	4 - 3	منخفض	منخفض	منخفض	
الكــرمــة	1 – 0	متوسط	متوسط	متوسط	متوسط
	2 – 1	متوسط	منخفض	متوسط	
	3 – 2	متوسط	منخفض	متوسط	
	4 - 3	منخفض	منخفض	منخفض	
حي القادسية	1 – 0	منخفض	منخفض	منخفض	منخفض
	2 – 1	متوسط	منخفض	منخفض	
	3 – 2	منخفض	منخفض	منخفض	
	4 - 3	منخفض	منخفض	منخفض	
شط العرب	1 – 0	متوسط	متوسط	متوسط	متوسط
	2 – 1	متوسط	متوسط	متوسط	
	3 – 2	متوسط	متوسط	متوسط	
	4 – 3	منخفض	منخفض	منخفض	
الأصمعي	1 – 0	متوسط	متوسط	متوسط	متوسط
	2 – 1	متوسط	متوسط	متوسط	
	3 – 2	متوسط	متوسط	متوسط	
	4 - 3	متوسط	متوسط	متوسط	
البريهة	1 - 0	منخفض	منخفض	منخفض	متوسط
	2 - 1	متوسط	منخفض	منخفض	
	3 - 2	متوسط	منخفض	متوسط	
	4 - 3	منخفض	منخفض	منخفض	
أبو الخصيب	1 - 0	متوسط	منخفض	منخفض	متوسط
	2 - 1	متوسط	منخفض	متوسط	
	3 - 2	متوسط	منخفض	متوسط	
	4 - 3	متوسط	منخفض	متوسط	
شط البصرة	1 - 0	متوسط	منخفض	متوسط	متوسط
	2 - 1	منخفض	منخفض	منخفض	
	3 - 2	متوسط	منخفض	متوسط	
	4 - 3	متوسط	منخفض	عالي	
القبلة	1 - 0	متوسط	متوسط	متوسط	منخفض– متوسط
	2 - 1	متوسط	متوسط	متوسط	
	3 - 2	منخفض	منخفض	منخفض	
	4 - 3	منخفض	منخفض	منخفض	
الفاو	1 - 0	متوسط	منخفض	متوسط	متوسط
	2 - 1	متوسط	منخفض	متوسط	
	3 - 2	متوسط	منخفض	متوسط	
	4 - 3	منخفض	منخفض	منخفض	

يؤدي تغير درجات الحرارة إلى تذبذب المحتوى المائي للتربة والذي يؤدي بدوره إلى حدوث تشويه في نظام التربة. تسبب درجات الحرارة العالية في فصل الصيف التي غالبا ما تتعدى قيمها 50 °C انكماشا في الترب التماسكية يؤدي إلى حدوث تشققات في الترب والتي تمتلئ بالماء خلال المواسم المطرية مما يسبب خفض سعة تحميل التربة. كذلك، فإن تغير درجات الحرارة اليومي يعمل على تفكك التربـــــة (19). تؤثر الأمطار في التذبذب الحاصل بالمحتوى الرطوبي للتربة، وتؤدي عملية الغسل إلى زيادة تركيز الأملاح التي تعمل كمادة رابطة في الطبقات السفلى مما يؤدي الى زيادة التماسك فيها, بينما تقل قوة تحمل الطبقات العليا المغسولة (20)، كما تقوم الرياح بدور مساعد مع ارتفاع درجات الحرارة بتبخر ماء التربة من الطبقات السطحية.

تعد غالبية ترب مدينة البصرة منقولة إما بواسطة الرياح أو بواسطة نهري دجلة والفرات ونهر الكارون، تتمثل فيها البيئات الرسوبية النهرية والمياه الضحلة والمصبات التي تساعد في تكوين الترب الانتفاخية كونها غنية بالايونات وذات نسب عالية من الأطيان. وقد لوحظ تباين تباين المحتوى المائي في منطقة الدراسة (جدول رقم 2)، والذي يؤثر بدرجة كبيرة في انتفاخ التربة، حيث تدخل المياه في التركيب البلوري لبعض المعادن الطينية (مجموعة السمكتايت)، مما يؤدي إلى اتساع المسافات بين طبقات هذه المعادن ومن ثم انتفاخها، وإذا وجدت هذه الأطيان تحت أسس المنشآت، فإنها تسلط ضغطا من الأسفل باتجاه الأعلى يؤدي إلى رفع أسس الأبنية والأرصفة والشوارع ويقابلها الانكماش عند فقدان الماء (21).

المعالجات المقترحة:

يمكن معالجة الترب المنتفخة بعدد من الطرائق التـــي اقترحتهـــا البحوث والدراسات وكما يأتي:

1.استبدال الترب المنتفخة بترب أخـــرى ذات خـــصائص غيـــر انتفاخية تحوي نسبا عالية من الحبيبات الخشنة ذات حجم حبيبـــي أكبر من 425 مايكرون مع حدل التربة على وفـــق المواصـــفات القياسية .

2. ترطيب التربة مسبقا لزيادة المحتوى الرطوبي فيها ، وملاحظة التغير الحجمي ثم حدلها ، وإقامة المنشآت عليها إذا كان التغييـــر الحجمي للتربة ضمن الحدود المسموح بها.

3. تؤدي إضافة الكلس للترب الطينية إلى تقليل قابليـــة الاحتفـــاظ بالماء وبالتالي تقليل الانتفاخ .
4. يقل جهد الانتفاخ في التربة عند إضافة الرماد المتطاير (Fly-ash) بسبب الأواصر المتكونة بينه وبين التربة (22)، وبإضـــافة الجبس ورمال الكثبان (23)، وكذلك عند إضافة خليط من رماد قشور الرز والكلس (24).
5. تؤدي إضافة مادة الكيروسين إلى زيادة ثباتية مجاميع التربـــة، إذ تعمل كمادة رابطة لدقائق الطين والكوارتز (25، 26).
6. استخدام عوازل للرطوبة مثل الألواح المعدنيـــة أو الحـــواجز المائية لتقليل تسرب المياه السطحية للتربة وتقليل الانتفاخ عنـــدما تكون المياه الجوفية عميقة .
7. معالجة التربة بحقن بعض المثبتات مثل الجير أو الاسمنت بين فراغات التربة حيث تساعد في تقليل حد السيولة ودليـــل اللدونـــة وبالتالي تقليل الانتفاخ .
8. استخدام الركائز لنقل ثقل المنشأ خلال الترب المنتفخـــة الـــى الترب غير المنتفخة (27).

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إزالة التلوث بالعناصر الثقيلة (الرصاص) من المياه باستخدام مادة حامض التانك المستخلصة من بعض النباتات الطبيعية

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الملخص باللغة العربية

تحظى مسألة إزالة المعادن الثقيلة من المياه العادمة بأهمية كبيرة قصوى لما ينتج عن وجود هذه المعادن من تلويث لموارد المياه بمواد ذات تأثير سمي على مختلف أشكال الحياة.
في هذه الدراسة، تم استخلاص حامض التانك القياسي من قشور الرمان والقلف ومخلفات الشاي الأسود على التوالي ، ولقد تم إجراء التشخيص الكمي والنوعي لحامض التانك المستخلص بواسطة جهاز كروماتوغرافيا السائلة ذات الأداء العالي (HPLC) وباستخدام حامض التانك القياسي.
وقد بينت النتائج الدور المهم الذي يسهم به حامض التانك المستخلص في إزالة التلوث بعنصر الرصاص من المياه الصناعية ، حيث أوضحت النتائج ترسيب الرصاص على شكل (معقد الرصاص – حامض التانك المستخلص) ، وقد بلغت نسبة ترسيب الرصاص على شكل معقد الرصاص – حامض التانك 86.13% لمستخلص قشور الرمان و61.33 % لمستخلص مخلفات الشاي الاسود ، أما مستخلص القلف فقد كانت النسبة 53.05%. وأفضل دالة حامضية (pH) لترسيب الرصاص مع حامض التانك هو 7.0. وتم تشخيص معقد حامض التانك مع الرصاص باستخدام طيف الأشعة تحت الحمراء (FTIR) بالمقارنة مع حامض التانك القياسي وملح الرصاص . وكانت النسبة المئوية لحامض التانك المستخلص من قشور الرمان والقلف والمخلفات الشاي الاسود هي (41.78 ، 35.23 ، 43.1) % على التوالي.

الكلمات المفتاحية: حامض التانك ، كروماتوغرافيا الفصل السائلة ذات الاداء العالي HPLC ، نترات الرصاص

Removal of pollution with heavy metals (lead) from water by using extracting tannic acid from some plants

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ABSTRACT

The issue of removal of heavy metals from wastewater had attracted a great concern due to their impacts as pollutants for water resources by toxic materials that harm several life patterns.
In this study, extraction of Tannic Acid from husks of pomegranate, bark and remnants of black tea were achieved. The quantitative and qualitative diagnoses were done for the extracted acid by HPLC apparatus.
Results showed the important role of Tannic acid in removing the pollution of lead our from the industrial water.

المقدمـــــــة

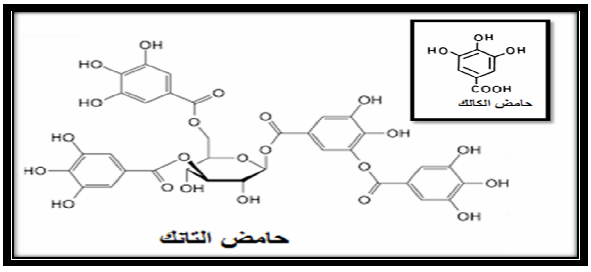
يعرف التلوث البيئي بأنه التغير في الصفات الكيميائية أو الفيزيائية أو الحيوية للبيئة، ويحدث بفعل انتقال الملوثات من مصادرها المختلفة بكميات مختلفة مسببة الاضرار الصحية والاقتصادية للإنسان وللكائنات الحية الأخرى (1).

تعد العناصر الثقيلة من الملوثات البيئية الخطرة وتكمن خطورتها في صفتها التراكمية في أجسام الكائنات الحية، ويحتاج الإنسان والحيوان لنسبة معينة من هذه العناصر التي قد يحصل على جزء منها عن طريق النباتات والسلسلة الغذائية (2)، لذلك فإن ارتفاع تراكيز هذه العناصر في النباتات عن الحدود المسموح بها يعرض حياة المستهلك للخطر، وتأتي هذه الزيادة في التراكيز لأسباب تعود إلى عوامل جيولوجية للتربة، أو نتيجة الاستخدام المفرط للأسمدة الكيمياوية والمبيدات الزراعية، وفي أغلب الأحيان تأتي هذه الزيادة نتيجة الري بالمياه الملوثة بمخلفات المعامل والمصانع والمبازل، إضافة لمخلفات الصرف الصحي (3).

