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Histopathological Changes Effects of *Lactobacillus Acidophilus* Filtrate and Cyclophosphomide Drug on Albino male mice

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Abstract: Probiotics are formulations containing microbial stimulants that have some beneficial influence on the maintenance of a balanced intestinal macrobiotic and on the resistance to infections. This study was designed to evaluate the effects of *lactobacillus acidophilus* at the concentrations (10^5, 10^{10} cfu/ml) and the effects of cyclophoamide drug at concentration (15 mg/kg) on the histopathological of stomach, liver and spleen tissues on albino male mice. The results showed congestion, degeneration and necrosis of spleenic, liver and stomach tissues when animals injected with cyclophosoamide, while the results of tissues which infected with *lactobacillus acidophilus* were showed normal shape of stomach, liver and spleenic tissues in comparison with negative and positive controls. However, in pre and post-treatment, stomach, liver and spleen sections were showed mild histpathological change when infected with *lactobacillus acidophilus* in comparison with negative control. It is concluded that *lactobacillus acidophilus* has not show histopathological changes on organs of mice.

Key words: *lactobacillus acidophilus*, Cyclophosomide, Histopathology. Spleen, Stomach, Liver.

التغيرات النسيجية المرضية الناتجة من تاثيرالبكتريا التعايشية *lactobacillus acidophilus* وعقار السيكلوفوسفامايد في ذكور الفاران المختبرية

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الخلاصة: بروبايوتك هو تركيب يحتوي على محفزات مايكروبية او،لها تأثيرات مفيدة في الحفاظ على توازن بكتريا الامعاء ومقاومة الاصابات المرضية. صممت هذه الدراسة لتقديرتأثيرات بكتيريا (*Lactobacillus acidophilus*) عند التركيزين (10^5 ،10^{10} cfu/ml) وتاثير عقار السايكلوفوسفومايد بتركيز (15mg/kg) على التركيب النسيجي للكبد والطحال والمعدة في ذكور الفئران البيض. أظهرت النتائج تقرحات وتغيرات انحلالية وتنخرات لانسجة الطحال والكبد والمعدة عندما حقنت الحيوانات بعقار السايكلوفوسفومايد بينما أظهرت نتائج الانسجة التي اصيبت ببكتريا *Lactobacillus acidophilus* عودة أنسجة المعدة، الكبد و الطحال إلى شكلها الطبيعي بالمقارنة مع السيطرة السالبة و الموجبة. على كل حال، أظهرت نتائج التداخل (قبل وبعد المعاملة) للمقاطع النسيجية لكبد والطحال والمعدة تغيرات طفيفة عندما اصيبت ببكتريا *Lactobacillus acidophilus* مقارنة مع السيطرة السالبة. نستنتج من ذلك أن المقاطع النسيجية للفئران المعاملة ببكتريا *Lactobacillus acidophilus* لاتظهر اي تغيرات نسيجية مرضية بعد المعاملة .

Keywords: *lactobacillus acidophilus* ، الكبد، المعدة، الطحال، الانسجة المرضية، عقار السايكلوفوسفومايد

Introduction

All warm-blooded vertebrates live in symbiotic association with a complex population of microorganisms which inhabits their gastrointestinal tract. One of the benefits which the host animal derives from this relationship is an enhanced resistance to infectious diseases. Thus conventional animals with a complete gut microflora are more resistant to infection than are germfree animals. The gut microflora stimulates mainly a local response at the gut wall. This mucosal immunity is an important element of the animal's immune status because it is responsible for the control of infections as well as inducing tolerance to environmental and dietary antigens (1). *Lactobacilli* commonly inhabit the gastrointestinal tract (GIT), oral, and vaginal regions of humans and animals. *Lactobacilli* have many important roles in industry. They contribute to the production of some cheeses, yogurt, and other products. The lactic acid produced by *Lactobacilli* inhibits the growth of other organisms and lowers the pH of the product in these

products (2). One of lactobacillus bacteria is *lactobacillus acidophilus*. *(L. acidophilus*) has been further characterized as a short Gram-positive rod (2-10μm), is homo fermentative and has optimal growth at temperatures of 37˚C-42˚C. One of the *Lactobacillus* species, is the *L. acidophilus* that is the most well known and is commercially distributed as a probiotic (3). The World Health Organization (WHO) defines a probiotic as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (4).

It was found that some drug likes ((mitomycin, cytosar, methotrexate) increased the frequency of micronucleus formation and sperm-head abnormalities, in addition to the reduction of metaphase index, and caused defects in histological section in many organs and such findings suggest that such drug is a mutagen (5). The purpose of this study is illustrating the effect of *L. acidophilus* on histopathological changes of liver, stomach and spleen in albino male mice.

Materials and Methods:

First: Bacterial Isolation from Stool Specimens:

Isolation method was performed in four steps ; at first one loopfull of stool sample was inoculated into DeMan Regosa Shharpe (MRS) broth and incubated overnight at 37C◦ for three times to increase the bacterial growth, then serial dilutions were made from 10^{-1} -10^{-5} and 1 ml from the last one was transferred by pouring to DeMan Regosa Shharpe (MRS) plates and incubate overnight at 37C◦ under anaerobic condition using gas generating kit. In the second step colonies were picked and cultivated on MRS-CaC O_3. After incubation, colonies surrounded by clear zone were collected and the morphological and microscopal examinations were performed (6). A third step, suspected isolated were transferred to asculine-cellobiose agar medium to obtain *Lactobacillus spp*., to detect theability to hydrolyze and convert asculine to asculietine, and then combines with iron ion to form dark green color that characterizing *Lactobacillus* colonies (7). Fourth step was performed by transferring green colonies to MRS-raffinose agar medium in which only the *L. acidophilus* have the ability to grow and ferment raffinose (8).

Second: Microscopical Examination of *Lactobacillus acidophillus* :

A loopfull of suspected colonies was fixed on a microscopic slide, then stained by Gram stain to examine cells grouping, shape, gram reaction,

and non spore forming (9), then biological tests were made depending on catalase test, gelatinase test, starch hydrolysis test, acid and cured production in litmus milk and carbohydrate fermentation test.

Third: Laboratory animals:

Albino Swiss male mice (*Mus musculs*) were the laboratory animals. They were supplied by the Biotechnology Research Centre (Al-Nahrain University). Their age at the start of experiments was 8-10 weeks, and their weight was 23-27 gram. They were divided into groups, and each group was kept in a separate plastic cage (details of these groups are given in the section of experimental design). The animals were maintained at a temperature of 23 – 25°C, and they had free excess to food (standard pellets) and water (*ad libitum*).

Fourth: Experimental Design

The experiments were designed to evaluate the histological effects of *L. acidophilus* on mice organs (stomach, spleen and liver), so the animals were divided into four groups (each group has 4 animals):

Group I: The animals were dosed with 0.1 ml of distilled water (negative controls).

Group II: The animals were injected intraperitonially with cyclophosoamide drug at dose of (15 mg/kg) (positive controls).

Group III: The animals were dosed with 0.1 ml of 10^5 cfu/ml of *lactobacillus acidophilus.*

Group IV: The animals were dosed with 0.1 ml of 10^{10} cfu/ml of *lactobacillus acidophilus.*

The tested materials were given orally for 7 days. Then the mice were sacrificed in day 8 for laboratory assessments.

Group V: The animals were dosed with interactions between (0.1 ml) of *L. acidophilus* (10^{10} cfu/ml) and (0.1 ml) of drug cyclophosoamide through two types of treatments, which were pre-treatment and post-treatment. In the pre-treatment group, the animals were dosed with a single dose/day of the *L. acidophilus* for six days, while in day 7, they were injected with cyclophosoamide. In the post-treatment group, the animals were injected with cyclophosoamide in day 1, while in days 2-7, they were dosed with the *L. acidophilus* (single dose/day). The animals were sacrificed in day 8 for laboratory assessments in both types of treatments. Also, both types of treatments were paralleled with control groups; the *L. acidophilus* was replaced with distilled water.

Fifth: Histopathological Study

Samples were obtained & cut into small pieces (2×2×2 mm.) then pre-fixed in 2.5% gluteraldehyde diluted in phosphate buffer PH (7.4).

After that specimen were rinsed in the same buffer for several times and left in PBS for 12 hrs, and the procedure of (10) was followed to prepare histopathological sections. The procedure is outlined as follows:

- **Washing:** specimen were rinsed in the same buffer for several times and left in PBS for 12 hrs**,** and then specimens were post-fixed in 1% osmium tetra oxide for 1 hr.
- **Dehydration:** The sample was dehydrated with ascending concentrations (50, 70, 90 and 99%) of ethanol. There was two hours for each concentration.
- **Clearing:** The sample was placed in xylene for two hours.
- **Infiltration**: The sample was first placed in paraffin-xylene (1:1) for 30 minutes at 57-58ºC, and then in paraffin alone for 2 hours at 60-70ºC.
- **Embedding**: The sample was embedded in pure paraffin wax (melting temperature: 57-58ºC) and left to solidified at room temperature.
- **Sectioning**: The paraffin block was sectioned (rotary microtome) at a thickness of 5 microns, and then the sections were transferred to a slide covered with Mayer's albumin. The slide was placed in a hot plate (35-40ºC) for 1-2 hours.
- **Staining**: The slide was first placed in xylene for 15-20 minutes, descending concentrations (90, 80 and 70%) of ethanol (two minutes for each concentration) and finally distilled water. After that, the slide was stained with heamatoxylin for 10-20 minutes and then washed with distilled water for 5 minutes. Then, the slide was placed in acidic alcohol for one minutes and washed with distilled water. After washing, the slide was placed in eosin stain for two minutes, and then in ascending concentrations (70, 80, 90 and 99%) of ethanol (two minutes for each concentration). Finally, the slide was cleared with xylene for 10 minute.
- **Mounting**: The slide was mounted with a Canada balsam and covered with a cover slip. Then, the slide was examined microscopically to inspect the histopathological changes.

Results and Discussion:

Animals dosed with 0.1 ml of distilled water (negative control):

- **Spleen**: section showing normal appearance of lymphoid follicle (white Bulb) with central arteriole (figure 1).

- **Liver**: section of liver showing normal appearance of parenchyma tissue (figure 2).
- **Stomach**: section showing normal structure of gastric mucosal tissue (figure 3).

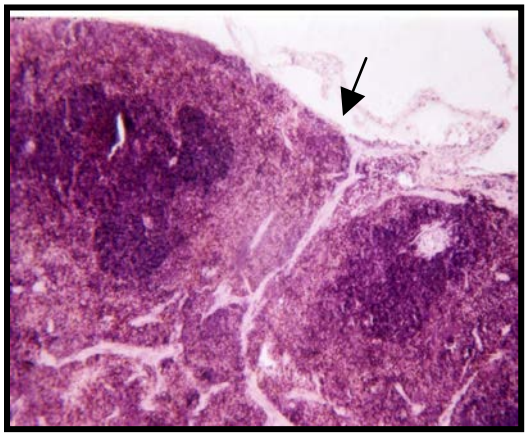

Fig 1: Section of spleen (negative control), showing normal appearance of lymphoid follicle (white bulb) with central arteriole (H&E)x200.

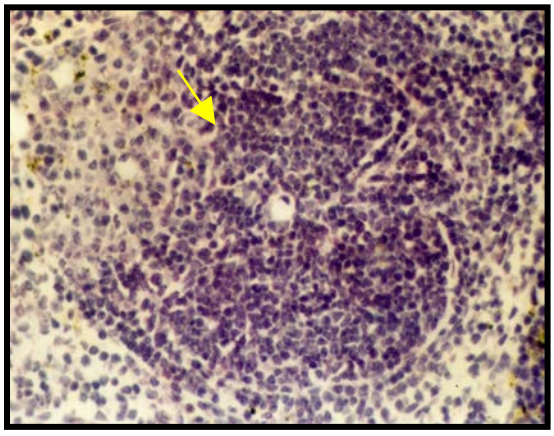

Fig 2: Section of liver (negative control), showing normal appearance of parenchyma tissue (H&E)x200.

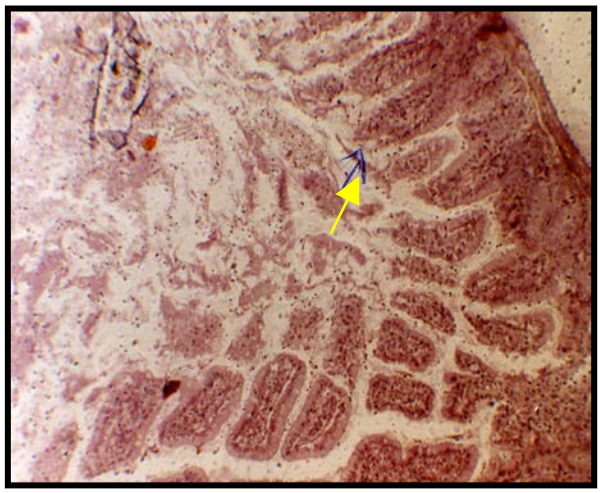

Fig 3: Section of stomach (negative control), showing normal structure of the gastric mucosa (H&E)x200.

Animals injected with 0.1 ml of cyclophosoamide (15 mg/kg) (positive control):

- **Spleen**: section of spleenic tissue showing congestion, degeneration &necrosis of splenic tissue, with haemorrhage (figure 4).
- **Liver**: section of liver tissue showing congestion, degenerative and necrosis of paranchymal tissue cells; with mild cells inflammation (figure 5).
- **Stomach**: section of stomach showing superficial degenerative changes in the gastric mucosa (figure 6).

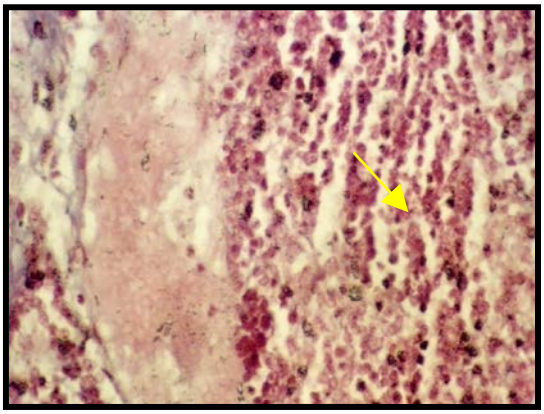

Fig 4: Section of spleenic tissue showing congestion, degeneration &necrosis of splenic tissue, with haemorrhage (H&E)x200.

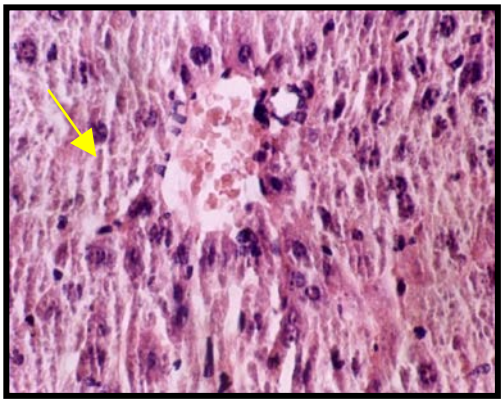

Fig 5: Section of liver tissue showing congestion, degenerative and necrosis of paranchymal tissue cells; with mild cells inflammation (H&E)x200.

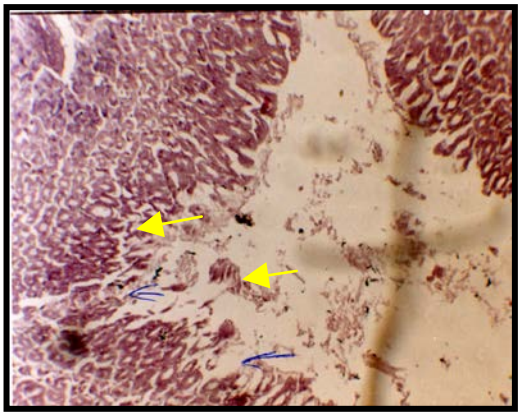

Fig 6: Section of stomach showing still mild degenerative changes of surface gastric mucosa (H&E) x200.

Liver, stomach and spleen sections of mice treated with drug showed congestion, degeneration and necrosis. It has been demonstrated that some drugs may cause abnormalities in lymphocyte receptors involved in mitogen recognition. Such effect may result in an inhibition of blastogenic index, mitotic index, and increase micronucleus formation, abnormality in histological section of some organs and chromosomal aberrations. The action of these drugs may act on the repair systems inside the cells, and as a result the cells loss the ability to repair the damaged DNA (11).

Animals dosed with 0.1ml of 10^5 cfu/ml of *lactobacillus acidophilus*:

- **Spleen**: section showing mild degenerative effect of splenic

parenchyma tissue with the presence of megakaryocyte i.e. the splenic return to near the normal (figure 7).

- **Liver**: section showing congestion, normal looking like of parenchyma tissue.
- **Stomach**: section showing degenerative of gastric mucosa, normal looking like of gastric mucosa (figure 6).

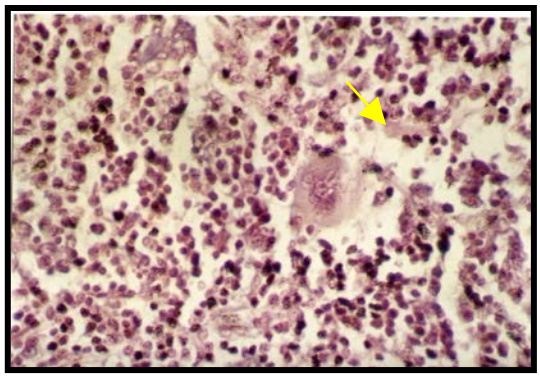

Fig 7: Section showing mild degenerative effect of spleenic parenchyma tissue with the presence of megakaryocyte i.e the splenic return to near the normal (H&E)x200.

These results indicate that lactobacilus spp. have no any pathogenic or toxic effect on animal's tissues. These results agreed with (12) and (13) who mentioned that lactobacilli is probiotics non pathogenic bacteria that modulate the host intestinal balance. The resident bacterial flora of the gastrointestinal tract may also be implicated in the pathogenesis of diseases such as inflammatory bowel disease (ulcerative colitis and Crohn disease). Any compound taken orally, entering the intestine through the biliary tract or by secretion directly into the lumen is a potential substrate for bacterial transformation. So the colonic microflora is important to health (14).

Animals dosed with 0.1ml of 10^{10} cfu/ml of L. *acidophillus*:

- **Spleen**: section showing rejoin of lymphoid tissue with infiltration of inflammatory cell and numerous megakaryocyte in other part shows widening of white bulb of macrophage of hyperplasia and numerous megakaryocyte.
- **Liver**: section shows congestion degeneration of hepatocytic cell (figure 5).

- **Stomach**: section shows degenerative changes with hyperplasia of lymphoid tissue apprevation (figure 6).

This results were appeared that the acidify the intestinal contents, inhibiting the growth of pathogenic bacteria, compete with other microorganisms for nutrients and receptors, or adhere to epithelial cells, producing antibacterial compounds, and affect the immune system (15). Also Ogawa et al. (2001) mentioned that preventive administration of probiotic lactobacilli may lead to enhance resistance to acute E. coli infection due to acceleration of a specific humoral immune response. The enormous numbers and diversity of the human gut microflora is reflected in a large and varied metabolic capacity, particularly in relation to xenobiotic biotransformation, carcinogen synthesis and activation(17).

Animals treated with interaction between 10^{10}cfu/ml of *lactobacillus acidophilus* and 15 mg/Kg of cyclophosoamide:

A- Pre-treatment:

1. **Animals treated with interaction between 0.1 ml distilled water and 0.1 ml cyclophosoamide (15 mg/Kg) (negative control):**
   - **Spleen**: Section showed wide of white bulb presence of germinal center with presence of numerous megakaryocyte with focal area of regeneration.
   - **Liver**: Section was showed congestion with a wide area of regeneration and necrosis of hepatocyte cells (figure 9).
   - **Stomach**: Normal structure of stomach with mucosal hyperplasia.

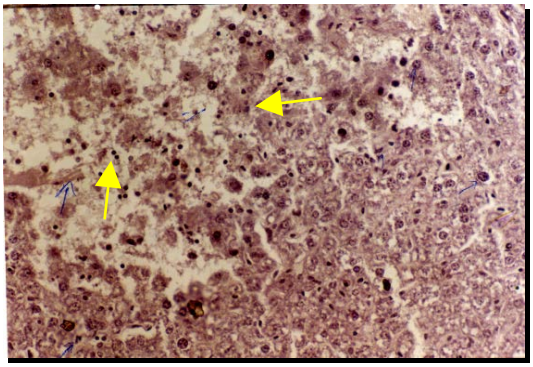

Fig 9: Section of liver from pre- treatment group (negative group), showing necrosis with degenerative changes and inflammatory cells infiltration (arrows) (H&E)X200.

Cyclophosphamide caused necrosis and congestion of the tissues resulting from the alkylating properties of this drug that lead to major disruptions in nucleic acid function and the inhibition of DNA synthesis. Cyclophosphamide-induced nucleic acid damage may lead to DNA mutations that result in cytotoxicity, carcinogenicity, teratogenecity, and reproductive toxicity following chronic exposure to CPH (18).

2. **Animals treated with interaction between 0.1 ml *lactobacillus acidophilus* (10^{10} cfu/ml) and 0.1 ml cyclophosoamide (15 mg/Kg):**

- **Spleen**: Section showed normal molecular splenic structure with presence of certain area of regeneration and processig.
- **Liver**: Section showed focal area degeneration change in histocyte cell with hematocyte mild cells and inflammatory cell function, (figure 11).
- **Stomach**: Normal looking structure histopathological of stomach.

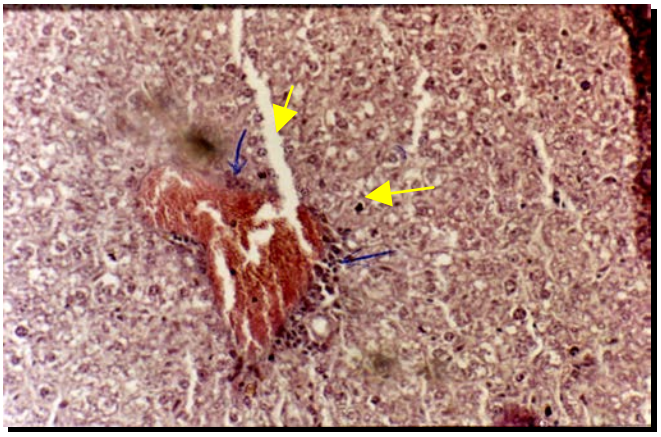

Fig 11: Section of liver from pre-treatment group was dosed with 0.1ml of 10^{10} cfu/ml *L. acidophilus* and 0.1 ml of cyclophosoamide showing congestion (arrows), mild degenerative changes with inflammatory cells infiltration (H&E)X200.

According of these results, jijoin and his colleges (2004), indicated that several strains of lactic acid bacteria (LAB) were reported to display stimulatory properties on cells of the innate immune system in-vitro. These include macrophages and natural (NK) cells that induce adjuvant activity at the mucosal surface and improve phagocytosis by increasing the proportion of lymphocytes and NK cell. Also, probiotic bacteria DNA can suppress systemic inflammatory responses to pathogenic bacterial DNA. The explanation of therapeutic efficacy of probiotic bacteria may be clear through its ability to modulate epithelial barrier function, with possible interaction with toll-like receptor 2 (TLR-2). TLR-2 recognizes bacterial lipoteichoic acid, zymosan and other different medical methods.

B- Post- treatment:

1. **Animals treated with interaction between 0.1 ml cyclophosoamide (15 mg/Kg) and 0.1 ml distilled water (negative control):**
   - **Spleen:** Section showed increase in splenic macrophage with white bulb and germinal center.
   - **Liver:** Section showed congestion with regeneration and necrosis of hepatocyte cells.
   - **Stomach:** Section showed focal area with distraction surface of mucosal, (figure12).

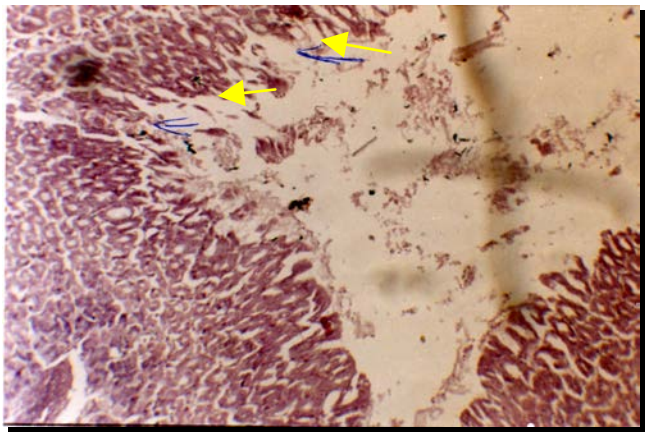

Fig 12: Section of stomach from post- treatment group (negative control), injected with 0.1ml of cyclophosoamide and dosed with 0.1 ml of distilled water, showing still mild degenerative changes of surface gastric mucosa (arrows) (H&E)X200.

2. **Animals treated with interaction between 0.1 ml cyclophosoamide (15 mg/Kg) and 0.1 ml *lactobacillus acidophilus* (10^{10} cfu/ml) (negative control):**
   - **Spleen**: Section showed increase in spleenic macrophage with white bulb and certain necrosis lymphoid tissue.
   - **Liver:** Section showed congestion with degenerative changing of hypatocyte cells, (figure 13).
   - **Stomach**: Normal looking structure appearance in stomach.

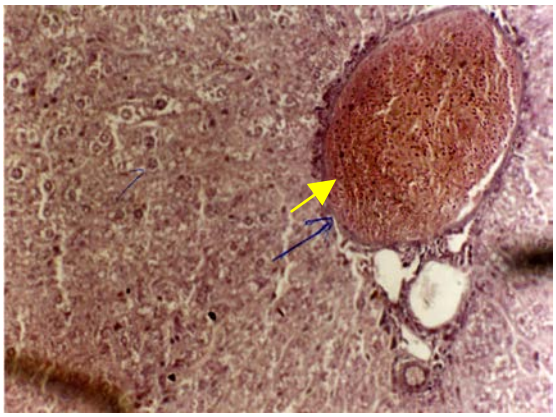

Fig 13: Section of liver from post- treatment group injected with 0.1ml of cyclophosoamide and dosed with 0.1 ml of *L. acidophilus*, showing normal look like appearance of liver tissue with still congestion of blood vessel in portal area (arrows) (H&E)X200.

From these results *L. acidophilus* had non pathogenic effects on tissues but it can elicit immune responses while protecting against microbial pathogens. The beneficial role of the normal flora is the prevention of other more pathogenic bacteria from gaining a foothold in the body. The gut bacteria seem to be responsible for the normal structure and function of the intestine: they degrade mucin, epithelial cells and carbohydrate fiber and their metabolism produces vitamins, especially vitamin K (20, 21).

The results showed normal appearance of liver tissues due to that the supplementation of *lactobacillus acidophilus* reduced the hepatocellular necrosis and inflammatory cell infiltration, which reflected by a decrease in liver enzymes (22).

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The effect of ethanol on some ecological and functional aspects of *Dictyostelium discoideum* beta- glucosidase

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Abstract: *Dictyostelium discoideum* has revealed as appropriate tool for studying the effect of many ecological factors. *Dictyostelium* can live in many places such as soil and moist leaf litter. Beta- glucosidase is one of the most important enzymes that used in degradation of food and takes part in other cell biology. Ethanol is a solvent that used widely rang for water insoluble substances in industry and medicine. In this study, the ecological effects of ethanol and its cellular role on *D. discoideum* beta-glucosidase were investigated. Cell line was generated for over-expressing beta- glucosidase protein and cell line with the first 83 amino acid from beta- glucosidase (a mutant version of this protein) was used. Beta- glucosidase over-expression cell line showed high level of growth rate, cells size, phototaxis and aggregation comparing to other studies cell lines even under high concentration of ethanol. These data support a role for Beta-glucosidase in resistance against the ecological factors like ethanol.

Key words: Ecology effects, *Dictyostelium*, Ethanol, lysosomal enzymes

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تاثير الايثانول على بعض الجوانب البيئية والوظيفية لبروتين الدكتيستليم بيتا كلوكوسيديز

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الخلاصة: تعتبر اميبا *Dictyostelium discoideum* الوسيلة الملائمة لدراسة تأثير العديد من العوامل البيئية. الدكتيستليم تستطيع العيش في عدة امكنة مثل التربة او تحت اوراق الشجر. يعتبرالبيتاكلوكوسيديزمن اهم الانزيمات الحالة والذي يساعد على تحلل الغذاء كذلك له دور في بايلوجية الخلية. يعتبر الايثانول من المذيبات التي تستعمل بشكل واسع في الصناعة والطب كمواد غير قابلة للذوبان في الماء. تمت دراسة التاثير البيئي للايثانول على الدكتيستيليم المطفرة . تم استخدام خلايا مطفرة احدهما تفرط في انتاج البيتاكلوكوسيديز والاخرى تحتوي على جزء من انزيم البيتاكلوكوسيديز. وقد بينت النتائج ان الخلايا المفرطة في انتاج البيتاكلوكوسيديز اظهرت مستوى اعلى من معدل النمو ولحجم الخلايا و الانتحاء الضوئي والتجمع الخلوي ونجحت بالبقاء تحت تاثير تراكيز متعددة من الايثانول. وتدعم هذه البيانات ان بروتين البيتاكلوكوسيديزله دورفي مقاومة الخلايا تجاه العوامل البيئية كالتعرض للايثانول.

Introduction

Interestingly, living microorganisms are capable to survive by adapting to adverse changes in their environment (1). Ethanol has a great effect than to any other solvent in water (2). As a solvent, ethanol is used in industry and medicine nevertheless it has a component of several intoxicating potions. The ethanol effects can depend on The ethanol effects can depend on many factors such as chronic exposure and dose, time-dependent which causes serious conditions. (2). Ethanol as a product from fermentations is characterize as an environmental change to the organisms (3).

D. discoideum is a unicellular eukaryotic microorganism and having rapid life cycle (4). *D. discoideum* can be considered as conversion fact in the evolution between multicellular and unicellular organisms.. Furthermore, it offers a perfect system for explaining development, gene expression morphogenic movement and cell differentiation (5). *Dictyostlium* lives as a single amoeba; however, after consumption of the food, cells develop chemically attractant to each other. Then, cells procedure aggregates, which go through a series of morphological variations (6). The most important advantages of the social behavior in *D. discoideum* are the protection from predators and long-range spore dispersal (7, 8). The greatest powerful degradative organelle in the eukaryotic cell is the lysosome. It has hydrolytic enzymes that achieve essential functions. There are several mutations that affect the lysosome in human which can lead to a diseased state with dramatic consequences. Interestingly, many of the lysosomal enzymes are exoenzymes; this means that they digest macromolecules from their ends (9). Beta-glucosidase is one of the essential lysosomal enzymes that hydrolyze glucosides. This enzyme works on bonds joining two glucose-substituted molecules (i.e., the disaccharide cellobiose) (10). Beta- glucosidase have a dramatic role in the consumption of cellulose in various organisms such as fungi and bacteria (11). Another role for this enzyme is the creation of oligomers and other complex molecules. Many studies has revealed that the enzyme activities increase during development and thus required RNA and protein synthesis (12) The *Dictyostlium* beta-glucosidase (GluA) associates with the lysosome as well as endosome and Golgi bodies. Additionally, cells over-expressing GluA had higher rates of growth, ATP level, endocytosis and phagocytosis (13). In this study, our analysis of these GluA proteins indicated that GluA plays a role in the

resistance of the cell against the effect of different concentration of ethanol.

Materials and Methods

Cells and culture conditions

There were three *D. discoideum* cell lines used for all of the experiments, wild-type strain AX4, GFP-GluA and GFP-GluAΔ84-821. All of these cell lines were developed axenically at 21^0C in shaking culture at 150 rpm in HL5 medium: 1% oxoid proteose peptone, 0.5% yeast extract, 1% glucose, 2.4 mM Na2HPO4, and 8.8 mM KH2PO4, pH 6.5. 300 mg/ml of streptomycin sulfate and 100 mg/ml of ampicillin (Sigma) was supplement. Furthermore, for the transfected cells, HL5 medium was supplemented with selective antibiotic 10 mg/ml of G418 (Invitrogen) (www.dictybase.org).

Creation of GFP tagged GluA and GFP-GluAΔ84-821 cell lines

Dictyostelium GluA cDNA were subjected to PCR via particular primers then, the resulting PCR yields were ligated into the TA vector (Invitrogen) and sequenced. The GluA PCR TA product was then digested and ligated into the pDneo2a-GFP vector. The resulting constructs, GFP-GluA, was sequenced for errors. Same thing we did to make GFP-GluAΔ84-821 but we use the first 83 amino acid of GluA instead of full length. Finally, constructs were then electro transfected into AX4 cells and grown cell with selected G418 antibiotic (Invitrogen).

Cell growth rate

The three cell line AX4, GFP-GluA, and GFP-GluAΔ84-821 cell lines were grown in HL5 medium until a density of $1\text{-}3\times10^4$ cells was achieved. Ethanol was added to the liquid medium (HL5) then the cellular growth were determined by counting the cell every 24 hours for five days by using special tool (hemacytometer) (Hausser Scientific, Horshman, PA).

Development Assay

All cells were harvested by centrifugation after growing them in HL5 medium on a rotary shaker (160 rpm) to 1x109 cells/ ml. Cells were washed with developing buffer then they were re-suspended in the same buffer at a density of 2×10^8 cells/ml. were taken and spread them on a100 mm KK2 plate by using a sterile glass spreader. All petri-dishes were wrapped with a wet paper towel, covered with plastic wrap, reversed, and incubated at 22°C. M 4201, 25× microscopes was used to photographs the multicellular development (Switzerland).

Cell size

All cells were washed with cold SB (soerensen phosphate buffer: 14.5 mM KH2PO4, 2.5 mM Na2HPO4, pH 6), then left on ice for 20 minutes after vortex them. A Nikon Eclipse 90i microscope equipped with 12V-100W halogen lamp was used to photograph. The Bright field filter was used and the diameter (px) of 50 cells was measured for each cell line and graphed by Using the Nikon software,

Slug formation and phototaxis

The phototaxis was done by grown the cells in HL5 medium until a density of 2-3×10^4 cells/ ml was obtained. Then cells harvested and grown in *Klebsiella aerogenes* bacteria on an SM plate. For the plates were left for 2-3 day until the bacteria was consumed then cells were washed by pipetting 2 ml of developmental buffer on the plate and collected in a tube. The cells were washed and re-suspended with the developing buffer. In2% non-nutrient agar plate, 2.5 x 107 cells were streaked in a 4-5 cm long straight line. The agar plate was then wrapped with aluminum foil and small hole was made into the aluminum foil near a light source. M 420 1, 25 microscope (Switzerland) was used for photograph the phototaxis.