يعد الرصاص من العناصر الخطرة التي تنتقل أحيانا من النبات إلى جسم المستهلك من إنسان أو حيوان عن طريق السلسلة الغذائية، وتكمن خطورته بصفته التراكمية، إذ إنه يسبب أضرارا فسيولوجية مثل التخلف العقلي والقصور في بعض الوظائف الحيوية للإنسان (4)، وبالرغم من خطورته ، إلا أن تأثيراته السمية لم تحظ بالاهتمام البحثي إلا في المائة سنة الأخيرة (5)، ويوجد في الطبيعة إما على شكل الرصاص اللاعضوي ويكون بشكل أملاح أو أكاسيد، أو بشكل الرصاص العضوي (6).

إن حامض التانك المستخلص من قشور الرمان والقلف ومخلفات الشاي الأسود يعتبر المادة الأساسية في تطبيقات بحثنا هذا من خلال قدرته على تكوين المعقدات مع أيونات العناصر المختلفة ومنها الرصاص، ولطريقة استخلاصه أهمية كبيرة في معالجة المياه الصناعية (7، 8)، ولاسيما المركبات العضوية والسامة والأصباغ/ التي لا يمكن فصلها بطرق التصفية كالترشيح والتعقيم، وحامض التانك يطلق عليه بالعفص الذي يتكون من استرات حامض الكالك مع نواة السكر السداسي (الكلوكوز)، ويوجد في العديد من النباتات بأجزائها المختلفة ، ويتميز حامض التانك بفعاليته المضادة للأكسدة والميكروبات والفيروسات ، ويتراوح لونه بين الأصفر إلى البني الفاتح . والوحدة البنائية لحامض التانك هي حامض الكالك (9).

لذا أرتأت الدراسة طريقة استخلاص بسيطة من مواد متوفرة تلقى كمخلفات تكون ذات كفاءة عالية وتكلفة قليلة لاستخدامها في إصلاح النظم المائية الملوثة نتيجة الاستخدام غير الصحيح والمعالجة القليلة. والشكل رقم (1) يوضح التركيب الكيميائي لحامض التانك .



شكل رقم (1): التركيب الكيميائي لحامض التانك

المواد وطرق العمل

الأجهزة المستخددمة:

1. جهاز الاشعة فوق البنفسجية – المرئية JAPAN 1800–Shimadzu recording Spectrophotometer UV، والمزود بمصباح الديتريوم للتشغيل في المنطقة الفوق البنفسجية (380–190) نانوميتر ومصباح التنغستن في المنطقة المرئية ولغاية 1100 نانوميتر باستعمال خلية مصنوعة من الكوارتز سعة (5 مل).
2. مطياف الأشعة تحت الحمراء لأنتقالات فورير (FTIR) من شركة Spectrolab Abbmb3000 .
3. جهاز الفصل الكروماتوكرافي للسائل العالي الاداء (HPLC) Shimadzu , JAPAN.
4. جهاز قياس التوصيلية Conductivity meter نوع WTW.
5. جهاز قياس الدالة الحامضية pH–meter نوع WTW .
6. مسخن كهربائي مع مازج مغناطيسي Hotplate stirrer model L –(81 LABINCO BV).
7. فرن كهربائي muffle furace.
8. طاحونة كهربائية مع ادوات زجاجية مختبرية مختلفة.

المواد المستخدمة:

1. حامض التانك القياسي المجهز من شركة BDH (poole,UK) نقاوة اكثرمن 99%.
2. ملح نترات الرصاص من شركة فلوكا.
3. هيدروكسيد الصوديوم (من شركة فلوكا).
4. حامض الهيدروكلوريك (من شركة هزارد) بنسبة 36%.

طرائق العمل:

تم الاعتماد على طريقة (10) مع بعض التحوير في نسب التراكيز والقياسات.

1– تهيئة النماذج النباتية:

تم اخذ ثلاثة انواع من النباتات (قشور الرمان ، القلف ، ومخلفات الشاي الاسود) ، تم غسل قشور الرمان والقلف على حدة وجففت بشكل جيد ، وبعد ذلك تم تكسيرها الى قطع صغيرة لتسهيل طحنها بشكل جيد لتكون مسحوق متجانس وحُفظت في حاويات نظيفة لحين الاستخدام.

2– عملية الاستخلاص:

للحصول على المستخلص المائي الساخن لقشور الرمان ومخلفات الشاي الاسود والقلف ، وُزنت 10 غرام من كل المواد المذكورة ووُضعت في دورق مخروطي حاوي على قطعة مغناطيسية لغرض التحريك والمزج وأُضيف لها 200 ملليلتر من الماء المقطر،أُجريت عملية التسخين وبدرجة 70 مئوي ولمدة ثلاث ساعات ، وبنفس الظروف تم استخلاص حامض التانك من تلك المواد النباتية .

تم تبريد المحلول لدرجة حرارة الغرفة ورشحت المحاليل للتخلص من المواد العالقة الغير ذائبة بأستخدام أوراق الترشيح.ومن ثم تشخيص مستخلصات حامض التانك من المواد اعلاه باستخدام تقنية كروموتكرافيا السائل عالي الاداء HPLC ومقارنة العمل مع حامض التانك القياسي.

وجرت عملية التشخيص باستخدام الظروف التشغيلية التي أعطت افضل نتائج وكانت هي الظروف المثلى للعمل بهذه التقنية بعد ان تم تهيئة النموذج السائل بأستخدام الماكروفلتر (0.45 مايكروليتر) لفلترة المستخلص النباتي ورُكز المحلول عشر مرات ثم أُضيف اليه 1 ملليلتر من الميثانول الذي نقاوته 99 % والمأخوذ من نفس الطور المتحرك ، رُج لغرض مجانسته قبل حقنه في الجهاز حيث حُقن 20 مايكروليتر من المحلول القياسي على عمود الفصل وباستخدام الظروف التشغيلية التالية تم التشخيص والتقدير الكمي للحامض المستخلص وهي:

1. الطور المتحرك (Mobile Phase) حيث كانت نسبة كحول الميثانول 30% الى الماء اللايوني 70%.
2. تم العمل بدالة حامضية pH تساوي 6.5.
3. معدل الجريان 1 مليلتر لكل دقيقة.
4. درجة الحرارة حيث اجريت بدرجة حرارة الغرفة.
5. استخدام عمود الفصل من نوع ODS_{C18} $_{5\mu m}$ (العرض 4.6 ملليمتر ، الطول 250 ملليمتر).
6. الطول الموجي المستخدم 265 نانوميتر.
7. المكشاف هو مكشاف الاشعة فوق البنفسجية– المرئية.

3– تحضير المحاليل:

حُضر محلول الخزن من حامض التانك القياسي بتركيز (5 ppm) ومنه حُضرت سلسلة من المحاليل ذات التراكيز المختلفة لبناء منحني المعايرة لحامض التانك القياسي.

4– قياس التوصيلية :

أجريت قياسات التوصيلية باستخدام محلول ملح نترات الرصاص ومعاملته مع المحلول الحامضي المستخلص من المواد النباتية حيث تم اضافته بشكل حجوم متساوية تصاعديا لغرض تحديد قيم التوصيلية والدالة الحامضية عند كل اضافة لحين الوصول الى قيمة توصيلية ثابتة . رُشحت المحاليل وجُففت الرواسب لغرض اجراء الحسابات الوزنية للمعقد الناتج . واجريت المقارنة مع حامض التانك القياسي.

5– قياس الامتصاصية :

باستخدام جهاز المطياف الضوئي تم ايجاد الأمتصاصية لمعقدات حامض التانك القياسي حيث أخذ تركيز $1x10^{-4}$ مولاري من الراسب المتكون من تجارب قياس التوصيلية لمعقد التانك مع ملح نترات الرصاص كمحلول خزن ومنه أخذت سلسلة من التراكيز لغرض اجراء منحني معايرة قياسي للمعقد المذكور ، وتم قياس الامتصاصية لكل تركيز.

النتائـــــــج والمناقشـــــة

دراسة الظروف المثلى:

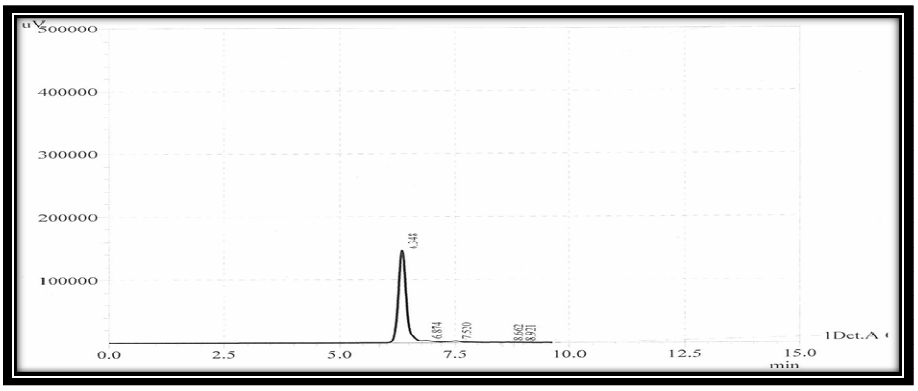
تم حقن 20 مايكروليتر من النموذج إلى العمود عند كل تحليل يتم إجراؤه.

وباختيار الطول الموجي : تم تحضير 5 مايكروليتر /ملليليتر من حامض التانك القياسي باستخدام الماء كمذيب وأخذ طيف . الحامض في المدى 190–400 نانوميتر مقابل المحلول الصوري الذي يمثل المذيب المستخدم إذا أعطى حامض التانك أعلى امتصاص له الطول الموجي 265 نانوميتر .