Data Analysis

One-way analysis of variance was used to test for differences among at least three groups.

Results

Growth rate

The overall effects of different concentration of ethanol on AX4, GFP-GluA, and GFP-GluAΔ84-821on cell growth rates were determined. The ethanol concentrations were (0, 0.5, 1, and 2 %). Figure 1 demonstrates cell growth rate as plotted on a graph comparing cells/ml (titer) and time in 5 days with different concentrations. In cell lines overexpressing GFP-GluA has higher growth rate rather than control in (0, 0.5, and 2 %) and there is a significant difference among them figure 1A, B, D. GFP-GluA cell line showed slightly increase of the growth rate of 1% with ethanol concentration figure 1C. GFP-GluAΔ84-821 growth rate seemed to be near to the control in all studied ethanol concentrations. In figure1, there is no significant difference between them figure 1.

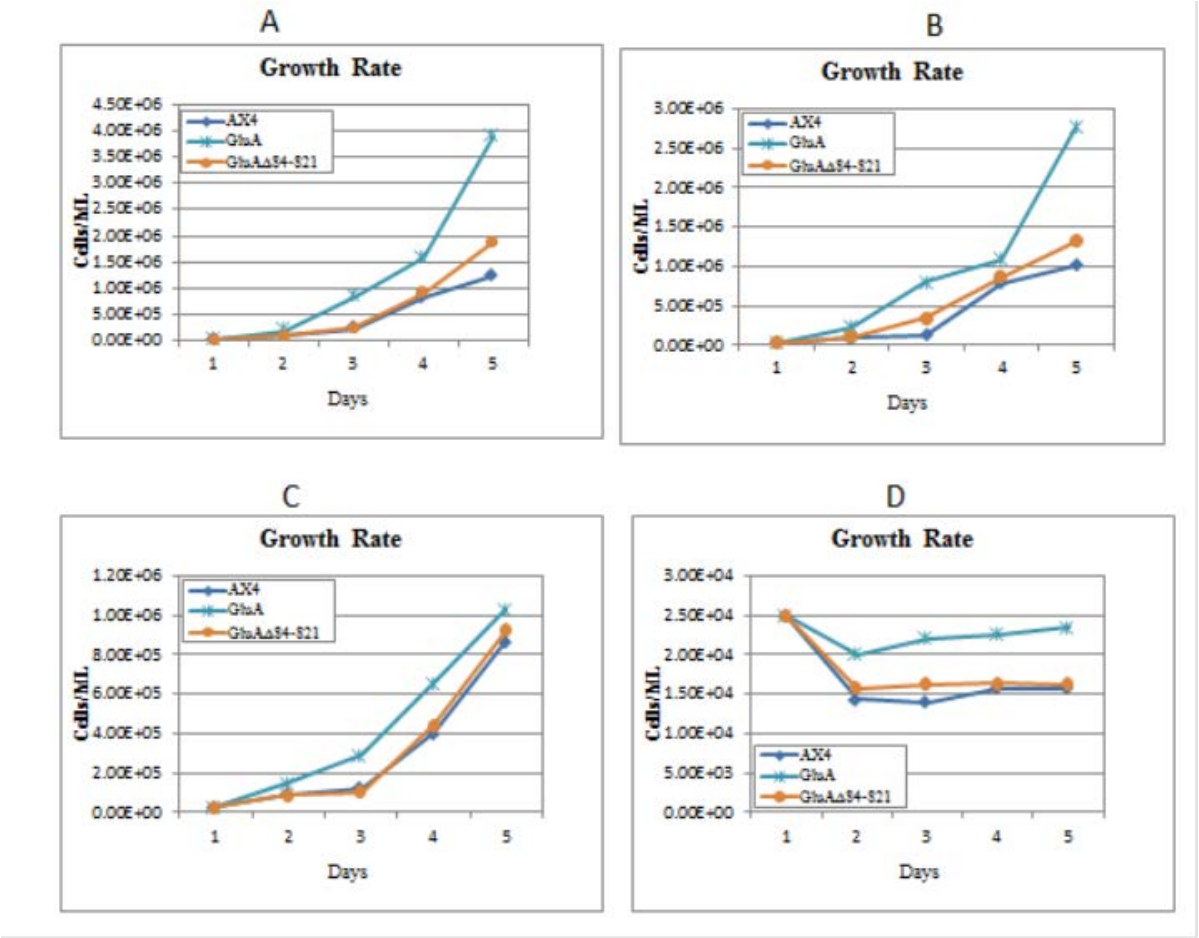

Figure 1. Analysis of the growth rate of AX4, GFP-GluA and GFP-GluAΔ84-821over-expressing cell lines in different ethanol concentration ecological condition. (A) Growth rate of cell lines under ethanol concentration (0%). (B) Growth rate of cell lines under (0.5%). (C) Growth rate of cell lines under (1%). (D) Growth rate of cell lines under (2%). GFP-GluA over-expressing cell line has an increase in growth rate compared to the other studied cell lines in all ethanol concentration.

Cells size

The average of cell size was investigated in all the cell lines through two concentrations (0, 1%). Figure 2A and B represents the graphical data regarding cellular size as measured by average diameter. Graphical averages from the bright line generated data for every cell type (AX4, GFP-GluA, and GFP-GluAΔ84-821) included the appropriate standard errors. Obviously, there is an apparent significant decrease in the

average cellular size of the AX4, GFP-GluA, and GFP-GluAΔ84-821 cell line in 1% concentration as compared to the average diameter of cell line with 0% concentration of ethanol the in wild-type AX4 cells.

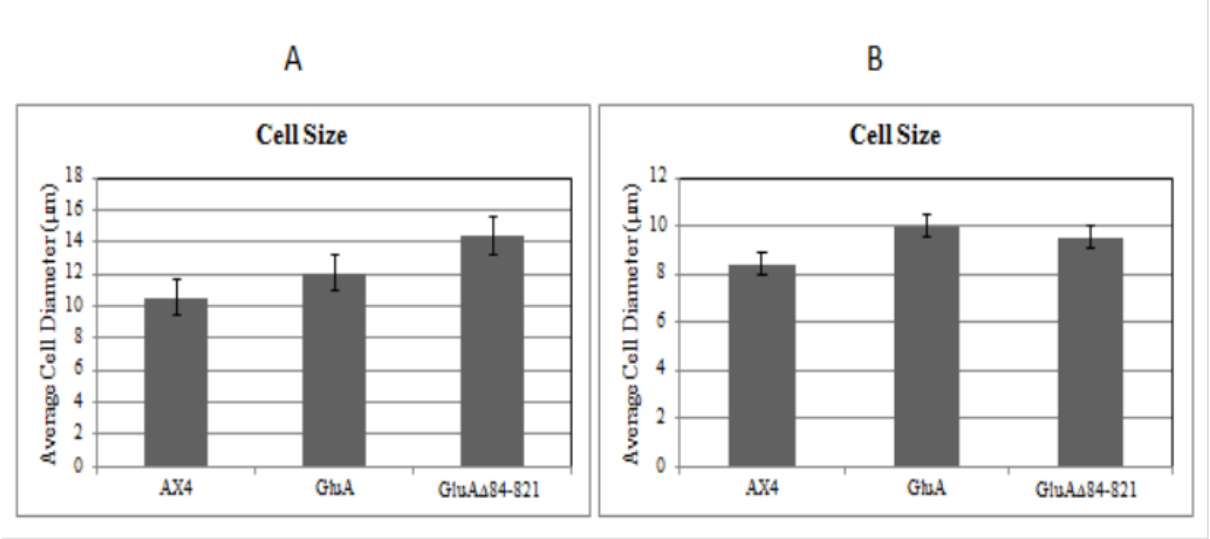

Figure 2. Graphical plots for the comparison of cell size levels in all cell lines. Graphical representation with standard error showed a decrease in cell size of the AX4, GFP-GluA, and GFP-GluAΔ84-821cell lines in 1% ethanol concentration.

Phototaxis Rate

To study the effect of ethanol as an ecological factor on cell lines culture in HL5 with different concentration of ethanol, *Dictyostelium* phototaxis behavior, photomicrographs for cell line has taken. All cell lines exposed to a single point source of light for 48 hours on nutrient-free agar. Figure 3A shows that both wild type and GFP-GluA cells migrated toward the light source in a linear way and developed fruiting bodies. Interestingly, GFP-GluA cell line migrates faster than other cell lines. Cells over-expressing GFP-GluAΔ84-821 did not develop; instead, these cells stayed in their places without forming fruiting bodies. The cell cultures in 1% ethanol showed low rate of phototaxis comparing to cells cultured in HL5 with 0% ethanol figure3A. They formed short-branched structures with limited movement figure 3B. All cell line cultured in 2% ethanol failed to made phototaxis figure 3C.

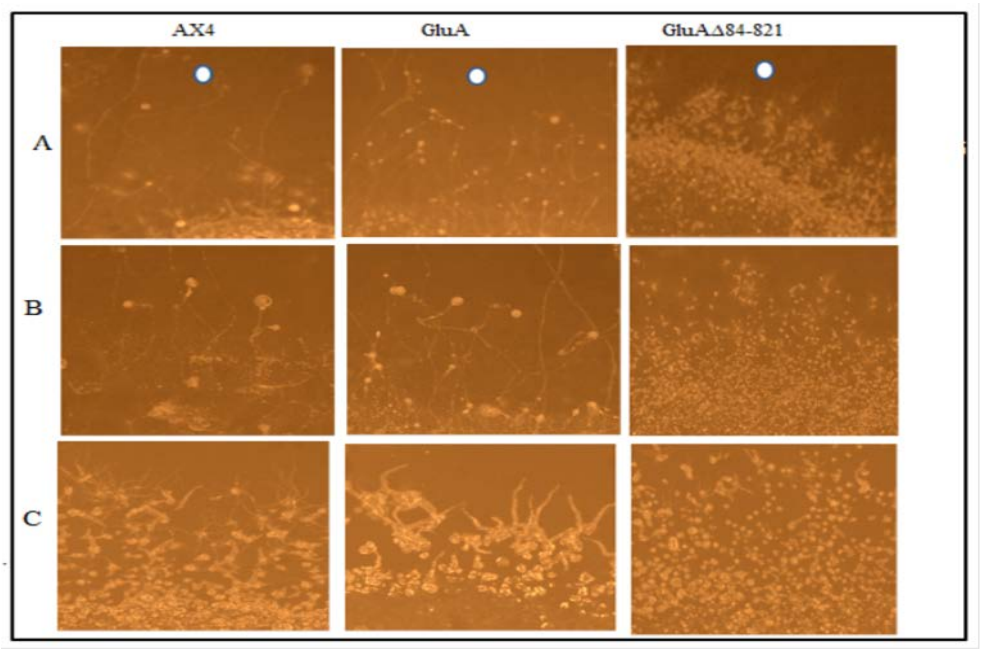

Figure 3. Over-expression of the AX4, GFP-GluA, and GFP-GluAΔ84-821 cell lines caused a differential effect on cellular and/or slug's phototaxis. (A) On 0.5% agar light-directed migration of slugs after 48 hours shows that AX4 and GFP-GluA slugs migrate normally and develop stalks with fruiting bodies. (B) AX4 and GFP-GluA reduce the amount of migration and formed short-branched structures with limited movement. (C) AX4, GFP-GluA cell line. GFP-GluAΔ84-821 cell line failed to made phototaxis.

Aggregation Assay

This test was done to address the effect of different concentration of ethanol on cell lines that over expression GFP-GluA and GFP-GluAΔ84-821on developmental pathways. To examine ethanol effects on developmental behavior, cells from all cell lines in mid-log phase were washed with developmental buffer. The cells then monitored microscopically and photographed to track the aggregation process. AX4 and GFP-GluA demonstrated normal aggregation and the aggregation of GFP-GluA cell bigger than AX4. GFP-GluAΔ84-821 cell line failed to aggregate (figure 4A). AX4 and GFP-GluA cell lines reduce the size of aggregation after culturing cells in HL5 with 1% ethanol figure4B. All cell lines failed to aggregate after culturing the cell lines in 2% ethanol figure 4˚C.

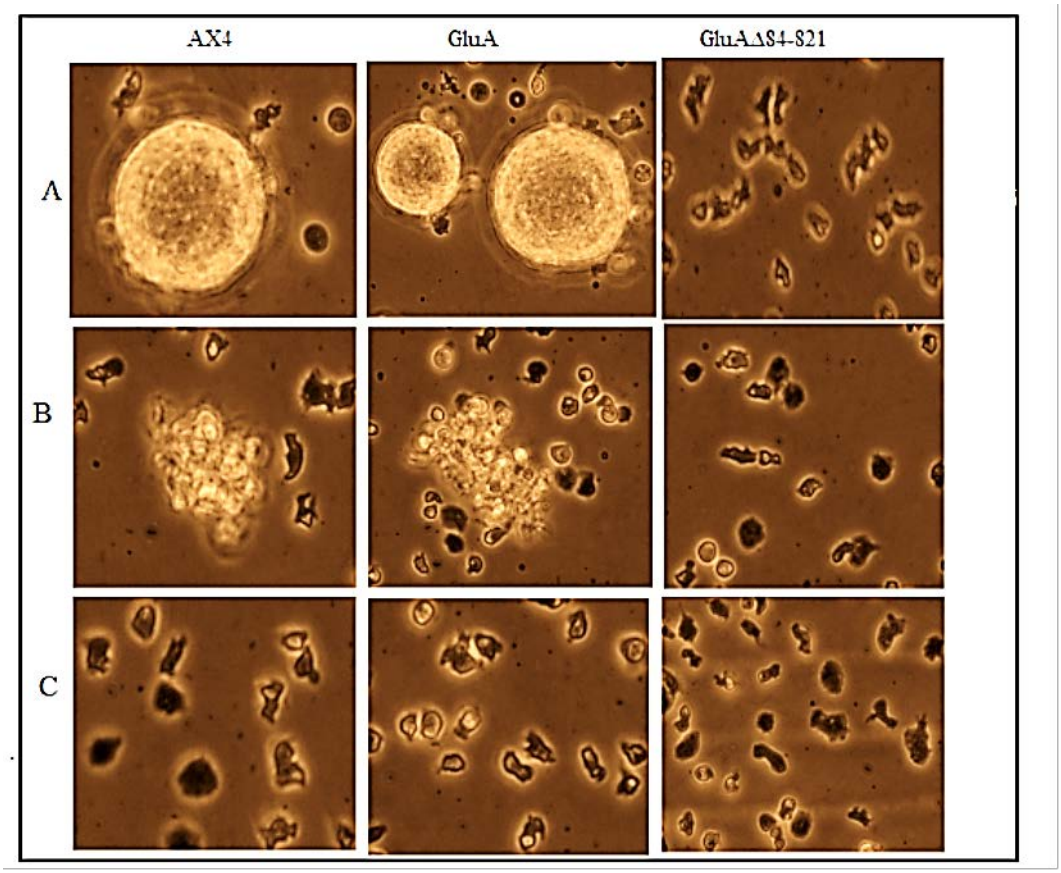

Figure 4 Ecological development analysis for Cell lines over-expression AX4, GFP-GluA, and GFP-GluAΔ84-821 under different concentrations of ethanol. (A) GFP-GluA over-expressing cell lines have wider aggregation compared to the AX4. (B) AX4 and GFP-GluA reduce the amount of aggregation in 1% ethanol concentration. GFP-GluAΔ84-821cell line failed to aggregate (C) All cell lines failed to aggregate.

Discussion

In this study we have analyzed the ecological effects of ethanol on the *D. discoideum* that overexpressed GluA protein to gain insight into its role in their resistance against ecological factors. It was demonstrated that the GluA protein localizes with lysosomes and endosome (13). Furthermore, over-expressing GluA protein causes significant defect in the rates of many cellular processes such as phagocytosis, pinocytosis and the rate of development. *Dictyostelium* has a perfect way to adapt to the environmental changes by using special mechanism to change their cell from unicellular to multicellular

case. Since the ethanol can accumulate as a result of fermentations so it represents an adverse environmental variation to organisms may have developed adaptive response (3). Interestingly, Ethanol can make physical changes of the environment of a cell (14) and in many cases ethanol alters the way by which the cell interacts with its environment. There were many studies deal with the molecular effects of ethanol in mammalian cells (15, 16). They have been found that the most effect of ethanol may create a membrane perturbation as a result of change in the lipid composition. There are new lines of studies to see the effect of ethanol on the cell growth in Tetrahymena (17). It has been reported that ethanol-inducing alterations caused in the disorder of the regular structure and the function of cell membrane. Nilson has found that the ethanol has many changes on Endocytosis and Proliferation of *Tetrahymena pyriformis*. He demonstrated that the critical effects of ethanol based on time, dose and a lag period (18). Ethanol effects the proliferation and decreased rate or capacity of cells to form food vacuoles. There was specific study to see the effect of ethanol on the *Escherichia coli* Plasma Membrane (19). The Effect of ethanol on spore germination and cell growth of *D. discoideum* has been studied by Hase (20). He found that the effect of ethanol on the cell growth was different between two strains of NC4 and A3 and the changes in the cell depended on the dose particular of the strain NC4 (20).

In this study, the effect of ethanol on *Dictyostelium* cell that over expression GluA protein was investigated. We found that ethanol did not affect the growth in low concentration. The growth rate and size of cells were slightly stable under low concentration. We suggest that cells made series of adaption action to live in that concentration. By increasing the concentration of ethanol from (1-2%); the cells change their strategy and the mechanism differ among the cell lines. There was reduction in the rate of growth. The cell that over expression GluA protein showed high resistance than the other cell lines. In high concentrations, however, both phototaxis and developing were affected by ethanol dose- dependently. In another study, the effect of ethanol on Tetrahymena showed that the high concentration of ethanol caused reduction in the cell line proliferation rate (21). GLuA showed more phototaxis rate compared to other cell lines, this could be due to lysosome enzymes have complex relationship with other

protein. Lysosome enzymes interact with Rab proteins which regulate the traffic and this increased the over expression of our genes, which in turns increased the rate of regulation and made the cells more resistance. In fact, cell plasma membrane is the main tool for contacting with their environment. Exposure to ethanol induces many change of plasma membrane by changing of the structure and function of the membrane.. Many studies of the ethanol effects on membrane such as the study of phospholipid reduction in the membrane after exposed to ethanol in *Bacillus subtilis* (22). Since the lysosome enzyme are released from the membrane and soluble fraction of the lysosome. On other hand, some lysosomes fused with plasma membranes during the vegetative growth (23). This may lead the overexpression of GluA to be more resistant to ethanol concentration. The cell line GFP-GluAΔ84-821 failed to aggregate and phototaxis in addition to that it has low growth rate and their cell size reduced more than the other cell lines. We think that the using of the first 84 amino acid might have abnormal binding which cause failed to produce the full enzyme length thus, make this cell line less resistance to the environment stress.

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Green synthesis of Magnetite Iron Oxide Nanoparticles using Al-Rawag tree (*Moringa oleifera* Lamarck) leaves Extract and Used in Tigris River Water Treatment

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Abstract: In this study, magnetite iron oxide nanoparticles were synthesized from aqueous ferrous chloride, ferric chloride and sodium hydroxide through a simple and eco-friendly route using *Moringa oleifera* leaves extracts, which acted as a reductant and stabilizer simultaneously. Characterizations of nanoparticles were done by using UV-visible, FT-IR, XRD, and SEM methods. The ultraviolet-visible spectrum of the aqueous solution containing magnetite iron oxide nanoparticle showed an absorption peak at round 430 nm. FT-IR graph showed peaks at 519 cm $^{-1}$ confirm the presence of Magnetite iron oxide nanoparticles in the synthesized samples. It is clear that the bioactive molecules present in the leaves extract of Al-Rawag tree interacted with the synthesized Magnetite iron oxide nanoparticles. The diffractogram exhibits six distinct diffraction peaks at 2θ values, as the (220), (311), (400), (422), (511) and (440) crystallographic planes of the inverse spherical magnetite crystal. The average crystallite size was evaluated by diffraction line broadening (d 311) using the Debye-Scherrer equation. The average size of Fe_3O_4 crystallites obtained from nanocomposite was about 40nm. The magnetite iron oxide nanoparticles synthesized by the help of Al-Rawag tree leaves extracts were scanned using SEM. It reveals that an iron oxide nanoparticle seems to be spherical in morphology.

The water treatment by magnetic iron oxide nanoparticles synthesize by Al-Rawag tree leaves extracts led to the reduction of the number of bacteria to 80% after twelve hours of treatment whereas water treatment by magnetic iron oxide nanoparticles for 24 hours led to kill all the bacteria in water.

Keywords: Green synthesis, Magnetite Iron Oxide Nanoparticles, Al- Rawag tree, Water treatment, **Tigris River**.

التخليق الصديق للبيئة لمنمنمات أوكسيد الحديد السوداء باستخدام مستخلص أوراق شجرة الرواق (مورينجا اوليفيرا لامارك) وأستخدامها في معالجة مياه نهر دجلة

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الخلاصة: في هده الدراسة تم تخليق منمنمات أوكسيد الحديد السوداء من المحلول المائي لكوريد الحديدوز، كلوريد الحديديك وهيدروكسيد الصوديوم بطريقة صديقة للبيئة باستخدام مستخلص أوراق شجرة الرواق (مورينجا اوليفيرا)، والتي تعمل كعامل مختزل و مثبت بالوقت نفسه. خصائص المنمنمات درست باستخدام مطياف الاشعة الفوق بنفسجية-المرئية، مطياف ناقل فورير للأشعة تحت الحمراء، حيود الاشعة السينية والمجهر الألكتروني الماسح. أظهر الفحص ان طيف الامتصاص للأشعة فوق البنفسجية-المرئية للمحلول المائي الحاوي على منمنمات أوكسيد الحديد السوداء له قمة امتصاص عند 430 نانومتر.

أظهر مخطط مطياف ناقل فورير للأشعة تحت الحمراء قمة عند 519 سم$^{-1}$ اثبتت وجود منمنمات اوكسيد الحديد السوداء في عينات التخليق. ومن الواضح ان الجزيئات الفعالة حيويا الموجودة في مستخلص أوراق شجرة الرواق متفاعلة مع منمنمات اوكسيد الحديد الاسود المخلق. اظهر مخطط الحيود ستت قمم حيود عند القيمة 2 ثيتا هي 220، 311 ، 400 ، 422 ، 511 و440 وان المخطط البلوري هو انعكاس للبلورات السوداء الكروية. أن معدل حجم البلورات قدر بوساطة خط الحيود الواسع (دي 311) باستخدام معادلة دبي-شرير. معدل حجم بلورات اوكسيد الحديد الموجودة في تركيب المنمنمات كان حوالي 40 نانوميتر. تم مسح منمنمات أوكسيد الحديد السوداء المخلقة بمساعدة مستخلص أوراق شجرة الرواق بوساطة المجهر الالكتروني الماسح ومنه يتبين ان شكل المنمنمات المخلقة كروي.

معاملة المياه بوساطة منمنمات أوكسيد الحديد السوداء المخلقة بوساطة مستخلص أوراق شجرة الرواق أدت إلى تخفيض عدد الجراثيم إلى 80٪ بعد اثنتي عشرة ساعة من المعاملة بينما معاملة المياه بواسطة منمنمات أوكسيد الحديد السوداء لمدة 24 ساعة أدت إلى قتل جميع الجراثيم في الماء.

الكلمات المفتاحية: التخليق الصديق للبيئة، منمنمات اوكسيد الحديد السوداء، شجرة الرواق ، معالجة المياه، نهر دجلة.

Introduction

Iron oxide nanoparticles play an important role in environmental remediation circles. As it removes both of organic and inorganic heavy metal pollutants from polluted water [1]. There are several chemical and physical methods available for synthesis of iron oxide nanoparticles. Those methods are used toxic or potentially hazardous as starting materials and more energy. However, it is still a big challenge to develop simple and reliable synthetic method for low-dimensional iron oxide nanostructures. The primary goal of nanotechnology is to improve simple ecofriendly method for synthesis of nanoparticles. The biomaterial such as microbes and plant extract can be used to prepare various types of nanoparticles and even nanorods. However, as some organisms are pathogens, it is risky to handle.

Microorganisms need maintenance of culture and controlled conditions such as temperature, pH and other factors for growth. Sometimes, the synthesis of nanoparticles utilizing plant parts could prove advantageous over other biological processes by eliminating the elaborate process of maintaining the microbial culture. Synthesis of iron oxide nanoparticles by using plant extract has been quite limited and few works have been reported. The reports are available for synthesis of hexagonal metallic iron, amorphous iron, and α-Fe_2O_3 by using tea extract [2, 3], and iron nanoparticles by using Aqueous *Sorghum bran* Extracts [4]. The tea extract mediated synthesized iron nanoparticles were found to be nontoxic when compared with iron nanoparticles prepared using conventional NaBH4 reduction protocols. The concentration of the tea extract in the reaction mixture plays an important role in the size and crystallinity (hexagonal metallic iron, amorphous iron, and R-Fe_2O_3) of the synthesized iron nanoparticles [3]. *Moringa oleifera* L., a wild herbaceous plant is very common in all tropical countries, including India. The stems are slender and often reddish in color, covered with yellowish bristly hairs especially in the younger parts. The leaves are oppositely arranged, lanceolate and are usually greenish or reddish; underneath measuring about 5 cm long *Moringa oleifera* (Moringaceae) is a small to medium evergreen tree widely distributed in Asia, Africa, and America. The plant is not only well known for high nutritional contents but also recognized for its therapeutic values [5]. The leaves of *M. oleifera* have been indigenously used for various medicinal purposes such as treating bronchitis, controlling glucose level, and reducing glandular swelling [6 , 7]. Numerous pharmacological investigations of *M. oleifera* leaves have been reported on anti-inflammation, anti-infection, antidiabetic, antioxidant, and antihyperlipidemic activities [8-13].

The present study aims to synthesize of magnetite iron oxide nanoparticles by ecofriendly method using Al-Rawag tree (*Moringa oleifera* Lamarck) leaves extract and used in tigers river water treatment.

Materials and Methods

Materials

The Al-Rawag tree were purchased from Regional Botany Garden Gherai'at, Baghdad. Ferric chloride hexa-hydrate ($FeCl_3.6H_2O$,AR), ferrous chloride tetra-hydrate ($FeCl_2.4H_2O$, AR) and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich Chemicals. All solutions were freshly

prepared using double-distilled water and kept in the dark to avoid any photochemical reactions.

Plant Material Collection

Al-Rawag tree leaves figure 1 were air dried for 10 days, and then kept in the hot air oven at 60°C for 24 to 48 h. The leaves were ground to a fine powder.

Figure 1. Al-Rawag tree (*Moringa oleifera* Lam.) leaves.

Synthesis of Magnetite Iron oxide Nanoparticles

Magnetite iron oxide nanoparticles were synthesized by dissolving $FeCl_2.4H_2O$ and $FeCl_3.6H_2O$ (1:2 molar ratio)in 500 mL of sterile deionized water and heated at 80℃ using magnetic stirrer. After 10 minutes, 25 mL of the aqueous solution of *Moringa oleifera* ethanolic extract was added to the mixture, immediately yellowish colour of the mixture changed to reddish brown colour. After 5 minutes, 100 mL aqueous solution of sodium hydroxide (26%) was added to the mixture with rate 3 ml/min for allowing the iron oxide precipitations uniformly. From the first addition of sodium hydroxide the reddish brown mixture changed to black suspended particles. The mixture was allowed to cool down to room temperature and the iron oxide nanoparticles were obtained by decantation, further dilution with sterile distilled water and centrifugation to remove heavy biomaterials of *Moringa oleifera*

ethanolic extract. The iron oxide nanoparticles were purified by dispersing in sterile distilled water and centrifugation for three times. The iron oxide nanoparticles after purification were dried overnight at 80℃ [14].

Characterization of magnetite iron oxide nanoparticles

Low concentration of the Magnetite (Fe_3O_4) nanoparticles was suspended in ethyl alcohol at room temperature, ultra-sonicated (Qsonica Sonicator Q500) (Tokyo, Japan) for 10 min and examined by X-ray diffractometer (Shimadzu, XRD-6000) (Tokyo, Japan) equipped with CuKαradiation source using Ni as filter at a setting of 30 kV/30mA. All XRD data were collected under the experimental conditions in the angular range $3^o \leq 2\theta \leq 50^o$.FT-IR spectra of *Moringa oleifera* leaf extract and magnetite iron oxide nanoparticles was obtained in the range of 4000-400 cm^{-1} with FT-IR spectrophotometer (IR-Prestige 21, Shimadzu) (Tokyo, Japan) using KBr pellet method. Scanning electron microscopy (SEM) analysis of the synthesized magnetite nanoparticles was done by SEM machine, Hitachi S-4500 (Tokyo, Japan). Thin films of magnetite nanoparticles were prepared on a carbon coated copper grid by just dropping a very small amount of the sample on the grid, extra solution was removed using blotting paper and then the film on the SEM grid were allowed to dry by putting it under a mercury lamp for 5 minutes. UV-vis spectroscopic studies were carried out using Shimadzu UV-1601 spectrophotometer (Tokyo, Japan) [15].

Tigers River water samples collection

Water samples were collected from Tigris River at Al- Adhamiya, Abu Nawas and Al-Doura sites in Baghdad, Iraq. Three samples taken from each site. The turbidity was measured using Milwaukee Mi415 Turbidity Meter, Martini Instruments, Inc., USA. [16]. Total count of bacteria in the water using the method of counting dishes and that planting 1 ml of the original or diluted sample at the center of Nutrient agar the plate incubated at 35ºC for 48 hours**.**

Determination the suitable amounts of MIONPS.

The test was down to determine the amount of magnetite iron oxide nanoparticles capable to killing the largest number of bacteria during the treatment of contaminated water experimentally by *E. coli*. The following concentrations were used 5 µg, 10 µg, 15 µg, 20 µg, 25 µg, 50 µg, 75µg and 1000µg / mL [17]. After

that the magnetite iron oxide nanoparticles particles are separated from solution by magnet bar.

Efficacy of iron oxide nanoparticles.

The synthesized nanoparticles were evaluated for their efficiency to treat raw water collected from Tigris River. Initial and final concentration of *E.coli* was measured by Plate Count Method as per the Standard Method for Water and Wastewater Analysis [18]. A dose of 1 gL^{-1} of nanoparticles was added to row water. The solution was stirred at 160 rpm at 37ºC for 12 and 24 hours. After treatment, the nanocomposites were magnetically separated and the supernatant was filtered through Whatman® Grade GF/C filter paper and the filtrate was analyzed for bacteria. The experiment was done in triplicates.

Results and Discussion

In the present study Al-Rawag tree cultivated from Seeds in nurseries in Baghdad, Iraq. Is recently introduced to Iraq from Egypt, Sudan, China and India for medicinal uses.

The UV Visible spectrum of magnetite iron oxide nanoparticles (MIONPs) in the aqueous Al-Rawag leaves extract is shows the absorption peaks at wavelengths of 430 nm Figure 2, which indicated the formation of magnetite iron nanoparticles.

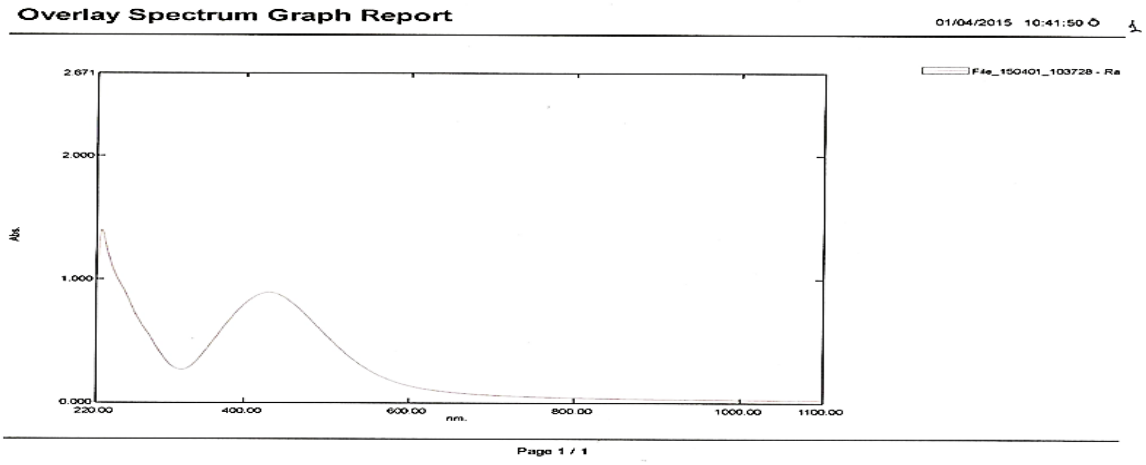

Figure 2. UV-visible absorption spectra of Magnetite iron oxide nanoparticles synthesized by Al-Rawag leaves extract.

This finding is in agreement with the studies [19, 20] in which found the absorption peaks of aqueous solution of silver nanoparticles found at range between 400-450 nm.

The FTIR spectra for Magnetite Iron oxide nanoparticles after treatment with the Al-Rawag leaves extract is shown in Figure 3. The FTIR spectra for Magnetite Iron oxide nanoparticles after treatment with the Al-Rawag leaves extract showed the peaks at 519 cm $^{-1}$. This confirms the presence of nanoparticles in the synthesized samples. The hydroxyl, sulphate and aldehyde group present in the Al-Rawag leaves extract are apparently involved in the bioreduction and stabilization of magnetite Fe_3O_4 -NPs. The results are in agreement with observation of several studies [21, 22].

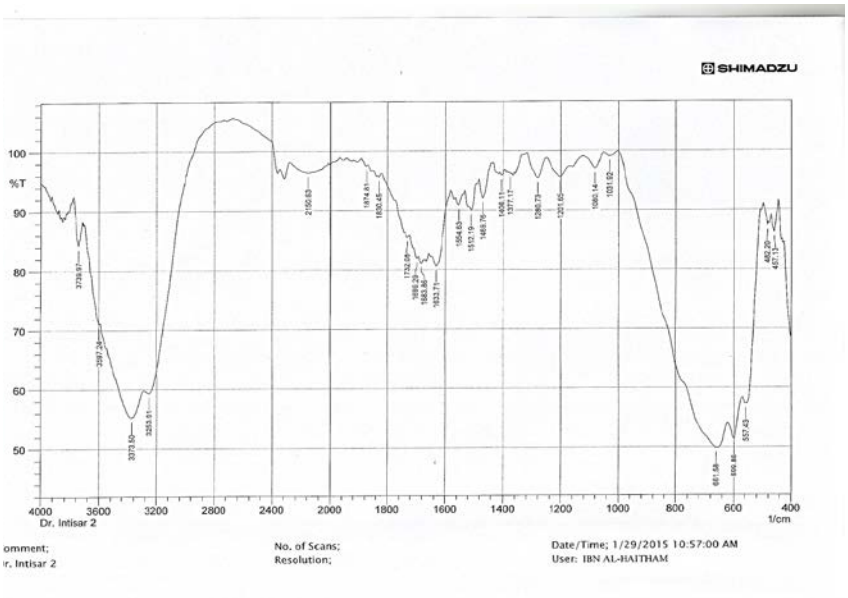

Figure 3. FTIR graph of Magnetite iron oxide nanoparticles synthesized by Al-Rawag leaves extract.