اما اختيار مكونات الطور المتحرك : فقد تم استخدام بعض المذيبات وبنسب مختلفة وذلك من خلال حقن 20 مايكروليتر من المحلول القياسي لحامض التانك بتركيز 5 مايكروغرام / ملليليتر وبمعدل سرعة جريان 1 ملليلتر .دقيقة وتم القياسي التحليلي عند طول موجي 265 نانومتر في درجة حرارة الغرفة .لقد أُجريت عملية التقدير الكمي والنوعي للحامض المستخلص بظروف تشغيلية مختلفة وتم فصل الحامض بوقت اسرع ألا ان عملية التشخيص والتقدير الكمي بهذه الظروف لم تحقق الفائدة اللازمة منها بالرغم من سرعة الفصل والتشخيص حيث كانت هذه الظروف ليست ذات جدوى من الناحية الاقتصادية في حالة استخدام الطور المتحرك بنسبة اعلى وهي 70% بدلا من 30% . أذ يظهر الكروماتوغرام قمة واضحة بتناظر جيد وبزمن احتجاز مقداره 6.348 دقيقة، ولدراسة تأثير وسط التحليل pH :تم استخدام ثلاثة أوساط من الدالة الحامضية (حامضية– متعادل – قاعدي) كما في الجدول رقم (1) ، لذا كانت الظروف التشغيلية المثبته في طريقة العمل هي الظروف المثلى (شكل رقم 2).

جدول رقم (1) : الدالة الحامضية والطور المتحرك وزمن الاحتجاز

ت	الطور المتحرك	الدالة الحامضية pH	زمن الاحتجاز t_R بالدقيقة
1	ميثانول :الماء اللا أيوني مع فوسفات البوتاسيوم الحامضية KH_2PO_4	5.0	6.025
2	ميثانول :الماء اللا أيوني مع خلات الامونيوم	6.9	6.314
3	ميثانول :الماء اللا أيوني مع فوسفات البوتاسيوم الحامضية KH_2PO_4	8.5	7.563

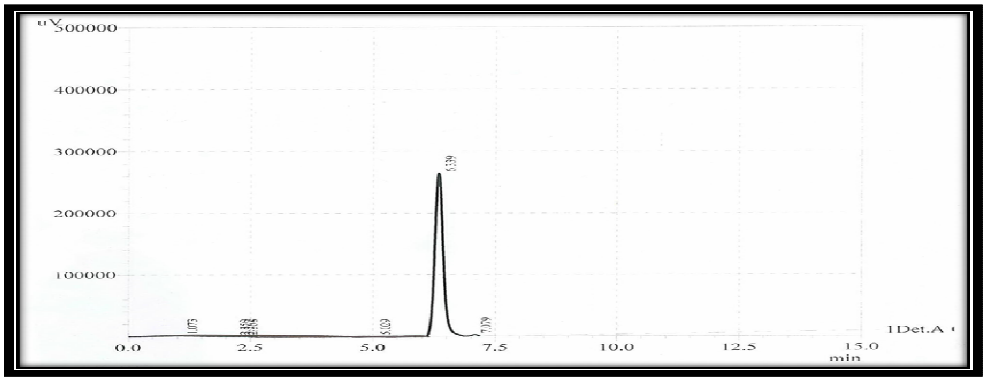


شكل رقم (2) : كرماتوغرام حامض التانك القياسي باستخدام الظروف المثلى

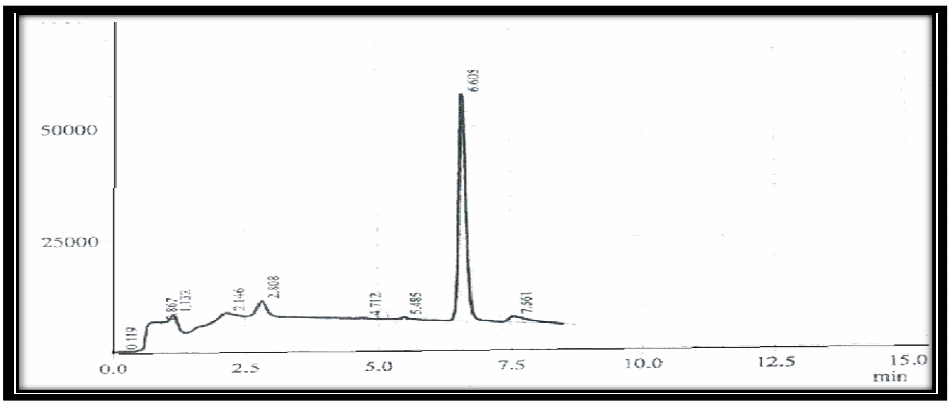
أجريت القياسات ايضا باستخدام جهاز الكروماتوغرافيا العالي الاداء (HPLC) للمستخلصات بالمقارنة مع حامض التانك القياسي . والأشكال (3–5) توضح القياس بجهاز HPLC لحامض التانك لمستخلص قشور الرمان، ومخلفات الشاي الأسود والقلف على التوالي. وقد تم حساب تراكيز حامض التانك المستخلص من المواد النباتية المذكورة من خلال معادلة الخط المستقيم للمنحني القياسي لحامض التانك ،والجدول رقم (2) يشير الى التراكيز والنسب المئوية للمستخلصات من النباتات مقارنة بحامض التانك القياسي.

جدول رقم (2) :التركيز والنسبة المئوية لكل من مستخلصات حامض التانك

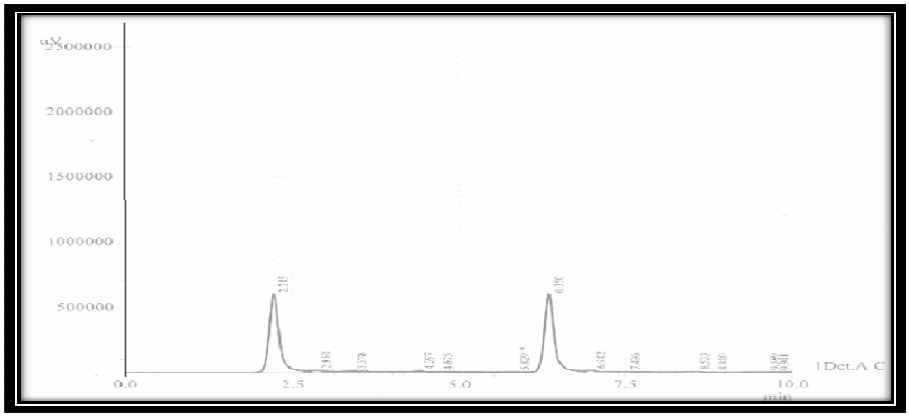
	المحلول القياسي	مستخلص قشور الرمان	مستخلص الشاي الاسود	مستخلص القلف
التركيز (PPM)	0.1	0.04	0.04	0.035
النسبة المئوية (%)	92	41.7	43.1	35.23



شكل رقم (3): كروماتوغرام حامض التانك المستخلص من قشور الرمان بتقنية جهاز الكروماتوغرافيا العالي الاداء (HPLC)



شكل رقم (4): كروماتوغرام حامض التانك المستخلص لحامض التانك المستخلص مخلفات الشاي الاسود بتقنية الكروماتوغرافيا العالي الاداء (HPLC)



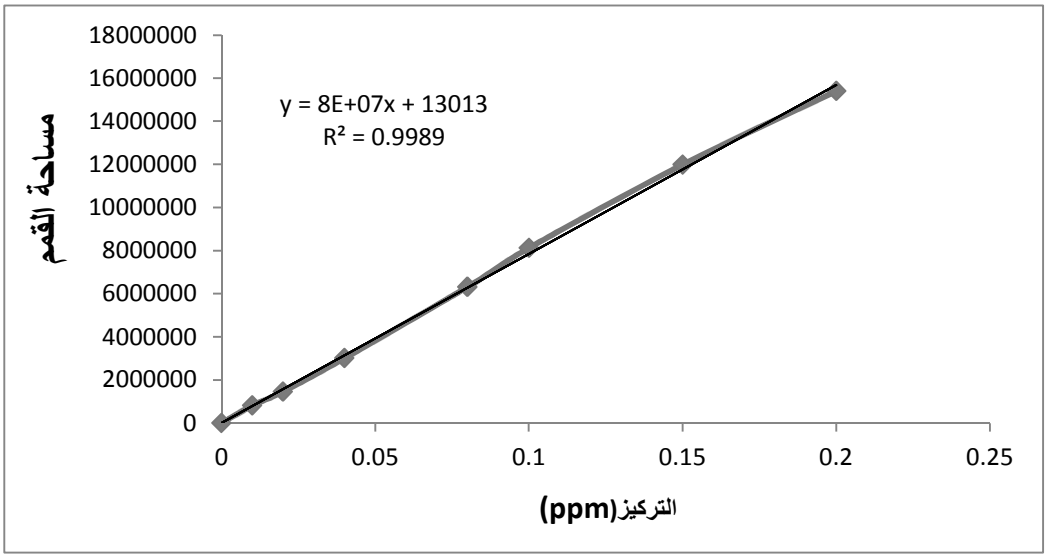
شكل رقم (5): كروماتوغرام حامض التانك المستخلص لحامض التانك لمستخلص القلف بتقنية الكروماتوغرافيا العالي الاداء (HPLC)

وفي دراسة سابقة (11)، تم قياس المركبات الفينولية باستخدام جهاز الكروماتوغرافيا العالي الأداء (HPLC) وكانت متوافقة مع نتائج الدراسة الحالية تقريباً لحامض التانك، والهدف من قياسات هذا الجهاز هو تحديد محتوى حامض التانك في المستخلصات النباتية، حيث تبين أن نسبة حامض التانك في مخلفات الشاي الأسود هي الأعلى مقارنة بالمستخلصات الأخرى (جدول رقم 3).

والشكل رقم (6) يوضح منحني المعايرة لحامض التانك القياسي، كما يوضح الجدول رقم (4) بعض الخواص البصرية المدروسة لمنحنى المعايرة لحامض التانك القياسي.