The XRD pattern of the Magnetite Iron oxide nanoparticles (MIONPs) is shown in figure 4.

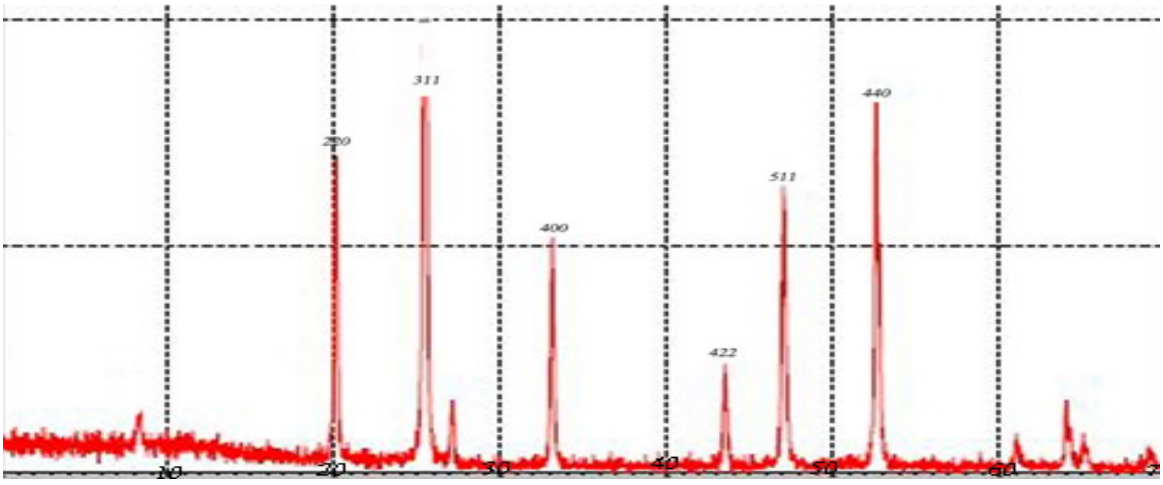

Figure 4. XRD patternof the Magnetite iron oxide nanoparticles synthesized by Al-Rawag leaves extract.

The results of the XRD pattern of the Magnetite Iron oxide nanoparticles (MIONPs), exhibits six distinct diffraction peaks at 2θ values. The diffraction angles of different peaks are corresponds to Fe_3O_4 nanoparticles. The X-ray power diffraction (XRD) results of nanoparticles confirmed that the synthesized product was a magnetite (Fe_3O_4). The results are in agreement with several studies dealing with green synthesis of MIONPs [23, 24].

The micrographs of scanning electron microscope of the magnetite iron oxide nanoparticles synthesized by Al-Rawag leaves extract is shown in figure 5.

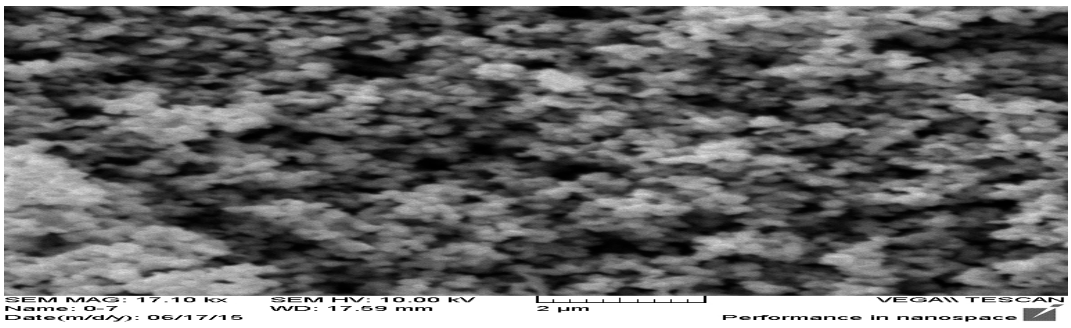

Figure 5. SEM image of Magnetite Iron oxide nanoparticles synthesized by Al-Rawag leaves extract.

The morphology of the particles was observed to be spherical. The results are in agreement with observation from SEM images [25].

The effect of Magnetite iron oxide nanoparticles (MIONPs) concentration and time of exposure on *E. coli* survival are shown in Table (1) and figure (6). The higher reduction percentage of *E. coli* survival was 100% by using 1mg/ml of MIONPs and after 24 hours of exposure, whereas the lowest reduction percentage of *E. coli* survival was 7% by using 5µg/ml of MIONPs and after 12 hours of exposure these finding is in agreement with [26, 27].

Table 1. Effect of MIONPs concentration and time of exposure on *E. coli* survival.

Concentration of MIOPNs*	Reduction percentage	
	12h.	24h.
5 µg /ml	7	10
10 µg /ml	10	13
15 µg /ml	16	25
20 µg /ml	21	37
25 µg /ml	27	48
50 µg /ml	54	67
75 µg/ml	73	85
1 mg/ml	91	100

MIOPNs*= Magnetite Iron Oxide Nanoparticles.

Figure 6. Antibacterial effect of magnetite iron oxide nanoparticles against *Escherichia coli* polluted water.

The results of Tigris River samples examination shows, the lowest turbidity and number of colony forming units were 70 NTU and 5×10^4 respectively at Adhamiya location. Whereas the highest turbidity and number of colony forming units were 100 NTU and 6×10^6 respectively at Ad-Dura area (Table 2).

Table 2. The sample collection from.

Sampling sites	Turbidity/NTU	Mean CFU/100ml
Al-Adhamiya	70	5×10^4
Abu Nawas	80	4×10^5
Al-Doura	100	6×10^6

The high level of turbidity and bacteria in Al-Doura site compared to others is attributed to the higher polluted materials discharged into the river from factories in Baghdad areas and moved down to the south at Al-Doura site.

The water treatment by magnetic iron oxide nanoparticles (MIOPNs) synthesis by Al-Rawag leaves extracts led to the reduction of the number of bacteria to 80% after twelve hours of treatment either water treatment by magnetic iron oxide for 24 hours led to kill all the bacteria in water (Table 3) , figure 7.

Table 3. Effect of magnetite iron oxide nanoparticles on microbes in Tigris River water samples.

Location of Samples	CFU/100ml before treatment	CFU/100ml after 12 hrs. treatment	CFU/100ml after 24 hrs. treatment
Al- Adhamiya	5×10^4	1×10^4	0
Abu Nawas	3×10^5	6×10^4	0
Al-Doura	6×10^6	12×10^5	0

*** The recorded value is mean value of 3 replicates.**

Figure 7. The separation of Magnetite iron Oxide Nanoparticles from water by Magnetic bar.

A-Tigris River water treated by (MIONPs), B- Control. The water treatment by magnetic iron oxide nanoparticles (MIOPNs) synthesis by Al-Rawag leaves extracts led to the reduction of the number of bacteria to 80% after twelve hours of treatment either water treatment by magnetic iron oxide for 24 hours led to kill all the bacteria in water.

The bacteria are used the iron oxide as a source of iron ions. The main mechanism by which these particles showed antibacterial activity might be via oxidative stress generated by ROS [26]. ROS, including superoxide radicals (O_2^-), hydroxyl radicals (^-OH), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), can cause damage to proteins and DNA in bacteria. In the present study, metal oxide (FeO) could be the source that created ROS leading to the inhibition of most of the pathogenic

bacteria found in Tigris River water samples. A similar process was also described by Kim *et al*., (2007) in which Fe_2^+ reacted with oxygen to create hydrogen peroxide (H_2O_2). This H_2O_2 consequently reacted with ferrous irons via the Fenton reaction and produced hydroxyl radicals which are known to damage biological macromolecules. Some authors have demonstrated that the small size of nanoparticles can also contribute to bactericidal effects. Lee *et al*., (2008) reported that the inactivation of *Escherichia coli* by iron nanoparticles could be because of the penetration of the small particles (sizes ranging from 10-80 nm) into *E. coli* membranes. Nano scale iron could then react with intracellular oxygen, leading to oxidative stress and eventually causing disruption of the cell membrane.

Studies on nanoparticles have also shown that antibacterial activity increased with decreasing particle size due to the higher surface area to volume ratio. Furthermore it shows better bactericidal activity in Gram-positive bacteria as compared to Gram-negative bacteria [25, 26, 27, 28].

For these reasons Magnetic Iron Oxide Nanoparticles (MIOPNs) synthesized by Al-Rawag leaves extracts was efficient in water treatment its kill all bacteria exists in water beside the ability of this material to absorbed some heavy metal, minerals, dyes and suspended materials such as clay, algi, fungi conidia etc, therefor cooperation in water purification.

Conclusions

Application of Magnetite Iron Oxide nanoparticle in water treatment showed potential effect against bacteria existed in Tigris River water. It is suggested that these may be used in future at large scale water purification. In particular, the use of magnetite nanoparticles as adsorbents in water treatment provides a convenient approach for separating and removing the contaminants by applying external magnetic fields.

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Measuring the levels of Malondialdehyde, Glutathione and Nitric Oxide in Sera of Men Blood Donors Infected with Toxoplasmosis

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Abstract: To estimate the oxidative stress status in patients with toxoplasmosis, 150 blood samples obtained from donors men of blood bank in Baghdad aged (18-55) years. Twenty three volunteers were positive for anti-*Toxoplasma gondii* IgG,IgM antibodies were evaluated by using ELISA (kit). Twenty normal healthy men of comparable age were considered as normal control. The results showed that the men infected with toxoplasma causes significant elevation of Serum malondialdehyde (MDA), and Nitric oxide (NO) levels compared with normal healthy control, while Serum glutathione (GSH) level for patients group was decreased significantly as compared with normal healthy group.

Key word : Toxoplasmosis - Malondialdehyde - Glutathione - Nitric oxide.

قياس مستويات Malondialdehyde ، الكلوتاثيون واوكسيد النتريك في مصول الرجال المتبرعين بالدم المصابين بداء المقوسات

هناء كامل حماد

جامعة بغداد- كلية العلوم للبنات

الخلاصة: لتقدير حالة االجهد التأكسدي لدى مرضى داء المقوسات الكوندية تم الحصول على 150 عينة دم من الرجال المتبرعين من مصرف الدم في بغداد الذين تتراوح أعمارهم بين (55-18) سنة وكان ثلاثة وعشرين متبرعا إيجابي لفحص المقوسات الكوندية، تم تقييم الأجسام المضادة باستخدام ELISA للكشف عن الاضداد المتخصصة المتمثلة بعدة فحص (kit) (Toxo IgG , Toxo IgM) . واعتبرت عشرين رجلا متقاربي الاعمار كمجموعة سيطرة ، وأظهرت النتائج أن الرجال الذين يعانون من الإصابة بداء المقوسات الكوندية يسبب ارتفاع كبير في مستوى malondialdehyde (MDA) ، وأوكسيد النيتريك (NO) في المصل مقارنة مع مستويات مجموعة السيطرة، بينما انخفض مستوى الكلوتاثيون (GSH) في مصل مجموعة المرضى بشكل ملحوظ بالمقارنة مع مجموعة السيطرة.

Introduction:

Toxoplasma gondii, an intracel-lular protozoan parasite, is the most common parasite found in devel-oped nations, infecting approxim-ately one-third of the world's popu-lation (1). The seroprevalence rate of toxoplasmosis is estimated to be between 20%-80% worldwide, tho-ugh latency is the most common state of infection (2). The Centers for Dis-ease Control and Prevention (2013) classified *T. gondii* as the leading cause of death by foodborne illness in the United States and it is con-sidered to be one of five neglected parasitic infections. Humans may remain infected for life and will stay asymptomatic unless immunos-uppression occurs (3). Humans beco-me infected with *T. gondii* through direct contact with o ocysts in cat feces or through eating meat con-taminated with the extra intestinal form of *T. gondii* (4). Oxid-ative stress may lead to cellular damage that can be confirmed by markers of cellular disruption. Meas-urement of lipid peroxidation prop-osed as the principle indicator of an agent indu-ced free radicals Reactive oxygen species degrade polyunsaturated lipi-ds, forming malondialdehyde (MDA) (5). This compound is a reactive aldehyde and is one of the many reactive electrophile species that ca-uses toxic stress in cells and forms covalent protein adducts which are referred to as advanced lipoxidation end products, in analogy to advanced glycation end-products (6). The prod-uction of this aldehyde is used as a biomarker to measure the level of ox-idative stress in an organism (7, 8). Glutathione (GSH) is a cysteine. Glu-tathione has antio-xidant properties since the thiol group in its cysteine moiety is a reducing agent and can be reversibly oxidized and reduced. It is one of the most important cellular antioxidants; it defends the cell agai-nst oxidative damage by undergoing reaction with free radicals and pero-xidase (9). Nitric oxide (NO) is a free radical, an uncharged molecule with an unpaired electron. NO plays multiple roles in both intracellular and extracellular signaling mechan-isms (10). NO is the product of arg-inine metabolism and one of the most effective O_2-free toxins.It was stated that nitric oxide levels in crease in *Tox-oplasma* infection and this increase is accepted as a physiological result of the immune response to *Toxoplasma* infection. The protective role of the increase in nitric oxide levels is noted in taking *Toxoplasma* infection under control, particularly in the chronic phase of the infection. It was

reported that the increase in nitric oxide against intra- cellular infection is necessary to control the host in toxoplasmosis (11). The aim of the present study was to evaluate the volume of oxidative stress by estim-ating the levels of Serum malon-dialdehyde, Glutathi-one and levels of nitric oxide which are indi-cators for the anti-oxidative status in men with to-xoplasmosis as compared with norm-al healthy control.

Materials and Methods:

Collection of Samples

The study was carried out on 150 blood samples from donor men of National blood transfusion center in Baghdad, age (18-55) year. We take 5 ml of venous blood and collected in polystyrene tubes and centrifuged at 500 × g for 15 min

Analysis of the Serum Samples

The Serum was then removed and stored at-20℃ until analysis by using more specific tests ELISA to detect antibodies specialized type of IgG &IgM (Biochech-USA) in acute and the chronic infe-ction by Electro Clia manner using a Roche Cobas e411. Positive samples were considered as the case group (23) men and the rest as the control (20) men. All of the samples were found IgM seronegative.

Determination of Malondialdehyde Level

As an indicator of lipid peroxi-dation, malondialdehyde serum level was determined at 532 nm using thio-barbituric acid according to the method of Satoh (12). Malondialdehyde conce-ntration was determined using 1,1,3,3 tetraethoxypropane as standard and ex-pressed as nmol/mg protein.

Determination of Glutathione Level

Glutathione level was measured using Tietz method (13). Glutathione in the supernatant was assayed at 412 nm by monitoring the absorbance of 5, 5-dithiobis 2-nitrobenzoic acid for 5 minutes. Glutathione level was determ-ined from a standard curve and expr-essed as nmol/mg protein.

Determination of NO Level

Serum nitrite plus nitrate conc-entration as an index of serum NO levels were determined by the method described previously (14). Quantifica-tion of nitrite and nitrate was based on the Griess reaction, in which chrom-ophore with a strong absorbance at (450) nm is formed by reaction of nitrite with a mixture of naphthyl ethy-lenediamine and sulphanilamide. The absorbance was measured in a spectro-photometer to give the nitrite conc-entration. For nitrate detection, a seco-nd sample was treated with

copporised cadmium in glycine buffer at pH (9.7) to reduce nitrate to nitrite, the concent-ration of which thus represented the total nitrite plus nitrate. A standard curve was established with a set of serial dilutions (10-8 –10-3mol/l) of sodium nitrite. All samples were assayed in duplicate NO system activity measured at 545 nm by a spectrophotometer

Statistical Analysis

The data were statistically analy-zed using t-test .P < 0.05 were conside-red as statistically significant.

Result &Discussion

Result shows the 23 (15.33 %) of 150 men were positive for anti *T. gondii* IgG antibodies while all of the samples were found IgM seronegative. *Toxo-plasma gondii* is an obligate intrac-ellular protozoan in birds and mam-mals. The life-long presence of dorm-ant stages of this parasite in the brain and muscular tissues of infected humans is usually considered asympt-omatic from the clinical point of view. (15). *T. gondii* has been recognized as a human parasite for almost a century, yet much is still unknown about host reactions to its chronic presence (16).

Free radical activity has been impl-icated in the pathogenesis of a variety of diseases, and many infectious dis-eases have been proved to be direct and indirect sources of large numbers of these biotoxic agents (17). The previous studies suggest that these changes reflect ability of *T.gondii* to induce oxidative stress .

Our study demonstrated there is statistically significant differences were found between the patients and the control group in terms of MDA, GSH, and NO parameters (P < 0.05). (Table 1).