جدول رقم (3) :قيم مساحة القمة المقابلة لتراكيز المحاليل القياسية لحامض التانك

التركيز (ppm)	معدل مساحة القمة	الانحراف القياسي النسبي x10-^{4}(RSD)	الخطأ النسبي المئوي E_{rel}	الموجود(ppm)	نسبة الاسترداد %
0.00	0.00				
0.01	812250	3.01	2.0	0.0098	98
0.02	1459786	2.98	1.5	0.0197	98.5
0.04	3011768	2.53	1.0	0.0396	99
0.08	6312378	3.097	1.25	0.0790	98.75
0.1	8122509	2.462	3.0	0.097	97
0.15	11984564	2.38	2.66	0.146	98
0.2	15399673	2.35	1.0	0.198	99



شكل رقم (6): المنحنى القياسي لحامض التانك

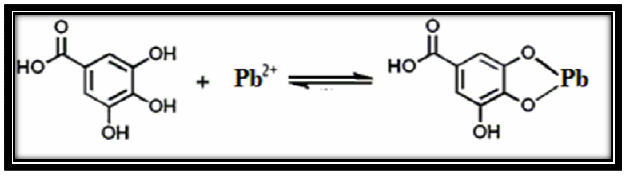
جدول رقم (4) : الخواص البصرية لمنحني المعايرة لحامض التانك القياسي

الخواص البصرية	القيم
اللون	أصفر فاتح جدا
الطول الموجي (نانومتر)	265
حدود قانون بير(مايكروغرام /ملليلتر)	0.01- 0.2
الامتصاصية المولارية(لتر/ مول .سم)	$1.360x10^{14}$
معامل الارتباط (r)	0.9994
حساسية ساندل (مايكروغرام / سم ^{2})	$2.126\ x10^{-5}$
الميل	$8.0\ x10^{6}$
نقطة التقاطع	13013
معادلة الاسترجاع(Y=mx+C)	$8*10^{6}x+13013$
معامل الاسترجاع (r^2)	0.9989
معامل التنوع (%)	99.89
حد الكشف (مايكروغرام/ملليلتر)	$9.675\ x\ 10^{-8}$
حد الكم(ملغرام /ملليلتر)	$3.225\ x\ 10^{-7}$
معدل الاسترداد(%)	98.3

التطبيـــــق

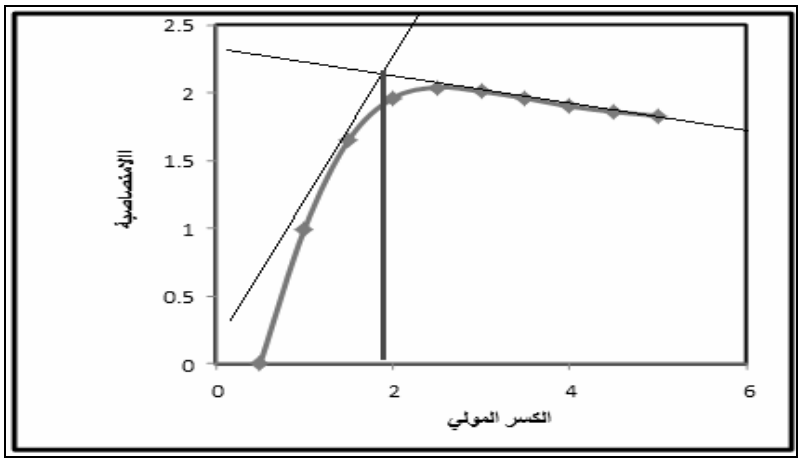
باستخدام نترات الرصاص وتفاعلها مع حامض التانك المستخلص في خطوة (2) من طرق العمل ، تمت إضافة أحجام متساوية من حامض التانك المستخلص إلى نترات الرصاص المحضرة بتركيز (20000 ppm) . وأجري التطبيق على الأنواع الثلاثة من المخلفات النباتية وتم عمل المقارنة مع حامض التانك كمحلول قياسي . وباستخدام جهاز قياس الدالة الحامضية والتوصيلية والمطياف الضوئي وجهاز مطياف الأشعة تحت الحمراء، بالإضافة إلى استخدام جهاز الفصل الكروماتوغرافي ذو الاداء العالي، تم دراسة التطبيق لإزالة التلوث بأملاح الرصاص من المياه الصناعية .

إن ارتباط املاح الرصاص مع حامض التانك يكون عن طريق وحدات حامض الكالك كما هو موضح بالمعادلة الكيميائية التالية:



شكل رقم (7): معادلة تكوين المعقد الرصاص مع حامض التانك

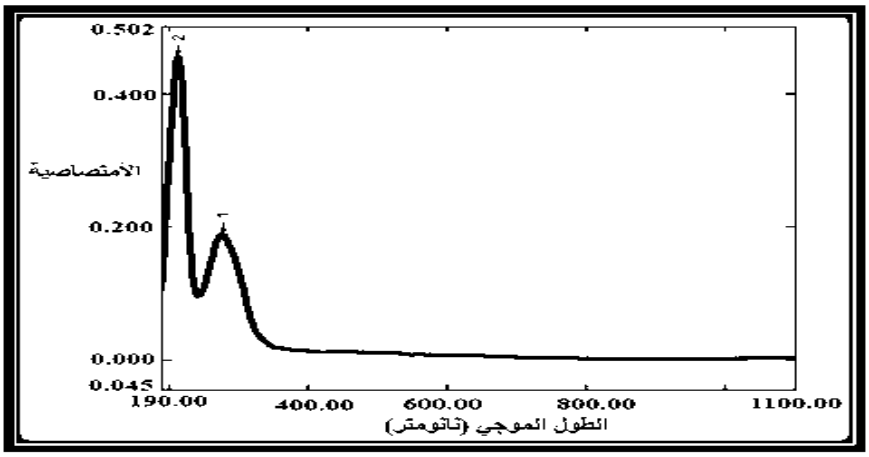
وبتطبيق معادلة النسبة المولية (mole ratio) يتم تحديد عدد جزيئات حامض التانك كمخلب يرتبط بعنصر الرصاص كأيون مركزي . والشكل رقم (8) يوضح النسبة المولية مقابل الامتصاصية للمعقد.



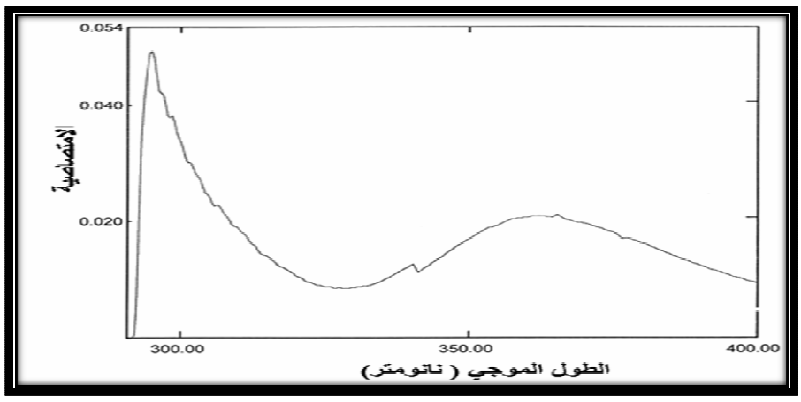
شكل رقم (8): النسبة المولية مقابل الامتصاصية للمعقد

يتبين من الشكل رقم (9) بعد رسم المماسين ورسم العمود من نقطة التقاطع ظهور الكسر المولي مساويا 2.0 مما يدل على أن عدد جزيئات الحامض المرتبطة بأيون الرصاص كانت بنسبة 1:2.

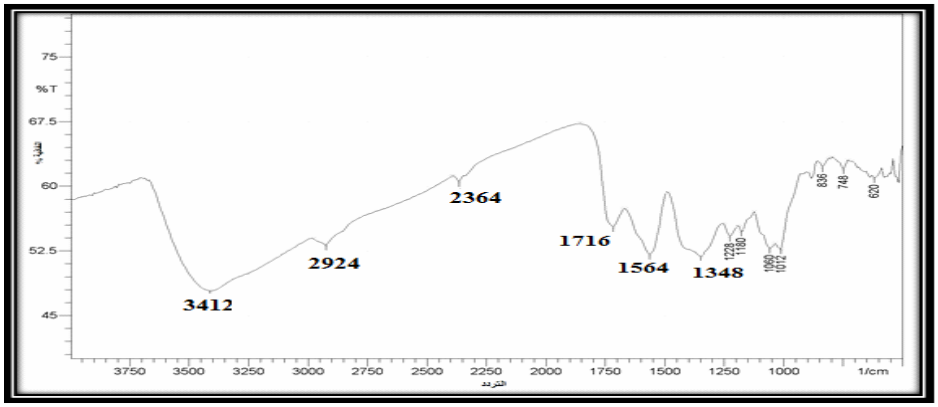
من خلال قياسات المطياف الضوئي ظهرت النتائج التي نوضحها بالاشكال التالية لحامض التانك القياسي وبعد تفاعله مع ملح نترات الرصاص (أشكال أرقام 9– 18).



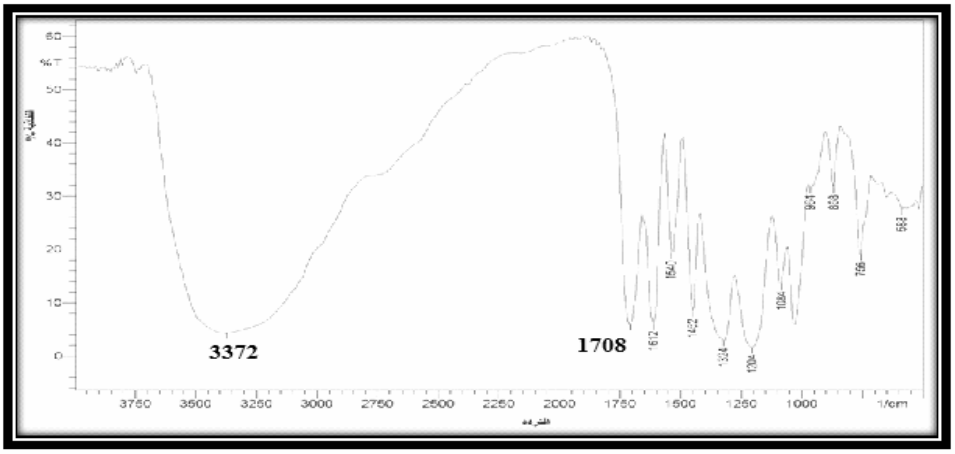
شكل رقم (9): الامتصاصية لحامض التانك القياسي (ظهور أعلى حزمة امتصاص لحامض التانك القياسي كانت 0.5 وعند طول موجي 200 نانوميتر)



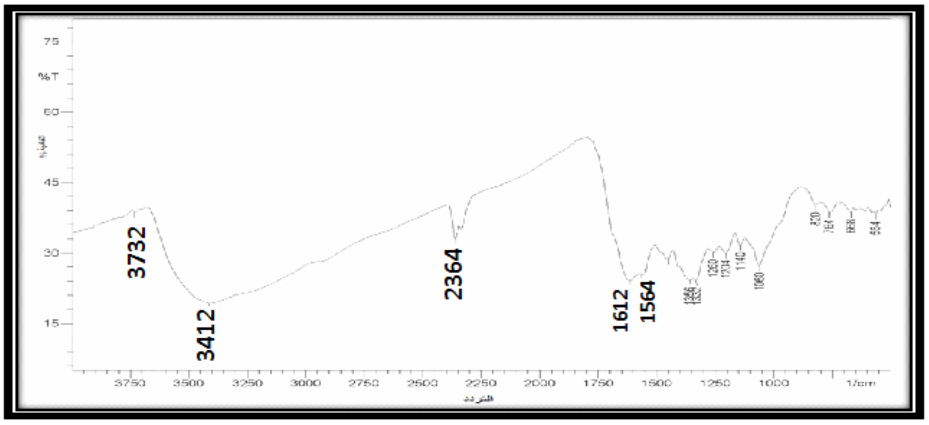
شكل رقم (10): الامتصاصية لملح نترات الرصاص مع حامض التانك القياسي (ظهور اعلى قمة امتصاص لمعقد نترات الرصاص مع حامض التانك كانت 0.05عند الطول الموجي 294 نانوميتر)