Table-1: Descriptive statistics of malondialdehyde, glutathione and nitric oxidebetween seropositive patients and healthy control

Parameter	Control group n=20 (Mean ±SD)	Toxoplasmosis group n=23 (Mean ±SD)	*P* value
Serum MDA level (n mol/ml)	4.39±0.605	25.685±1.274	<0.001
Serum GSH level (n mol/ml)	5.185±0.065	2.461 ±0.062	<0.001
Serum NO level (n mol/ml)	19.59±0.645	29.508±0.621	<0.001

In this study, Malondialdehyde level in men of the case group was significantly higher than in the control (25.685 ± 1.274. 4.39 ± 0.605) , p < 0.001.Lipid peroxidation is the process of oxidative degeneration of polyun-saturated fatty acids membranes of tissues because of free radical gen-eration. A common marker of lipid pe-roxidation is malondialdehyde which has been frequently used as markers of oxidative stress in response to different agents such as infection (18). In this study, malondialdehyde serum level had increased in men of the case gro-up. The increased lipid peroxidation shows that *T. gondii* infection-induced ROS are not totally scavenged by the antioxidant enzymes in tissues. Num-erous studies have shown that malondialdehyde level increased in serum of toxoplasmosis patients (19 , 20).

Glutathione level in serum of men of the case group was significantly lower than in the control (2.461 ± 0.062. 5.185 ± 0.065) , P < 0.001. A significant depletion of glutathione were noted in the present study in serum of men infected with *T. gondii* which was the result of high oxidative stress and glutathione over-use by the cells. Our finding is in agreement with the results of the previous reports in which the infection with *T. gondii* caused a decrease in the level of the depleted glutathione in serum of toxoplasmosis patients.(21) Several studies have shown that decrease of antioxidant enzyme activities in *T. gondii*- infected patients are associated with a depletion of glutathione and an increase of lipid peroxidation, all of which can lead to oxidative stress and finally cell death (22). Glutathione is the most significant component, which directly quenches ROS such as lipid peroxides and plays major role in xen-obiotic metabolism ,when an indivi-dual utilized for conjugation making it less available to is serve as antioxidant (23).

The results have showed increased level of serum Nitric Oxide in the *T. gondii* patients as compared with normal healthy control (29.508 ± 0.621.19.59 ± 0.645) , P< 0.001. NO is the product of arginine metabolism and one of the most effective O2 free toxins (24). There are also previous studies reporting an increase in the NO level in parasitic diseases(25). It can be stated that the NO level increase as a defensive mechanism to protect the patient against the harmful effects of the parasite.

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Efficiency of insect predator *Chrysoperla carnea* against *Bemisia tabaci* on eggplant crop under greenhouse conditions

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Abstract: The study was aimed to measure the effectiveness of predator *Chrysoperla carnea* in reducing the population of whiteflies *Bemisia tabaci* on eggplant crop in greenhouses. In 2013, the lacewings predators were released on eggplant crop in greenhouse at rate of 20, 40, 80, 100, 150 and 200 eggs / 2m distance; 25, 50 and 75 larvae / 2m distance; and 5 larvae / plant in comparison with the insecticide Esters as well as the control treatment. Results were measured after seven days. The highest efficiency was achieved by releasing 50 larvae /2 m (58%) without significant differences from the efficacy of insecticide - Aster (62%) and both were did not differ significantly from the effect of 75 larvae / 2m , 100, 150 , 200 eggs /2 m and 5 larvae / plant. Weakest results were by releasing 20 eggs / 2m, but it did not differ significantly from 40 and 80 eggs / 2m. Generally the efficiency of the predator increased with increasing of nymphs' population density at a decreasing rate with the increasing of population density during the season. At the following season (2014) the release of 25 first instar larvae of predators (*C. carnea)* / plant achieve 34% reduction in the pest nymphs (*B. tabaci*) and 38% reduction in the pest eggs (*B. tabaci*); at the release of 50 larvae /plant the reduction in the nymphs was 69% while it was 70% for the eggs, releasing of 200 larvae has 89% and 82% reduction in the nymphs and eggs, respectively. The population density rate of adult predator reached at the end of the season to 40 adult/ plant, which achieve %38 reduction in the population density of whiteflies adult.

Key words: *Chrysoperla carnea ; Bemisia tabaci*; biological control

تقييم فاعلية المفترس الحشري اسد المن الاخضر في خفض سكان الذبابة البيضاء على نبات الباذنجان تحت ظروف البيوت البلاستيكية

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الخلاصة: هدفت الدراسة الى قياس فاعلية المفترس اسد المن في خفض سكان افة الذباب الابيض على نبات الباذنجان في البيوت البلاستيكية . ففي عام 2013 اطلق بيض المفترس بمعدل 20 و 40 و 80 و 100 و 150 و 200 بيضة/ 2م طول ويرقات المفترس بالطور الثاني بمعدل 25 و 50 و 75 يرقة / 2م طول و بواقع 5 يرقات / نبات وقورنت مع مبيد الاستر علاوة على معاملة السيطرة وقيست النتائج بعد سبعة ايام. اظهرت النتائج ان اعلى كفاءة للمكافحة بهذا المفترس كانت بإطلاق 50 يرقة/2م (58%) دون فارق معنوي عن استعمال مبيد الاستر (62%) والاثنان لم يختلفا معنويا عن فاعلية اطلاق 75 يرقة/2م و100 و150 و 200 بيضة /م و 5 يرقات/نبات . اضعف النتائج كانت باطلاق 20 بيضة / 2م الا انها لم تختلف معنويا عن اطلاق 40 و 80 بيضة /2م. بشكل عام ارتفعت كفاءة المفترس مع ارتفاع الكثافة العددية للحوريات بمعدل متناقص مع زيادة الكثافة العددية خلال الموسم. وفي الموسم التالي 2014 أطلقت 25 يرقة في الطور الاول و هذا ادى الى خفض حوريات الأفة الى 34% و خفض بيوض الأفة الى 38% وايضاً أدى اطلاق 50 يرقة / نبات الى خفض حوريات الأفة الى 69% و70% للبيض و اطلاق 200 يرقة ادى الى خفض حوريات الأفة الى 89% و82% للبيض. ان نسبة كثافة سكان المفترس وصلت في نهاية الموسم الى 40 يرقات /نبات والذي ادى الى خفض سكان افة الذباب الابيض الى 38%.

Introduction

Bemisia tabaci (Homoptera: Aleyrodidae) has been known as the most devastating agricultural pests worldwide and a serious pest in glasshouses in Iraq. This species can damage a broad range of crops, both **:** directly, by having a high reproduction rate and a short generation time, the large numbers of offspring can affect plants negatively through feeding. This feeding cause's leaf chlorosis, leaf withering, consequently, reduces the plant growth rate and yield (1). Indirectly, they are act as a vector of different phytopathogenic viruses (2). A small population of whiteflies could be sufficient to damage hosts considerably. Furthermore, the insect secretes large quantities of honeydew that cause a reduction in photosynthetic potential when colonized by fungi.

Biological control through natural enemies is an effective strategy to control *B. tabaci*. Many predators can attack whiteflies *B. tabaci* efficiently. *Chrysoperla carnea* is one of these promising predators (3). Predator, *C. carnea* (Stephens) (Neuroptera; Chrysopidae), is known as an opportunistic feeder of soft bodied insects including whitefly (4). One

larva of *C. carnea* could consume 510.8 whitefly pupae during its life span (5).

The purpose of this study was to evaluate the effectiveness of *C. carnea* in reducing population density of whitefly on eggplant crop under greenhouse conditions.

Materials and methods

Predator mass Rearing**:**

The adults were maintained in rearing glassy canes 8 x 14 cm. The upper aperture was blocked with fabric clothes and supply with food (yeasts, sugar and distal water at ratio 4, 7, and 10 respectively) (6). Adults supplied via cotton swabs, with the water and artificial diet that mentioned daily until pupation. The eggs laid by females were harvested daily. Newly hatched larvae were fed eggs of *Ephestia* spp. The rearing of the predator was carried out at temperature 28^{o}C with 16:8 (light: dark) (7; 8)

Greenhouse experiments**:**

The field experiments were carried out under greenhouse conditions cultivated with eggplant crop in Baghdad- Iraq to control the whitefly *B. tabaci* by using the predator *C.carnea.* There were three treatments namely, chemical (by using Astra pesticide), biological by releasing the predator *C. carnea* and none treated as a negative control. The numbers of nymphs of flies were counted per leaf, before and after 7 days of application (releasing), in laboratory, using the stero-microscope. The efficacy percentage was calculated according to Henderson and Tilton formula (9).

$$\% \text{ efficacy } = \frac{\text{n in co before treatment x n in t after treatment}}{\text{n in co after treatment x number in t before treatment}} \text{ x } 100$$

Where
n= number
co= control
t= treated

Experiment during season 2013

The natural enemy was released as eggs in an average of 20, 40, 80, 100, 150, and 200 eggs/ 2m distance, and as larvae at second instar in an average of 25, 50, and 75 egg/2m distance and 5 larvae/ plant. In addition to two controls / the positive control (Spraying Aster pesticide) and negative control without any treatment were tested.

Experiment during season 2014

In this season only the first larval instar of the predator (*C. carnea*) were released, at an average of 25, 50, and 200 larvae/ plant.

Statistical analysis

All experiments were assigned to randomized complete design, using SASS version 20 software programs, where the mean values compared using Duncan's multiple range test. Standard error was calculated by using Microsoft excel 2007 program. Each treatment repeated three times.

Results and Discussion

The results revealed that the highest percentage of the efficacies were achieved by releasing 50 larvae /2 m (58%) without significant differences from the efficacy of insecticide -esters (62%) and both did not differ significantly from the effect of releasing 75 larvae / 2m and 100, 150 , 200 eggs /2 m and 5 larvae / plant. The weakest result was by releasing 20 egg/2m, which did not differ significantly if compared with releasing 40 and 80 egg/ 2m treatments (fig. 1). It has been noticed that the efficacy of the predator increased when the number of nymphs increased and then the average of the predator efficacy decreased due to decline in pest population (fig. 2). In this case, where the tendency is decreasing gradually until those equal, similar to the second type of functional response to the variety of prey densities, a pattern shown by a lot of predators to prey densities variety of which are determined by predator saturation occurs and time of treatment, a type certified to reverse density (inversely density dependent) (10).

In the second year 2014, releasing of the first larval instar of the predator at average of 25 larvae/ plant, caused reduction in population of pest nymphs to 34% and pest eggs to 38%. The efficacy of the predator (*C. carnea*), when released at an average of 50 larvae/ plant were 69 % for nymphs and 70% for eggs. The reductions were 89% for nymphs and 82% for eggs after one week of releasing 200 larvae of the predator. Under weekly monitoring the number of adult predators per one house was 40 adults which reduced the density of pest population up to 38% (fig. 4).

In similar studies (11) demonstrated the same results of the study where the *C. carnea* considerably decreased the whitefly density on eggplant crop. (12) found that the predators reduced whitefly densities on canola crop up to 52 and 27.5% by releasing 10000 and 150000 egg, respectively. It seems that the lowest number of predator that can achieve noticeable reduction is 100 egg/ plant or more, as figured out by (13), where 50 egg/ plant did

not reduce the thrips density on mango trees; whereas, 100-200 egg/plant significantly reduced thrips density. (14) recorded the highest reduction in the density of whitefly on cotton by releasing 3000 egg/ acre of *C. carnea* after four week of releasing. (15) succeeded in controlling aphids by releasing one predator insect per three aphids in greenhouse cultivated with capsicum.

Rearing of *C. carnea* in the glass cages proved better management method in comparison with others methods; this will save labor health and environment and will be more sustainable than other type's pest managements. As mentioned by (16), releasing one larva at the second instar per 5 aphids *Pentalonia nigronervos* is enough to reach 78% predation rate.

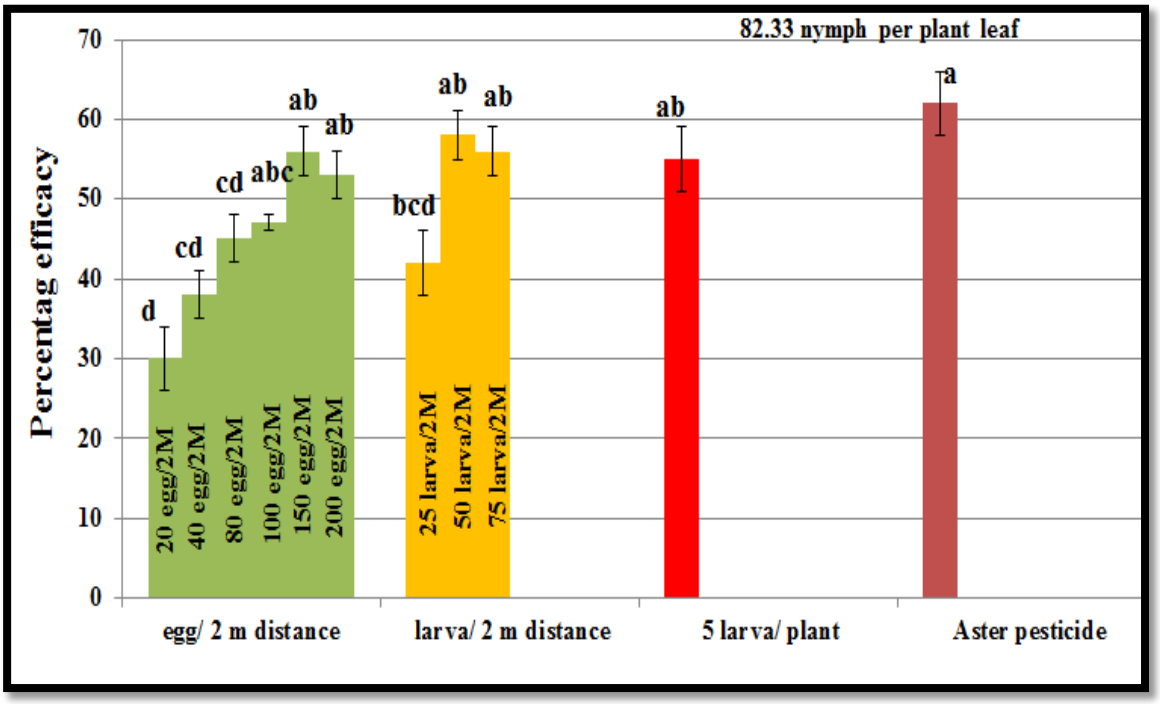

Figure 1: The percentages of efficacy of Aster pesticide and different stage and level of predator *Chrysoperla carnea.*

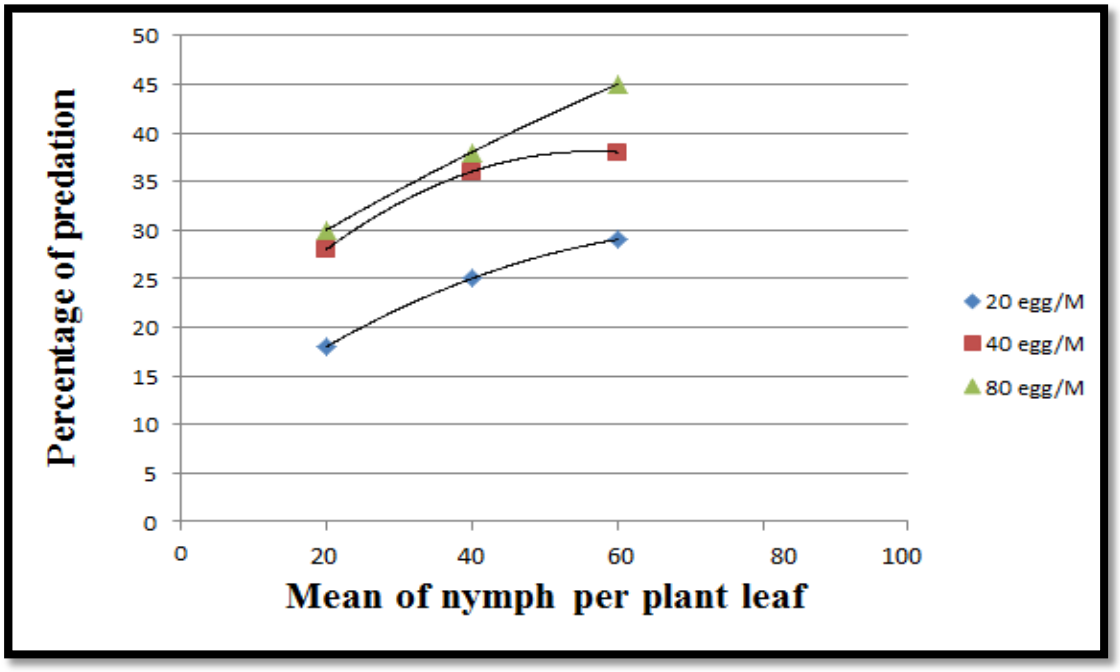

Figure 2: The percentages of efficacy of the predator *Chrysoperla carnea* at different nymph pest densities of *Bemisia tabaci*.

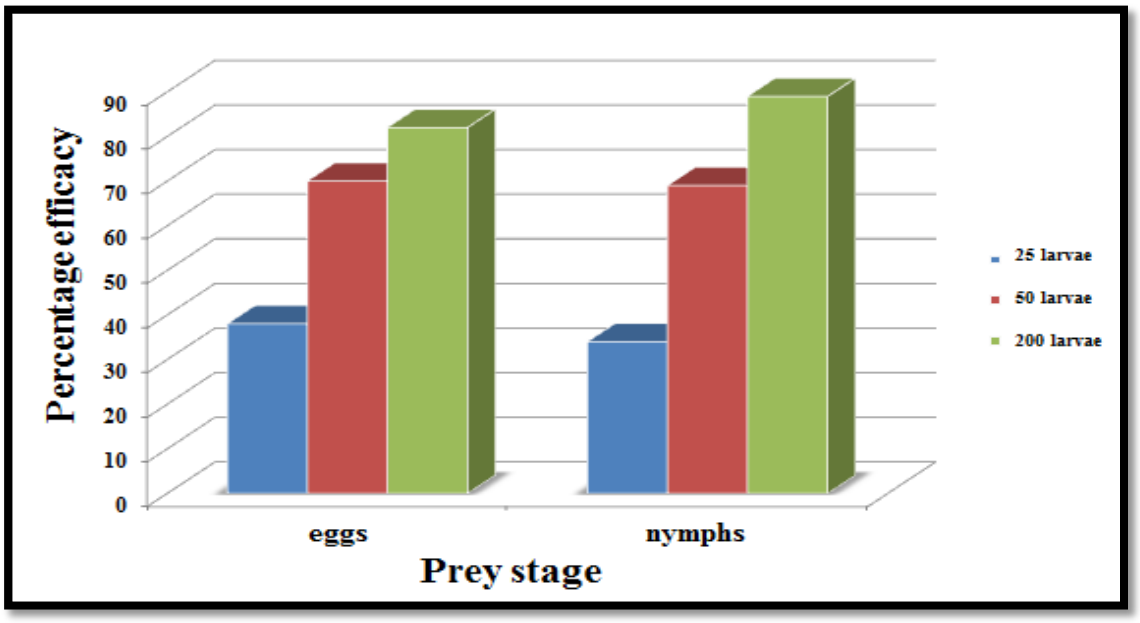

Figure 3: The percentages of the predation efficacy of the first larval instar of *Chrysoperla carnea*.

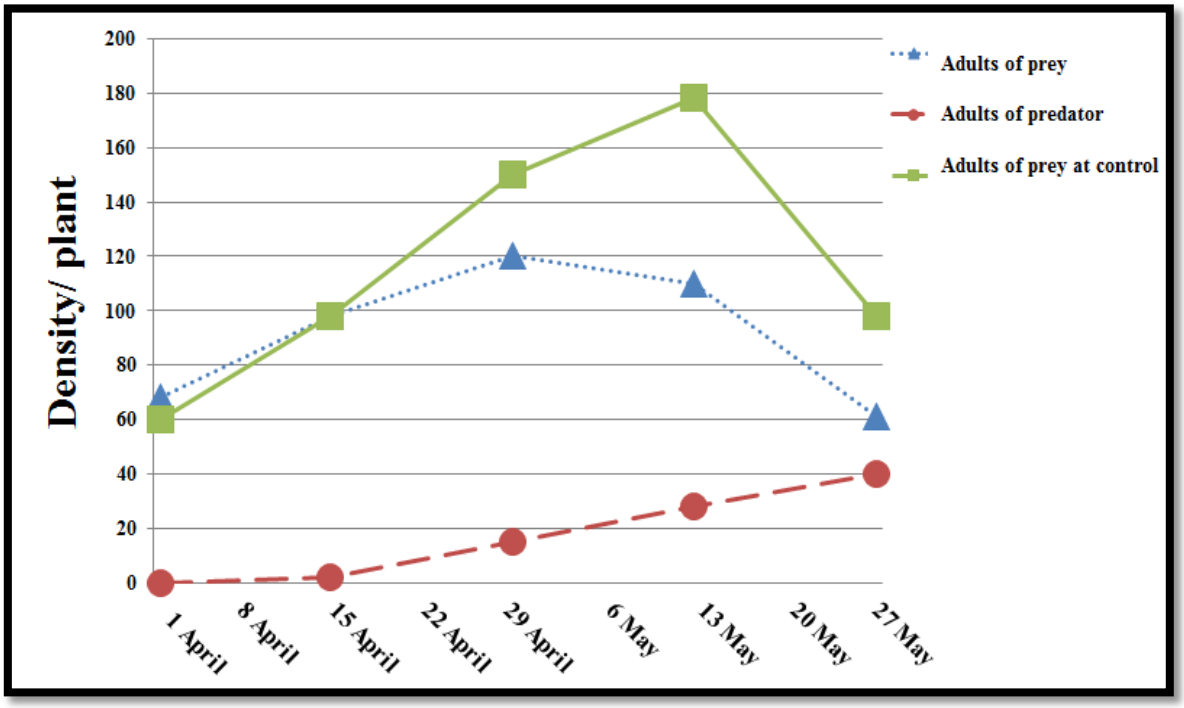

Figure 4: The prey density of whiteflies (*Bemisia tabaci*) adults and predator density of green lacewing (*Chrysoperla carnea*) adults after two months of releasing larvae of the predator

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Study the Protective Effect of Radish (*Raphanus sativus*) Seeds Extract against Harmful Effects of Sodium Nitrite on Some Physiological and Histological Parameters in Male Rabbits

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Abstract: The aim of this study is to investigate the protective effect of Radish (*Raphanus sativus*) seeds ethanolic extract 70% against harmful effects of sodium nitrite ($NaNO_2$) on some physiological and histological parameters of liver and kidney in male rabbits. Twenty five adult male rabbits were used in this study, divided into five groups (five rabbits in each group) and intubated daily for 30 days. Group T1: intubated orally 20 mg/kg $NaNO_2$, Group T2: intubated orally 20 mg/kg $NaNO_2$ + 50 mg/kg of ethanolic extract from *Raphanus sativus* seeds, Group T3: intubated orally 20 mg/kg $NaNO_2$ + 100 mg/kg of ethanolic extract from *Raphanus sativus* seeds, Group T4: intubated orally 20 mg/kg $NaNO_2$ + 200 mg/kg of ethanolic extract from *Raphanus sativus* seeds as well as Group C: control intubated orally distilled water. In rabbits intubated with $NaNO_2$, the results showed that there were increasing in the level of liver enzymes (AST, ALT and ALP) and kidney functions parameters (Uric acid and Creatinine) in comparison with normal rabbits (control). In rabbits intubated with $NaNO_2$ + 50,100,200 mg/kg of ethanolic extract of *Raphanus Sativus* seeds, this study explained that there were decreasing in the level of liver enzymes (AST, ALT and ALP) and kidney functions parameters (uric acid and creatinine) in comparison with rabbits intubated with $NaNO_2$ only. Histological sections of liver and kidney of sodium nitrite treated group revealed congestion of blood vessels, infiltration, fatty degeneration, While histological examination findings of sodium nitrite + *Raphanus sativus* seeds extract treated groups revealed healing of liver and kidney tissue. In conclusion according to results obtained from this study that alcoholic extract of *Raphanus sativus* seeds in dose (50,100,200 mg/kg) have protective role against harmful effect of sodium nitrite on liver and kidney in male rabbits.

Keywords: Radish seeds, Sodium nitrite, Liver, Kidney, Rabbits.

دراسة التاثير الواقي لمستخلص بذور الفجل (*Raphanus sativus*) ضد التأثيرات الضارة لنتريت الصوديوم على بعض المؤشرات الفسلجية والنسجية في ذكور الارانب

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الخلاصة: هدفت الدراسة الحالية الى معرفة التأثير الواقي للمستخلص الكحولي 70% لبذور الفجل ضد التاثيرات الضارة لنتريت الصوديوم $NaNO_2$ على بعض المؤشرات الفسلجية والنسجية للكبد والكلى في ذكور الارانب. استعملنا في الدراسة الحالية 25 ارنب ذكر بالغ قسمت الى 5 مجاميع (5 ارانب في كل مجموعة) وجرعت فمويا يوميا لمدة 30 يوم. المجموعة T_1: جرعت فمويا ب 20 ملغم/كغم من مادة نتريت الصوديوم، المجموعة T_2: جرعت فمويا ب 20 ملغم/كغم من مادة نتريت الصوديوم واعطيت المستخلص الكحولي لبذور نبات الفجل بجرعة 50 ملغم/كغم، المجموعة T_3: جرعت فمويا ب 20 ملغم/كغم من مادة نتريت الصوديوم واعطيت المستخلص الكحولي لبذور نبات الفجل بجرعة 100 ملغم/كغم، المجموعة T_4: جرعت فمويا ب 20 ملغم/كغم من مادة نتريت الصوديوم واعطيت المستخلص الكحولي لبذور نبات الفجل وبجرعة 200 ملغم/كغم، بالاضافة الى المجموعة C: مجموعة السيطرة جرعت فمويا بجرعة من الماء المقطر مساوية لحجم الجرعة المعطاة لمجاميع المعالجة. اوضحت نتائج الدراسة الحالية في الارانب المعاملة بمادة نتريت الصوديوم حصول ارتفاع في مستوى انزيمات الكبد (ALP, AST, ALT), مؤشرات وظائف الكلى (حامض اليوريك, الكرياتينين) بالمقارنة مع مجموعة السيطرة. في الارانب المعاملة بمادة نتريت الصوديوم والمستخلص الكحولي لبذور نبات الفجل 200,100,50 ملغم/كغم نلاحظ حصول انخفاض في مستوى انزيمات الكبد (ALP ,AST, ALT), مؤشرات وظائف الكلى (حامض اليوريك, الكرياتينين) بالمقارنة مع الارانب المعاملة بمادة نتريت الصوديوم فقط. بينت المقاطع النسجية للكبد والكلى للمجموعة المعاملة بنتريت الصوديوم ظهور احتقان الاوعية الدموية، ارتشاح، تنكس دهني. بينما بينت نتائج الفحص النسجي للمجاميع المعاملة بنتريت الصوديوم والمستخلص الكحولي لبذور نبات الفجل وجود حالة اصلاح لانسجة الكبد والكلى. اعتمادا على نتائج الدراسة الحالية نستنتج ان المستخلص الكحولي لبذور نبات الفجل بجرعة (50 و100 و 200 ملغم/كغم) يمتلك الدور الوقائي ضد التاثيرات الضارة لنتريت الصوديوم على الكبد والكلى في ذكور الارانب.

كلمات مفتاحية بذور الفجل ، نتريت الصوديوم ، الكبد ، الكلى ، الارانب.

Introduction

Preservatives defined as a natural or synthetic substance that are added to fruits, vegetables, cosmetics and pharmaceuticals, for increasing their shelf life and maintaining their quality and safety by inhibiting the fermentation, acidification, microbial contamination and decomposition, and preservative also added for crude meats, fish and poultry product [1]. Preservatives may be added to the food including nitrate and nitrite, benzoic acid and monosodium glutamate [2]. In the market, there are many foods containing different types of preservatives. These chemicals may be of risk for human consumption [3]. Humans are exposed to nitrite through food and drinking water [4]. Nitrites are of great importance and concern to man and animals because they have mutagenic, carcinogenic , teratogenic and embryotoxic activities [5]. In relation to reproductive toxicity, nitrites may induce abortion in experimental animals[6]. Nitrite cause harmful

effect on the respiratory function as a result of acute intoxication and being responsible for methaemoglobinemia (MetHb) [7]. Nitrite methaemoglobinemia is potent process for free radical generation [8]. Nitrite and other food preservative may react with amines of the foods in the stomach and formation of nitrosamines and free radicals, These products may increase lipid peroxidation, which has adverse effect to various organs involving liver and kidney [9]. Antioxidants have been reported to prevent oxidative damage caused by free radical and may prevent the occurrence of disease. It can interfere with the oxidation process by reacting with free radicals, chelating, catalytic metals and also by acting as oxygen scavengers [10]. Antioxidants are found in varying amounts in foods such as vegetables, fruits, seeds [11]. Many plants contain substantial amounts of antioxidants such as vitamin C, vitamin E and flavonoid and carotenoids [12].

Materials and Methods

Plant materials

The plant materials (seed) were obtained from commercial sources from Baghdad and then deposited to be identified and authenticated at the National Herbarium of Iraq Botany directorate in Abu-Ghraib. The plant materials were extracted according to [13].

Phytochemical analysis:

detection of alkaloids, saponins, tannins, terpenoids, and flavonoids according to [14],[15].

Animals and experimental design

In this study, 25 adult male rabbits were kept in conditioned room (22-25°C) with providing proper ventilation and had free access to water and standard pellet diet along the experimental period. Rabbits were left for two weeks for adaptation with the experimental conditions. Animals treated daily for 30 days. They were randomly divided into five groups, (5 rabbits/group). Group C (control): administered distilled water orally, Group T1: Animals in this group were administered 20 mg/kg B.W of sodium nitrite orally by gavage needle, Group T2: Animals in this group were administered 20 mg/kg B.W of sodium nitrite plus 50 mg/kg B.W of ethanolic extract from (*Raphinus sativus*) orally by gavage needle, Group T3: Animals in this group were administered 20 mg/kg B.W of sodium nitrite plus 100 mg/kg B.W ethanolic extract from (*Raphinus sativus*) orally by gavage needle. Group T4: Animals in this group were administered 20 mg/kg B.W of sodium nitrite plus 200 mg/kg B.W ethanolic extract from

(*Raphinus sativus*) orally by gavage needle.

Blood sample collection

After 30 day of experiment fasting blood samples were collected from animals by cardiac puncture technique. Serum was separated by centrifugation of clotted blood at 4000 rpm for 15 min and then serum was kept at appendrof tube in deep freeze for biochemical study that include**.**

Determination of liver enzymes and kidney function parameters

Serum liver enzyme and kidney function parameters were determined using the linear kit**.** Serum aspartate aminotransfrease (AST) activity measured according to[16]**,**serumalanine aminotransfrase (ALT) activity was measured according to [17]**,** serum alkaline phoasphatase (ALP) activity was measured according to [18]**,** serum uric acid was measured according to [19], serum creatinine was measured according to [20]**.**

Histololgical examination

At the end of the experiment , the treated animals were sacrificed liver and kidney removed and then fixed in 10% formalin for 24 hours. The tissue was routinely processed and sectioned by rotary microtome and stained with hematoxylin and eosin for histological studies. Histological study was according to [21].

Statistical analysis

The statistical analysis system – SAS (2012). Least significant difference LSD was used to compare significant between means in this study

Results

A. Phytochemical Analysis

The results of phytochemical analysis explained that *Raphanus sativus* seeds contain some of active ingredients that include: Alkaloids, Saponins, Tannins, Terpenoids and Flavonoids, these active phytochemical detected by using of two reagents for each active compound as shown in Table (1).

Table (1): Phytochemical analysis of *Raphanus sativus* Seeds

Active ingredients	Reagents or Tests
Alkaloids	Mayer's Reagent (+) , Wagner's reagent (+)
Saponins	foam test (+) , Mercuric chloride test (+)
Tannins	Ferric chloride test (+) , Lead acetates test (+)
Terpenoids	Chloroform-H_2SO_4 (+) , Anace-aldihyde reagent (+)
Flavonoids	Magnecium test (+) , Sulphuric acid test (+)

B. Biochemical Analysis

Serum Aspartate Transaminase(AST) Activity (IU/l)

Table (2) illustrates that the mean of (AST) activity (IU/l) in control and treated groups through the experimental period. The level of serum AST was close in all groups at the zero time. Table{2} explains that the serum AST increases significantly ($P \leq 0.05$) after 30 days in sodium nitrite treated Group (T_1) as compared with the control (C) and sodium nitrite+ *Raphanus Sativus* seeds extract treated groups (T_2, T_3, T_4).There was significant decrease in the level of AST in sodium nitrite+ *Raphanus Sativus* seeds extract treated groups (T_2, T_3, T_4) as compared with sodium nitrite treated group (T_1) and non-significant increase in AST level in (T_2, T_3, T_4) as compared with control group.

Table (2): Effect of 70% *Raphanus sativus* seeds alcoholic extract on aspartate transaminase (AST) activity (IU/l) in control and sodium nitrite treated rabbits.

Stage	Treatment rabbit groups					LSD value
	Cont.	T1	T2	T3	T4	
Pre	a 20.0 ± 0.83	A 19.0 ± 0.79	a 19.58 ± 0.92	a 19.54 ± 1.4	a 19.78 ± 0.82	4.92 NS
Post	b 19.52 ± 0.94	A 26.6 ± 1.5	b 21.1 ± 0.85	b 19.7 ± 0.83	B 19.72 ± 0.94	4.06 *
LSD value	3.79 NS	5.01 *	5.66 NS	4.72 NS	4.16 NS	----
* (P≤0.05), NS: Non-significant.						

***Values are expressed as mean ± SE n=5rabbit/group, small letters denote differences between groups, P≤0.05 Vs control.**

Serum Alanine Transaminase (ALT) Activity (IU/l)

Table (3) explains (ALT) activity (IU/l) in control (C) and treated groups (T_1, T_2, T_3,T_4) . The results have revealed that there was a significant increase (P≤0.05) in the serum ALT after 30 days in animals treated with sodium nitrite (T_1) as compared with pretreated and with sodium nitrite + *Raphanus sativus* seeds extract 50,200 mg/kg B.W (T_2, T_4) treated group . There was a significant decrease in the level of ALT in animals groups treated with sodium nitrite 20 mg/kg B.W+ *Raphanus sativus* seeds extract 50,200 mg/kg B.W (T_2, T_4) as compared with sodium nitrite treated group (T_1), and no significant decrease in level of ALT in (T_2, T_3, T_4) as compared with control group.