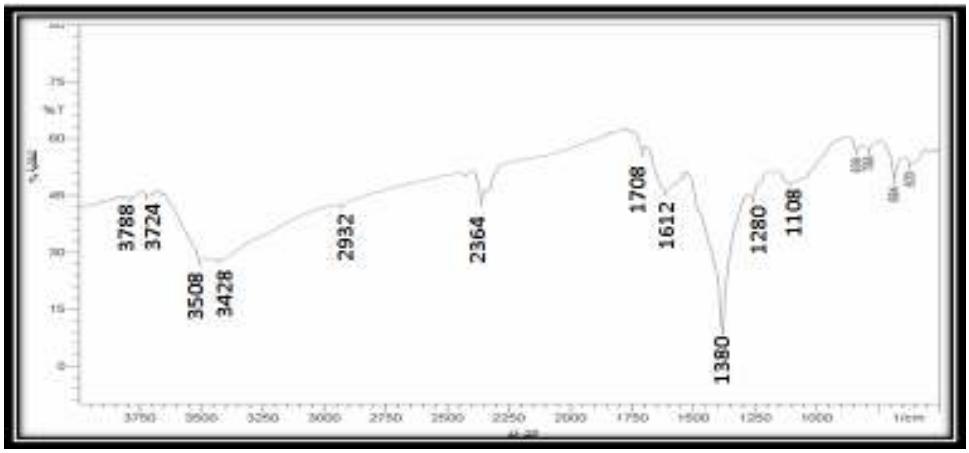
شكل رقم (11): طيف الاشعة تحت الحمراء لمعقد نترات الرصاص مع حامض التانك المستخلص من قشور الرمان



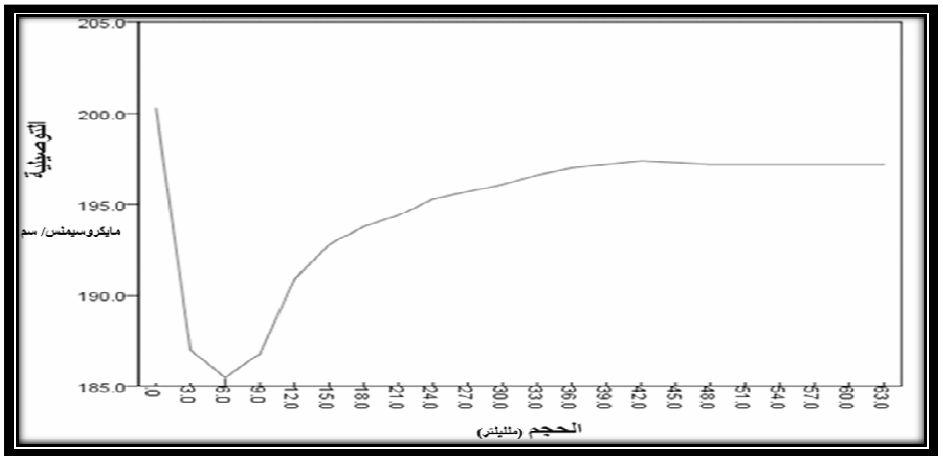
شكل رقم (12): طيف الاشعة تحت الحمراء لحامض التانك القياسي



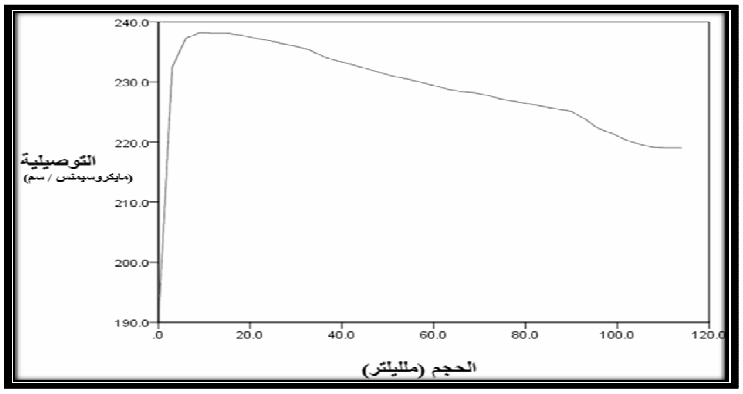
شكل رقم (13): طيف الاشعة تحت الحمراء لمعقد حامض التانك المستخلص من نفايات الشاي



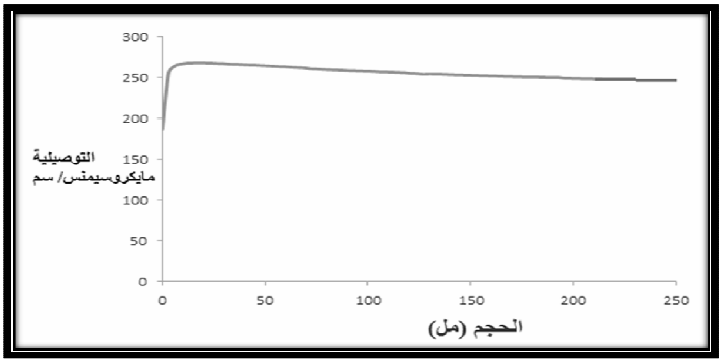
شكل رقم (14): طيف الأشعة تحت الحمراء لمعقد نترات الرصاص مع حامض التانك المستخلص من القلف



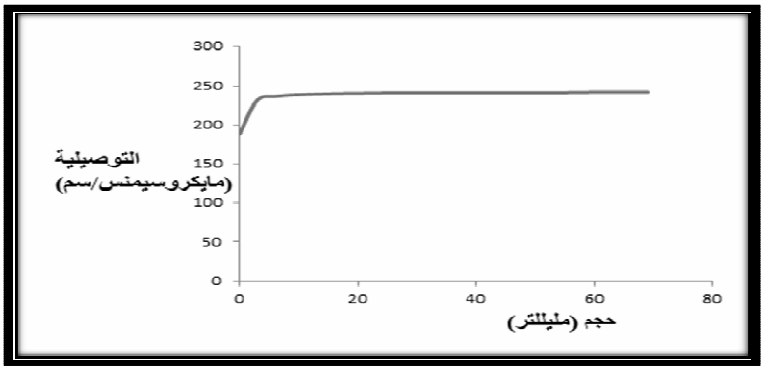
شكل رقم (15): التوصيلية لملح نترات الرصاص مع حجوم حامض التانك المستخلص من القلف



شكل رقم (16): التوصيلية لملح نترات الرصاص مع حامض التانك لمستخلص مخلفات الشاي الاسود



شكل رقم (17): التوصيلية لملح نترات الرصاص مع حامض التانك لمستخلص قشور الرمان



شكل رقم (18): التوصيلية لملح نترات الرصاص مع حامض التانك

تم دراسة قياس امتصاصية المعقد بين الرصاص ومستخلص حامض التانك للمواد النباتية باستخدام المطياف الضوئي للاشعة فوق البنفسجية – المرئية وأغلب البحوث درست عنصر الحديد (12، 13) مع هذه المستخلصات والتي لا تتوافق نتائجها مع الدراسة الحالية.

وقياسات مطياف الاشعة تحت الحمراء بينت وجود الاختلاف بالمجاميع الفعالة مابين محلول حامض التانك القياسي ومحلول المعقد بعد الارتباط مع المستخلصات.

أظهر الشكل رقم (11) طيف الأشعة تحت الحمراء (FTIR) لمعقد نترات الرصاص مع حامض التانك المستخلص من قشور الرمان، حيث يلاحظ من الشكل صغر قمة OH– عند العدد الموجي 3500 سم$^{-1}$ مما يدل على اتحاد مجموعة –OH مع عنصر الرصاص واستحداث قمة واضحة عند العدد الموجي 3100سم$^{-1}$ تمثل CH.

يبين الشكل رقم (12) ظهور أعلى قمة امتصاص عند العدد الموجي 3500 سم$^{-1}$ تعود لمجموعة OH – وهي مجموعة فعالة في حامض التانك والمسؤولة عن الارتباط بأيونات العناصر الأخرى، كما بين الشكل رقم (13) طيف الأشعة تحت الحمراء لمعقد حامض التانك المستخلص من نفايات الشاي وأظهرت المجموعة الفعالة OH– عددا موجيا عند 3412 سم $^{-1}$.

ثم يأتي شكل رقم (14) موضحا طيف الأشعة تحت الحمراء (FTIR) لمعقد نترات الرصاص مع حامض التانك المستخلص من القلف، حيث تظهر القمة عند العدد الموجي 3504 سم $^{-1}$ دلالة على وجود ارتباط ذرة O من OH– مع الرصاص لتكوين المعقد، وظهور قمة واضحة 2932 سم $^{-1}$ تدل على CH_2- ، أما القمة CO– فقد ظهرت عند 1708 سم $^{-1}$.

من خلال قياسات مطياف الأشعة تحت الحمراء، توافقت النتائج مع دراسة (14).

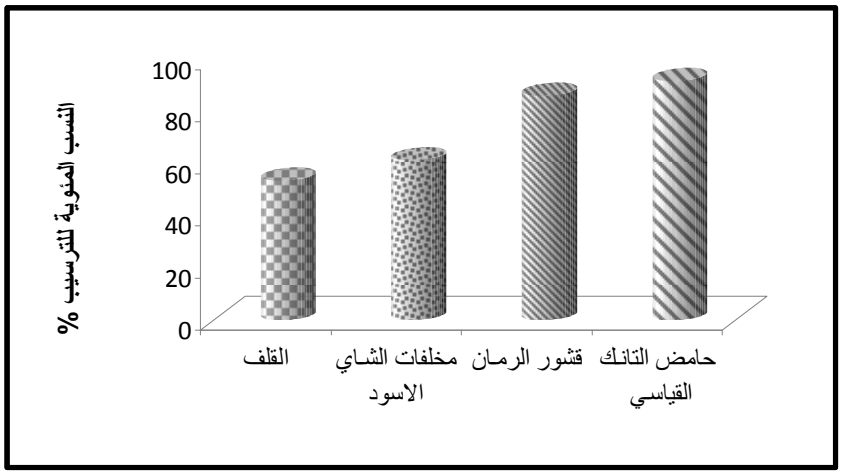
أما قياسات التوصيلية مقابل حجوم حامض التانك المستخلص فقد أظهرت الاختلاف ما بين المستخلصات.

ويمثل شكل رقم (15) قيم التوصيلية بالمايكروسيمنس لملح نترات الرصاص مقابل حجم التانك المستخلص من النبك، وقد أوضحت التجربة استقرار التوصيلية عند قيمة (48 ملليلتر) من حجم حامض التانك المضاف، مما يدل على اتحاد أيونات الرصاص مع حامض التانك المستخلص وبالتالي ثبات قيمة التوصيلية.

في حين مثل شكل رقم (16) قيم التوصيلية بلماكيروسيمنس/سم لملح نترات الرصاص مقابل حجم التانك المستخلص من مخلفات الشاي، حيث أوضحت التجربة استقرار وثبات التوصيلية عند حجم (108 ملليلتر) من حجم حامض التانك.

أما شكل رقم (17) فيمثل قيم التوصيلية بالماكيروسيمنس/سم لملح نترات الرصاص مقابل حجم التانك المستخلص من قشور الرمان، وبينت التجربة استقرار وثبات التوصيلية عند حجم (202 ملليلتر) من حجم حامض التانك، ويمثل شكل رقم (18) قيم التوصيلية بالماكيروسيمنس/سم لملح نترات الرصاص مقابل حجم التانك القياسي، حيث أوضحت التجربة استقرار وثبات التوصيلية عند حجم (60 ملليلتر) من حجم حامض التانك.

يتبين من الشكل رقم (19) النسب المئوية المتكونة من حامض التانك المستخلص من المواد النباتية بالمقارنة مع النسبة المئوية للراسب المتكون من حامض التانك القياسي وأيون العنصر الثقيل (الرصاص) ، حيث لوحظ أن أعلى نسبة مئوية كانت عند استخدام حامض التانك القياسي 91.8 %، كما يوضح الجدول رقم (5) قيم الامتصاصية والانحراف القياسي النسبي والخطأ النسبي المئوي المقابلة لتراكيز محاليل معقد حامض التانك مع الرصاص، والجدول رقم (6) يوضح الخواص البصرية لمنحنى المعايرة لمعقد حامض التانك مع الرصاص.



شكل رقم (19): النسب المئوية للترسيب بالمقارنة بين المستخلصات وحامض التانك القياسي

جدول رقم (5) :قيم الامتصاصية المقابلة لتراكيز محاليل معقد حامض التانك مع الرصاص

التركيز (ppm) $X10^{-5}$	الامتصاصية	الانحراف القياسي النسبي (RSD)	الخطأ النسبي المئوي E_{rel}	الموجود(ppm)	نسبة الاسترداد %
0.00	0.00				
1.0	0.0218	0.04587	7.900	0.9 21	97.2
1.5	0.0329	0.22948	1.733	1.474	98.2
2.0	0.045	0.38491	0.700	1.986	99.3
2.5	0.054	0.18518	3.64	2.409	96.4
3.0	0.064	3.125	2.66	2.920	97.3
3.5	0.078	1.28205	1.657	3.442	98.3
4.0	0.087	2.29885	0.250	3.990	99.7
4.5	0.097	1.63092	0.222	4.455	99.0

جدول رقم (6) : الخواص البصرية لمنحني المعايرة لمعقد حامض التانك مع الرصاص

الخواص البصرية	القيم
اللون	عديم اللون
الطول الموجي (نانومتر)	295
حدود قانون بير(مايكروغرام /ملليلتر)	$(1.0\text{-}4.5)x10^{-5}$
الامتصاصية المولارية(لتر/ مول .سم)	7.121
معامل الارتباط (r)	0.99
حساسية ساندل (مايكروغرام / سم ^{2})	15404.65
الميل	0.0215
نقطة التقاطع	0.0012
معادلة الاسترجاع(Y=mx+C)	0.0215*x+0.0012
معامل الاسترجاع (r^2)	0.9991
معامل التنوع (%)	99.9
حد الكشف (مايكروغرام/ملليلتر)	21.35
حد الكم(ملغرام /ملليلتر)	71.16
معدل الاسترداد(%)	99.91

المصـــــــادر

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التركيب الدقيق للغلاف المحي والمح في بعض مراحل الخلية البيضية لسمكة الــشبوط *Barbus grypus* Heckel,1843 في بغداد

أفين رمضان محسن (1) عبد الحكيم أحمد الراوي (1) نـهلة عبد الرضا البكري (2)

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البريد الاليكتروني: aveenramadhan2015@gmail.com

الملخص باللغة العربية

جمعت عينة من إناث أسماك الشبوط من نهر دجلة في بغداد واستخرجت مبايضها. وقد أظهرت النتائج لبعض مراحل الخلية البيضية أن المنطقة الشعاعية Zona radiate (الغلاف المحي) في مرحلة المح الابتدائي Primary Yolk Stage ذات تركيب متعدد الــصفائح Multilamellar وتخترقها بصورة عرضية قنيات (قنوات) الثقوب Pore canaliculi العديدة التي تترتب شعاعيا لمركز البيضة وتكون لولبية الــشكل تقريبــا ، حيث يتكون الغلاف المحي في هذه المرحلة من صفاحات كثيفة إليكترونيا Electron dense lamellae عددها إحدى عشرة صفاحة متبادلــة مع إحدى عشرة صفاحة أقل كثافة إليكترونيا Lower electron density lamellae، وظهرت أيضا في سايتوبلازم خلية البيــضة الــصفائح المحية Yolk platelets والأسناخ القشرية Cortical alveoli.

الكلمات المفتاحية: أسماك الشبوط، الغلاف المحي، الخلية البيضية.

Fine structure of vitelline envelope and yolk in some oocyte stages in Shabout *Barbus grypus* Heckel,1843 in Baghdad

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ABSTRACT

A sample of female Shabbout were collected from Tigris River in Baghdad and their ovaries were extracted. Results showed for some oocyte stages that zona radiata (vitelline envelope) in primary yolk stage with multi-Multilamellar installation, which penetrated transversely by pore canaliculi (pore canals) that is radially to the center of the egg and is almost a spiral shape. The vitelline envelope consisted of eleven electron dense lamellae mutual with eleven lower electron density lamellae, and yolk platelets and cortical alveoli were appeared in oocyte cytoplasm.

المقدمـــــــة

تعتبر سمكة الـشبوط *Barbusgrypus* Heckel (Shabbout) 1841 أحد أسماك التغذية المهمة في المياه العذبة العراقية، وهـي تنتمي إلى عائلة الشبوطيات Cyprinidae (1).

هناك علاقة وثيقة في الاسماك العظمية بين الخلايا الجريبية وخلية البيضة المتمثلة بالزغيبات Microvilli التي تنشأ من خلية البيضة والخلايا الحبيبيـة، و تمتـد عبـر المنطقـة الـشعاعية Zona radiata(zr) من خلال قنوات الثقوب Pore canals ، وتـؤدي الزغيبات دورا أساسيا في عملية تبادل المغذيات والهورمونات بين خلية البيضة والخلايا الحبيبية (2). وتحاط خلية البيضة Oocyte في أغلب الأسماك بخلايا جريبية Follicular cells تتألف مـن طبقتين :الداخلية وهي طبقة الخلايـا الحبيبيـة Granulosa cell layer، والخارجية تتألف من صف واحد أو اثنـين مـن خلايـا قرابية Theca cells ، ويفصل الطبقتان الحبيبية والقرابيه غـشاء قاعدي Basement membrane ، فضلا عن طبقة لا خلوية مـا بين طبقة الخلايا الحبيبية وغشاء الخلية البيضية تدعى بالمنطقـة الشعاعية Zona radiata ، وتنشأ الزغيبات Microvilli من خلية البيضة والخلايا الحبيبية، وتقوم بإفراز مادة المنطقـة الـشعاعية فتتكون قنوات حول الزغيبات تـدعى بقنـوات الثقـوب Pore canals(3) .

لقد أطلق على المنطقة الـشعاعية تـسميات عديـدة منهـا Egg capsule، Chorion، Zona pellucida، Primary membrane، Egg shell، Vitelline envelope، Vitelline membrane، Radiata membrane، و تعد غلافـا ابتـدائيا Primary envelope للبيضة الناضجة ، فضلا عـن الأغلفـة الثانوية Secondary envelopes والأغلفـة الثالثيـة Tertiary envelope مثل الغلاف الجلاتيني (Jelly coat) (4).

يتشكل المـح yolk فـي خليـة البيـضة علـى ثلاثـة أنمـاط: 1- الحويصلات المحيـة Yolk vesicles(تحتـوي بروتينـات سكرية) .

2- الكريات المحية Yolk globules(مؤلفة من بروتينات دهنية وكاربوهيدرات ومواد أخرى).

3- القطيرات الدهنية Oil droplets (مؤلفة من كليسريدات وكمية قليلة من الكوليسترول) .

إن وقت ظهور هذه الأنواع الثلاثة من المـح يختلـف بـاختلاف النوع. وتصبح الحويصلات المحية فيمـا بعـد أسـناخ قـشرية Cortical alveoli، و تنشأ الأنماط الثلاثة من خليـة البيـضة ، حيث تصنع في الشبكة الاندوبلازمية الخشنة وجهاز كولجي (5) . ونظرا لعدم وجود معلومات حول تركيب الغلاف المحي والمح في بيضة سمكة الشبوط ، جاءت هذه الدراسة لدراسة هذه السمكة لما لها من أهمية اقتصادية في العراق و دول الجوار.

المـــواد وطرق العمـل

تم جمع 15 أنثى من أسماك الشبوط مـن نهـر دجلـة ببغـداد، واستخرجت المبايض منها ، ثم أخذت الأجزاء الأمامية والوسطى والخلفية من المبيضين الأيمن والأيـسر، وللتحـضير للدراسـة بـالمجهر الالكترونـي النافـذ Transmission Electron Microscope ، ثبتت العينات في محلـول Glutrealdehyde بتركيز 2.5% المخفف بمحلـول دارئ الفوسـفات Phosphate buffer solution، وأكملت عملية التثبيت باستخدام رابع أوكسيد الاوزميوم Osmium tetraoxide اعتمادا على ما ورد في (6) ، ثم فحصت العينـات بـالمجهر الإلكترونـي النافـذ مـن نـوع Philips CM10 باستعمال فولتية عالية 60 كيلوفولت.

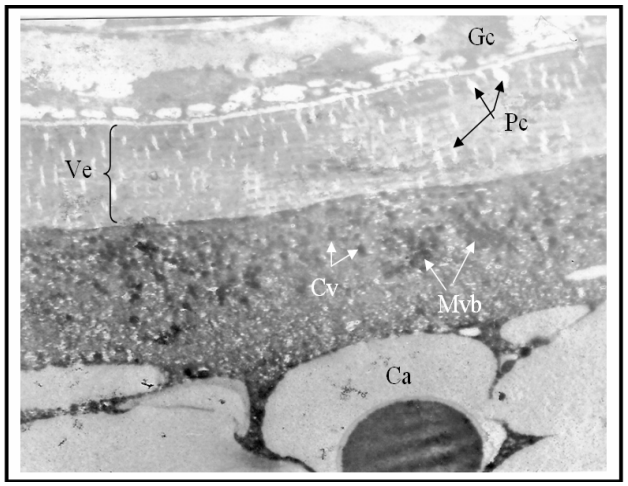
النتـــــائج

أظهرت نتائج الفحص بالمجهر الالكتروني النافذ لبعض مراحـل الخليـة البيـضية لـسمكة الـشبوط أن المنطقـة الـشعاعية (الغلاف المحي) في مرحلة المـح الابتـدائي Primary Yolk Stage ذات تركيب متعدد الصفائح Multilamellar وتخترقهـا بصورة عرضية قنيات (قنـوات) الثقـوب Pore canaliculi (canals) العديدة التي تترتب شعاعيا لمركز البيضة وتكون لولبية الشكل تقريبا ، ويتكون الغلاف المحي فـي هـذه المرحلـة مـن صفاحات كثيفة إليكترونيا Electron dense lamellae عددها إحدى عشرة صفاحة متبادلة مع إحدى عشرة صفاحة اقل كثافـة إليكترونيا Lower electron density lamellae.

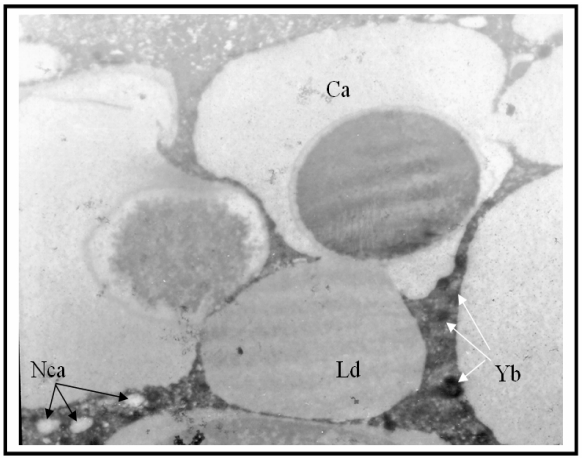
كذلك لوحظ وجود العديد مـن الحويـصلات المطليـة Coated vesicles ضمن السايتوبلازم المحيطي Peripheral ooplasm التي تندمج لتكون الأجسام متعددة الحويصلات Multivesicular bodies ، ثم تزداد بالحجم لتصبح فيما بعد أجساما محية Yolk bodies ، وتتحول بعدها إلى صفائح محية Yolk platelets في المراحل اللاحقة (شكل رقم 1).

كما لوحظ أيضا وجود أسناخ قشرية ناشـئة Nascent cortical alveoli منتشرة عشوائيا في السايتوبلازم وهذه تكـوّن الأسـناخ القشريةCortical alveoli التي تظهر بوصفها حويصلات غشائية ذات أحجام مختلفة وتركيب مختلف، فبعـضها يظهـر بوصـفها حويصلات غشائية متجانسة نصف شفافة الكترونيا وذات تحبـب دقيق والبعض الأخر يحوي حويصلة غشائية كثيفة اليكترونيا داخل حويصلة غشائية متجانسة نصف شفافة اليكترونيا ذات تحبب دقيق (شكلي رقم 2، 3).

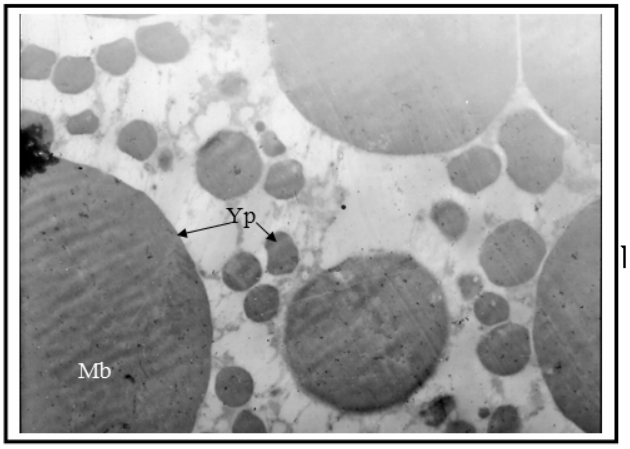
كما وجدت بعض القطيرات الدهنية Lipid droplets مـا بـين الاسناخ القشرية، فضلا عن وجود العديد مـن الأجـسام المحيـة Yolk bodies الـصغيرة المنتـشرة بـين الاسـناخ القـشرية (شكل رقم 2).



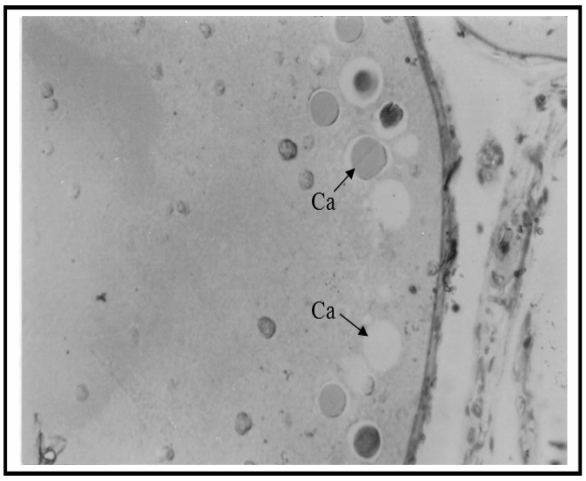
شكل رقم (1): صورة بالمجهر الإليكتروني النافذ في مبيض سمكة الشبوط توضح الجدار الخارجي لجزء من جريبة مبيضية في مرحلة المح الابتدائي ، (Ca) cortical alveoli أسناخ قشرية ، (Cv) coated vesicles حويصلات مطلية، (Gc) granulosa cell خلايا حبيبية، (Mvb) multivesicular bodies أجسام متعددة الحويصلات ، (Pc) pore canaliculi (canals) قنيات (قنوات) الثقوب (Ve) vitelline envelope غلاف محي .(ملون خلات اليورانيل وسترات الرصاص ، قوة تكبير X2600).



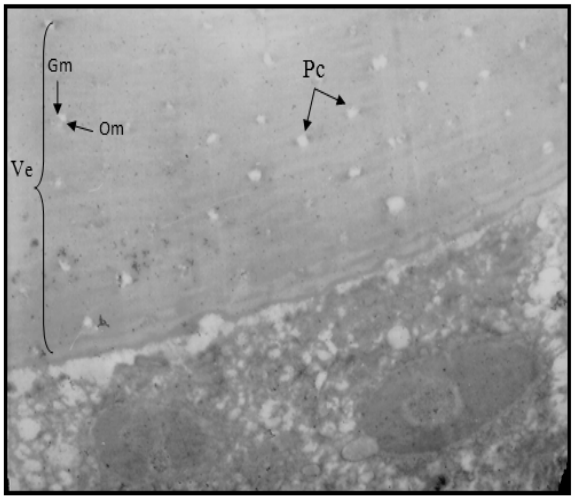
شكل رقم (2): صورة بالمجهر الاليكتروني النافذ في مبيض سمكة الشبوط توضح جزء من السايتوبلازم المحيطي لجريبة مبيضية في مرحلة المح الابتدائي cortical alveoli (Ca) اسناخ قشرية lipid droplets(Ld) قطيرات دهنية nascent cortical alveoli (Nca) اسناخ قشرية ناشئة yolk bodies (Yb) اجسام محية .(ملون خلات اليورانيل وسترات الرصاص، قوة تكبير X3400)



شكل رقم (4): صورة بالمجهر الاليكتروني النافذ في مبيض سمكة الشبوط توضح جزء من سايتوبلازم جريبة مبيضية في مرحلة المح الثالثي ، main body (Mb) الجسم الرئيس ، yolk platelets (Yp) صفيحات محية. (ملون خلات اليورانيل وسترات الرصاص، قوة تكبير X4600).



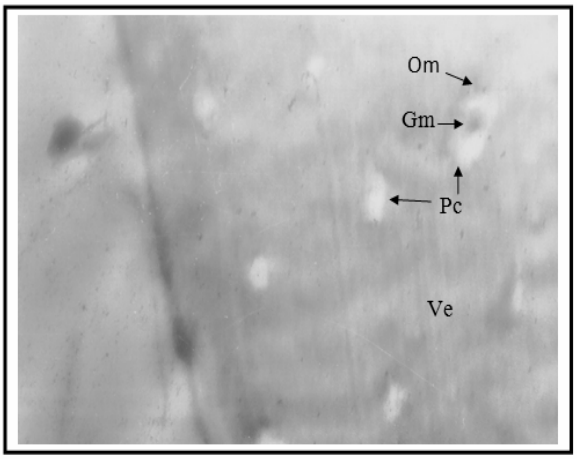
شكل رقم (3): خلية بيضية في مرحلة الأسناخ القشرية ، (Ca) cortical alveoli أسناخ قشرية .(ملون أزرق المثلين)



شكل رقم (5): صورة بالمجهر الاليكتروني النافذ في مبيض سمكة الشبوط توضح جزءا من جريبة مبيضية في مرحلة النواة المهاجرة، granulosa (Gm) microvilli، زغيبات الخلايا الحبيبية ،oocyte microvilli(Om) زغيبات الخلية البيضية pore canaliculi (canals) (Pc) قنيات (قنوات) النقوب ، (Ve) vitelline envelope غلاف محي. (ملون خلات اليورانيل وسترات الرصاص ، قوة تكبير X5800).

وظهرت الصفيحات المحية Yolk platelets بمختلف الأحجام في مرحلة المح الثالثي Tertiary Yolk Stage، الصغيرة منها تكون ذات محتوى متجانس ، والأكبر تحتوي على كتلة غامقة ذات خطوط مستقيمة بوسط الصفيحة تدعى بالجسم الرئيس Main body الذي يظهر تركيبة من المح البلوري بشكل خطوط مستقيمة (شكل رقم 4).

ظهر الغلاف المحي في مرحلة النواة المهاجرة Migratory Nucleus Stage تحت المجهر الاليكتروني النافذ أكثر انضغاطا و لوحظت زغيبات الخلية البيضية Oocyte microvilli أرفع من زغيبات الخلية الحبيبية Granulosa microvilli ضمن القنية النقيبة الواحدة، و يمكن ملاحظة انكماش بعض الزغيبات من قنيات النقوب فتبدو فارغة (شكلي رقم 5، 6).



شكل رقم (6): صورة بالمجهر الاليكتروني النافذ في مبيض سمكة الشبوط توضح الغلاف المحي لجريبة مبيضية في مرحلة النواة المهاجرة ، (Gm) granulosa microvilli زغيبات الخلايا الحبيبية، (Om)oocyte microvilli، زغيبات الخلية البيضية، (Pc)pore canaliculi (canals)قنيات(قنوات) الثقوب، (Ve) vitelline envelope غلاف محي .(ملون خلات اليورانيل وسترات الرصاص، قوة تكبير X 25000).

المناقشـــــــــة

تتكاثر سليفات البيوض Oogonia لتتحول إلى خلايا بيضية Oocytes تنمو تدريجيا إلى جريبات Follicles ، ثم تدخل مرحلة تكوين المح Vitellogenesis ثم مرحلة النضج Maturation ، بعدها تخرج البيضة من المبيض بعملية الإباضة Ovulation ، وهذه المراحل متشابهة في الأسماك الطرفية التعظم (7– 9).

حددت في سمكة الشبوط تسع مراحل لتكوين الخلايا البيضية وهي :(مرحلة سليفات البيوض Oogonia stage ،مرحلة النويات المحيطية المبكرة Early perinucleolar stage ، مرحلة النويات المحيطية المتقدمة Advanced perinucleolar stage، مرحلة الأسناخ القشرية Cortical alveoli stage ، مرحلة المح الابتدائي Primary yolk stage ، مرحلة المح الثانوي Secondary yolk stage ، مرحلة المح الثالثي Tertiary yolk stage ، مرحلة النواة المهاجرة Migratory nucleus stage ، ومرحلة الإماهة Hydration stage) (10).

لقد أظهرت نتيجة الفحص بالمجهر الاليكتروني النافذ أن المنطقة الشعاعية Zona radiata في مرحلة المح الابتدائي Primary Yolk Stage تكون ذات تركيب متعدد الصفائح Multilamellar مؤلف من صفاحات كثيفة اليكترونيا Electron dense lamellae متبادلة مع صفاحات أقل كثافة اليكترونيا Lower electron density lamellae تخترقه قنيات (قنوات) الثقوب Pore canaliculi canals العديدة ، وهذا ما أشار إليه (11). ربما تكون تلك الصفاحات نتيجة ترسب الغلاف المحي ما بين زغيبات الخلية البيضية Oocyte microvilli بشكل صفائح متبادلة موازية لطبقة الخلايا الحبيبية ، أما في سمكة *Epinephelus diacanthus* تكون المنطقة الشعاعية ذات منطقتين : داخلية zona radiate interna (ZRI) وخارجية zona radiate externa (ZRE) (12)، وفي سمكة Zebra fish تتكون المنطقة الشعاعية في مرحلة الأسناخ القشرية (13).

إن تسمية قنوات الثقوب Pore canals تعد غير دقيقة لأنه لا يمكن مشاهدتها إلا بالمجهر الاليكتروني، لذا رأينا إن تسميتها بقنيات الثقوب Pore canaliculi تكون أدق وذلك أسوة بالقنيات الموجودة ضمن الصفاحات العظمية Bone lamellae ، وقد أوضح (14) أن ظهور الصفائح في المنطقة الشعاعية يكون بسبب الاختلاف في اتجاه الليفات الدقيقة Microfibrils وفي مستوى القطع، وليس بسبب اختلاف التركيب الكيميائي لتلك الصفائح ، وجاءت هذه النتيجة مخالفة لما جاء به (14)، إذ ظهرت الصفائح في المنطقة الشعاعية و لجميع المقاطع ، كما أشار(4) إلى أن اختلاف الكثافة الاليكترونية لتلك الصفائح يعود إلى الاختلاف في تركيبها ، ففي سمكة *Oryzias latipes* تكون الطبقة الخارجية الكثيفة الكترونيا للمنطقة الشعاعية غنية بالسكريات المتعددة، بينما الطبقة الداخلية تكون غنية بالمواد البروتينية . وبصورة عامة تفرز المنطقة الشعاعية من خلية البيضة وليس من الخلايا الحبيبية (15، 16) ، ولكن النظرية الكلاسيكية تشير إلى أن المنطقة الشعاعية تتكون بوساطة خلية البيضة أو الخلايا الجريبية أو بوساطة كليهما ، ففي سمكة *Synbrancus marmoratus* تتكون المنطقة الشعاعية الداخلية والخارجية بوساطة خلية البيضة (17) .

وقد بينت دراسة (17) أن خلية البيضة والخلايا الجريبية في مرحلة النويات المحيطية تكونان زوائد زغيبية Microvillar processes تمتد إلى الفسحة حول المحية Perivitelline space ، وتقوم خلية البيضة بإفراز مواد كثيفة تمثل بداية ظهور الغلاف المحي Vitelline envelope ، وتلك الزغيبات تؤدي دورا أساسيا في عملية تبادل المغذيات والهورمونات بين خلية البيضة والخلايا الحبيبية (2).

إن الاختلاف في تركيب المنطقة الشعاعية بين أنواع الأسماك العظمية يعود إلى اختلاف الموطن أو التغذية ، لذا فأسماك المياه البحرية تمتلك منطقة شعاعية أكثر سماكة وتعقيدا من أسماك المياه العذبة (18)، ففي سمكة *Synbrancus marmoratus* يصل سمك الغلاف المحي إلى 11 مايكرومتر، وله أكثر من 30 طبقة عند البيضة الناضجة (17) ، وفي سمكة *Gadus morhua* يتكون الغلاف المحي من ثلاث طبقات :طبقة خارجية ضيقة مثقبة Porous layer، وطبقة وسطى متجانسة Homogenous layer ، وطبقة داخلية سميكة وحلزونية المظهر Helicoidal layer تترتب بشكل صفاحات Lamellae (14) ، كما أن المنطقة الشعاعية الداخلية (Zona radiata interna) في البيضة الناضجة لسمكة Zebra fish *Branchydanio rerio* مكونة من ست عشرة صفاحة كثيفة الكترونيا Electron dense lamellae متعاقبة مع خمس عشرة صفاحة أقل كثافة الكترونيا Lower density electron lamellae (18)، أما بيضة سمكة الكارب *Cyprinus carpio* فتتميز بمنطقة شعاعية سميكة – 10.0 (10.2مايكرومتر) وذات تركيب معقد مؤلف من أربع طبقات (19).

كما أكدت نتائج الفحص الدقيق أن زغيبات الخلية البيضية تكون أدق وارفع من زغيبات الخلايا الحبيبية ضمن القنية الثقبية الواحدة، وهذا مشابه لما وصف في سمكة (Zebra fish) (7، 20) ، كما وجد انكماش بعض الزغيبات في قنوات الثقوب فتظهر فارغة وذلك في مرحلة النواة المهاجرة ، وهذا الانكماش قد يكون لقلة حاجة خلية البيضة للإدخال الخلوي للمواد الغذائية وغيرها من المواد بسبب قرب نضج البيضة وهذا ما أشار إليه (21) .

وبينت النتيجة الحالية وجود اسناخ قشرية ناشئة Nascent cortical alveoli تؤدي إلى تكوين اسناخ قشرية Cortical alveoli وهذا يتطابق مع (21) ، وان الاسناخ القشرية تظهر بعضها بوصفها حويصلات غشائية متجانسة ذات تحبب دقيق والبعض الأخر يحوي حويصلة غشائية كثيفة داخل حويصلة غشائية متجانسة ذات تحبب دقيق، وهذا ما تمت الإشارة إليه في (14، 15، 22).

كما لوحظ وجود عمليات إدخال خلوي Endocytosis ضمن السايتوبلازم المحيطي Peripheral ooplasm متمثلة بالعديد من الحويصلات المطلية Coated vesicles التي تندمج لتكون أجساماً متعددة الحويصلات Multivesicular bodies و تزداد بالحجم لتصبح أجساما محية Yolk bodies ، ثم إلى صفيحات محية Yolk platelets ، وهذه النتائج تتوافق مع ما أشار إليه (21)، وهو ماتم ملاحظته أيضا في سمكة *Gadus morhua* (14).

كذلك أظهرت هذه النتيجة وجود صفيحات محية وبأحجام مختلفة، الكبيرة منها تتألف من الجسم الرئيس Main body ذي التركيب البلوري من المح Crystalline yolk ، أما الصغيرة منها فتكون

ذات تركيب متجانس من المح ، وقد أشار إلى ذلك أيضا كل مـــن (23، 24) ، ويمكن الاستنتاج بأن الصفيحات المحية الكبيرة هـــي الكريات المحية نفسها التي تشاهد تحت المجهر الضوئي للبيـــضة في مراحل تكوين المح، كذلك الصفيحات المحية الـــصغيرة هـــي الحبيبات المحية نفسها.

تكون الصفيحات المحية في الخلايا البيضية لسمكة *Branchydanio rerio* Zebra fish ذات تركيب بلوري Crystalline arrangement من المح ، وخلال مرحلة تكوين المح Vitellogenesis تفقد الأجسام المحية كتلتها البلورية وتصبح متجانسة (23)، أما في سمكة *Epinephelus diacanthus* تكون الكريات المحية yolk globules ذات منطقتين :داخلية كثيفة الكترونيا electron dense inner layer وخارجية اقل كثافة lighter outer layer (12) ، وفي أغلب الأسماك طرفية التعظم يترسب البروتين المحي على شكل كريات محية ، وهي إما تبقى محافظة على شكلها خلال مراحل تكون البيضة، أو تندمج مركزيا لتكون كتلة مستمرة من المح السائل، ولكن في بعض الأنواع كما في سمكة Zebra fish يخزن المح في البيضة بشكل صفيحات بلورية Crystalline platelets .(25)

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