Table (3): Effect of 70% *Raphanus sativus* seeds alcoholic extract on alanine transaminase (ALT) activity (IU/l) in control and sodium nitrite treated rabbits

Stage	Treatment rabbit groups					LSD value
	Cont.	T1	T2	T3	T4	
Pre	ab 22.3 ± 0.84	b 20.7 ± 0.93	ab 22.3 ± 0.77	a 24.06 ± 1.4	b 20.0 ± 0.86	3.63 *
Post	ab 23.0 ± 1.5	a 25.0 ± 1.09	b 21.6 ± 1.2	ab 23.0 ± 1.52	b 21.4 ± 0.97	3.75 *
LSD value	3.07 NS	3.66 *	3.85 NS	3.91 NS	2.78 NS	----
* (P≤0.05), NS: Non-significant.						

***Values are expressed as mean ± SE n=5rabbits/group, small letters denote differences between groups, P≤0.05 Vs control.**

Serum Alkaline Phosphatase (ALP) Activity (IU/l)

The results of (ALP) activity (IU/l) were clarified in Table (4).The results manifested that there was a significant (P≤0.05) increase in serum level ALP in animals treated with sodium nitrite (T_1) as compared with control and with sodium nitrite + *Raphanus sativus* seeds extract (T_2, T_3, T_4) treated groups. There was no significant increase in serum level ALP in animal groups treated with sodium nitrite + *Raphanus sativus* seeds extract (T_2, T_3, T_4) as compared with control but there was a significant decrease in serum level ALP in T_2, T_3, T_4 groups as compared with sodium nitrite treated group(T_1).

Table (4): Effect of 70% *Raphanus sativus* seeds alcoholic extract on alkaline phosphatase (ALP) activity (IU/l) in control and sodium nitrite treated rabbits

Stage	Treatment rabbit groups					LSD value
	Cont.	T1	T2	T3	T4	
Pre	a 92.0 ± 3.8	a 89.0 ± 2.6	a 91.0 ± 4.7	a 90.1 ± 3.8	a 89.8 ± 3.6	12.49 NS
Post	b 90.0 ± 3.6	a 157 ± 12.4	b 92.8 ± 4.1	b 94.5 ± 5.2	b 106 ± 7.4	19.73 *
LSD value	8.83 NS	16.42 *	11.09 NS	9.61 NS	9.64 *	----
* (P≤0.05), NS: Non-significant.						

***Values are expressed as mean ± SE n=5rabbits/group, small letters denote differences between groups, P≤0.05 Vs control.**

Serum Uric Acid Concentration (mg/dl)

The mean value of serum Uric acid concentration (mg/dl) of control and T_1, T_2, T_3, T_4 groups was explained in Table {5}. After 30 days of sodium nitrite intubation in T_1 group, a significant increase (P≤0.05) in serum level uric acid concentration was observed as compared with the control and there was no significant increase as compared with sodium nitrite + *Raphanus sativus* seeds extract (T_2, T_3, T_4). There was no significant decrease in level of uric acid in sodium nitrite + *Raphanus sativus* seeds extract treated groups (T_2, T_3, T_4) as compared with sodium nitrite treated group (T_1), and no significant increase as compared with control group.

Table (5): Effect of 70% *Raphanus sativus* seeds alcoholic extract on serum uric acid concentration (mg/dl) in control and sodium nitrite treated rabbits

Stage	Treatment rabbit groups					LSD value
	Cont.	T1	T2	T3	T4	
Pre	a 3.1 ± 0.52	a 3.2 ± 0.69	a 3.4 ± 0.58	a 3.1 ± 0.47	a 3.8 ± 0.66	0.88 NS
Post	b 3.2 ± 0.61	a 4.2 ± 0.66	a b 3.44 ± 0.49	a b 3.4 ± 0.50	a b 3.9 ± 0.47	0.94 *
LSD value	0.72 NS	0.92*	0.77 NS	0.54 NS	0.73 NS	----
* (P≤0.05), NS: Non-significant.						

***Values are expressed as mean ± SE n=5rabbits/group, small letters denote differences between groups, P≤0.05 Vs control.**

Serum Creatinine Concentration (mg/dl)

Table {6} explains the mean of serum creatinine concentration (mg/dl) of control and T_1, T_2, T_3, T_4 groups . The results showed a significant increase (P≤0.05) in serum level creatinine in sodium nitrite treated group (T_1) as compared with control and sodium nitrite + *Raphanus sativus* seeds extract treated groups (T_2, T_3, T_4).There was a significant decrease in serum level creatinine in sodium nitrite + *Raphanus sativus* seeds extract treated groups (T_2, T_3, T_4) as compared with control and sodium nitrite treated group(T1) .

Table (6): Effect of 70% *Raphanus sativus* seeds alcoholic extract on serum creatinine concentration (mg/dl) in control and sodium nitrite treated rabbits

Stage	Treatment rabbit groups					LSD value
	Cont.	T1	T2	T3	T4	
Pre	a 3.0 ± 0.27	a 3.0 ± 0.33	a 3.1 ± 0.28	a 3.2 ± 0.51	a 3.3 ± 0.49	0.41NS
Post	b 3.1 ± 0.40	a 3.8 ± 0.27	c 2.5 ± 0.47	c 2.6 ± 0.42	c 2.6 ± 0.55	0.53*
LSD value	0.39NS	0.42*	0.33*	0.39*	0.40*	----
* (P≤0.05), NS: Non-significant.						

***Values are expressed as mean ± SE n=5rabbits/group, small letters denote differences between groups, P≤0.05 Vs control.**

C. Histological Examination

1. Histology of liver in control group (C)

Histological examination of liver found that the liver is divided into lobules and each lobule consists of radially arranged strands of hepatopcytes which extend from the central vein. Blood sinusoids separated the hepatocytes from each other. Portal area which consists of portal vein, hepatic artery, bile duct are distributed at the corners of the lobules as shown in Figure (1).

2. Histology of liver in 20 mg/kg sodium nitrite treated group (T_1)

Histological examination detected several changes in liver of 20 mg/kg sodium nitrite treated group. These changes are congestion of central vein, infiltration of inflammatory cells, and fatty degeneration as shown in Figure (2).

3. Histology of liver of 20 mg/kg sodium nitrite + 50 mg/kg *Raphanus sativus* seeds extract treated group(T_2)

In some regions of liver tissue some degree of repairs appears like less congestion and infiltration as shown in Figure (3).

4. Histology of liver of 20 mg/kg sodium nitrite + 100 mg/kg *Raphanus sativus* seeds extract treated group(T_3)

In this group, many repairs were found in liver tissue as less congested central vein, absent of infiltration and absent of degeneration as shown in Figure (4).

5. Histology of liver of 20 mg/kg sodium nitrite + 200 mg/kg *Raphanus sativus* seeds extract treated group (T_4)

Histological examination of this group detected several regenerations like appear of hepatic lobules, normalize central vein and blood sinusoids, presence of radially arranged hepatocytes, absent of congested hepatic blood vessels and infiltration as well as regeneration of hepatocytes as shown in Figure (5).

6. Histology of Kidney in Control Group (C)

Histological examination of kidney explained that kidney consists of two regions, outer cortex and inner medulla. The cortex consists of numerous Bowman's capsule, proximal and distal convoluted tubules. The medulla consists from longitudinal and transverse section of the loops of Henley and collecting tubules as shown in Figures (6),(7).

7. Histology of kidney in 20 mg/kg sodium nitrite treated group (T_1)

Histological examination detected several changes in kidney of 20 mg/kg sodium nitrite treated group; these changes were:- congestion of blood vessels and infiltration of inflammatory cells as shown in Figures (8),(9).

8. Histology of kidney in 20 mg/kg sodium nitrite + 50 mg/kg *Raphanus sativus* seeds extract treated group (T_2)

Histological section of kidney of T_2 group explained there was absence of congestion in some region of kidney tissue and continuous infiltration of inflammatory cells as shown in Figure (10).

9. Histology of kidney in 20 mg/kg sodium nitrite + 100 mg/kg *Raphanus sativus* seeds extract treated group (T_3)

Histological examination detected several repairs in kidney tissue such as normal renal glumeruli, normal proximal and distal convoluted tubule and absence of congestion and infiltration as shown in Figure (11).

10. Histology of kidney in 20 mg/kg sodium nitrite + 200 mg/kg *Raphanus sativus* seeds extract treated group (T_4)

(T_4) treated group showed continuous of normal appearance of kidney tissue as well as absence of infiltration and congested blood vessels as shown in Figure (12).

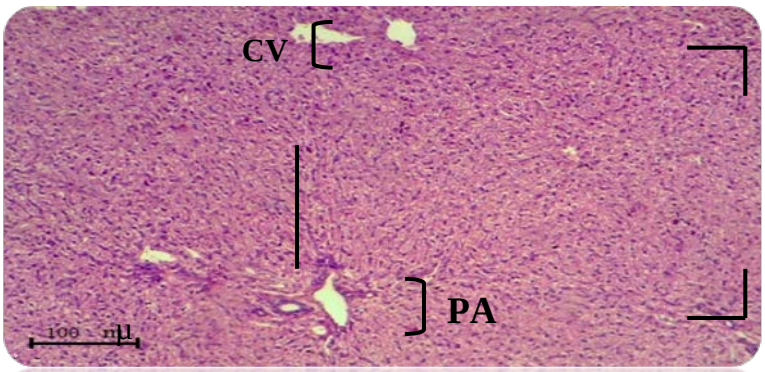

Figure {1}: Section in the liver of control group (C) shows hepatic lobule L, central vein CV, portal area PA and readily arranged hepatocyte (H & E).

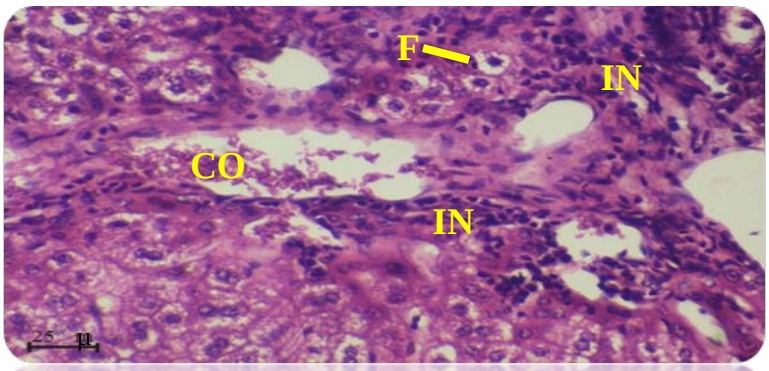

Figure {2}: Section in the liver of 20 mg/kg sodium nitrite treated group (T_1) shows congestion CO, infiltration IN and fatty degeneration F (H & E).

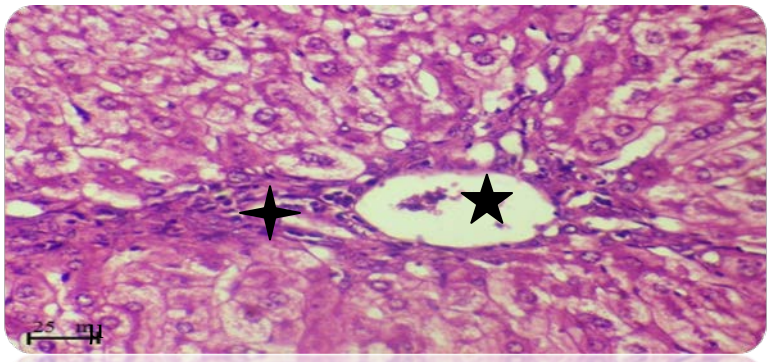

Figure {3}: Section in the liver of 20 mg/kg sodium nitrite + 50 mg/kg *Raphanus sativus* seeds extract treated group(T_2) shows less congestion and less infiltration (H&E).

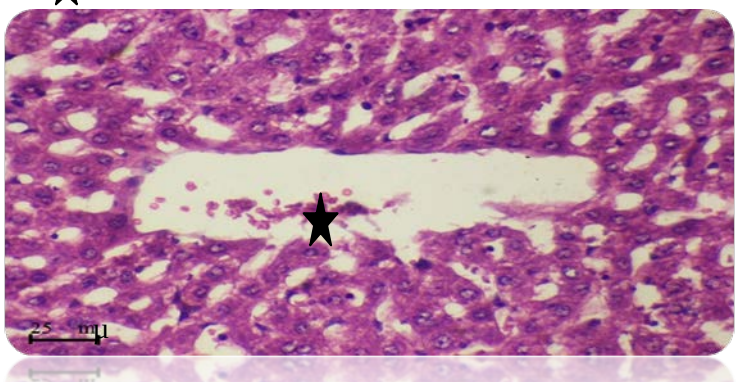

Figure {4}: Section in the liver of 20 mg/kg sodium nitrite + 100 mg/kg *Raphanus sativus* seeds extract treated group(T_3) shows less congestion and normal liver tissue (H & E).

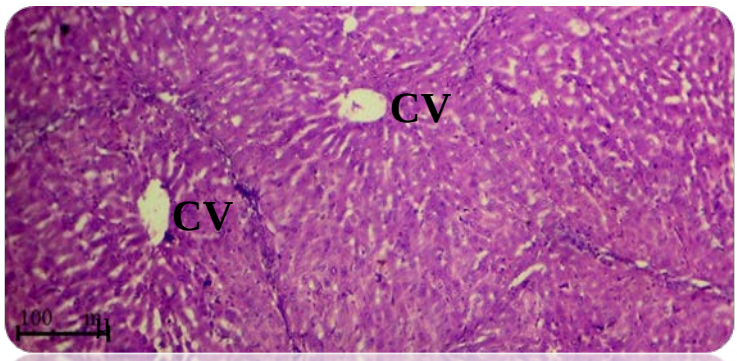

Figure {5}: Section in the liver of 20 mg/kg sodium nitrite + 200 mg/kg *Raphanus sativus* seeds extract treated group (T_4) shows normal liver parenchyma (H & E).

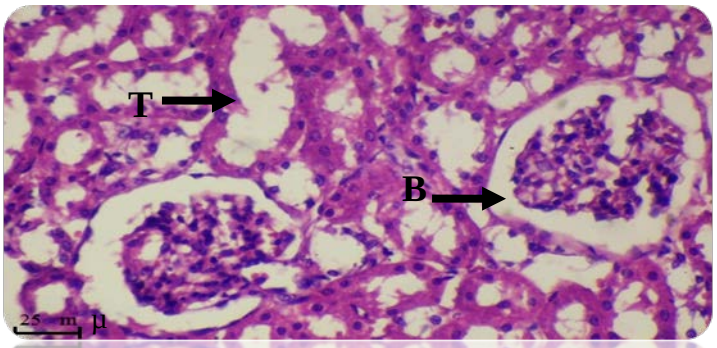

Figure {6}: Section in the kidney of control group (C) shows normal region of medulla (H & E).

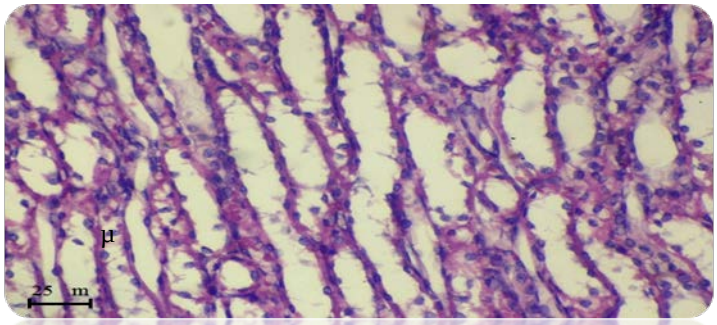

Figure {7}: Section in the kidney cortex of control group (C) shows normal bowman's capsule B and renal tubules T (H & E).

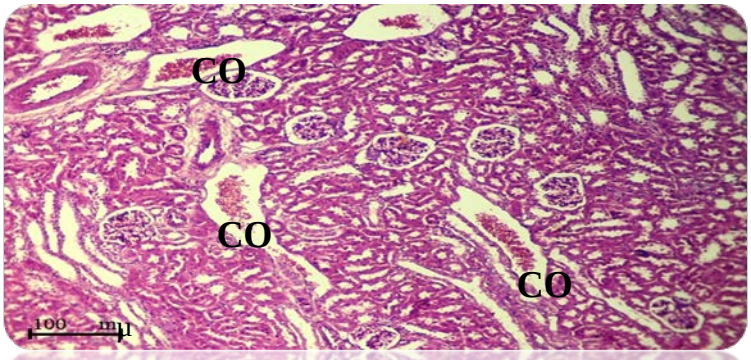

Figure {8}: Section in the kidney cortex of 20 mg/kg sodium nitrite treated group (T_1) shows congestion CO of blood vessels (H & E).

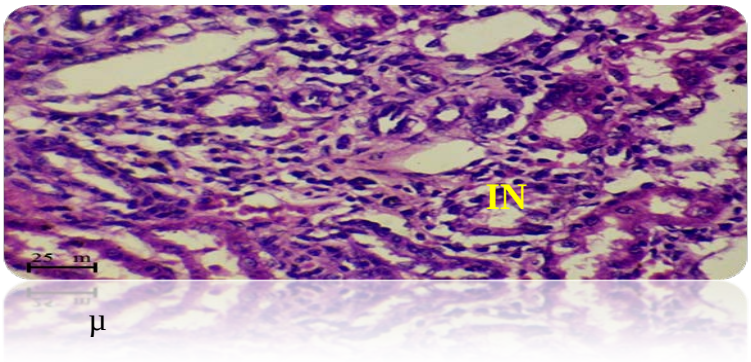

Figure {9}: Section in the kidney cortex of 20 mg/kg sodium nitrite treated group T_1) shows infiltration IN (H & E).

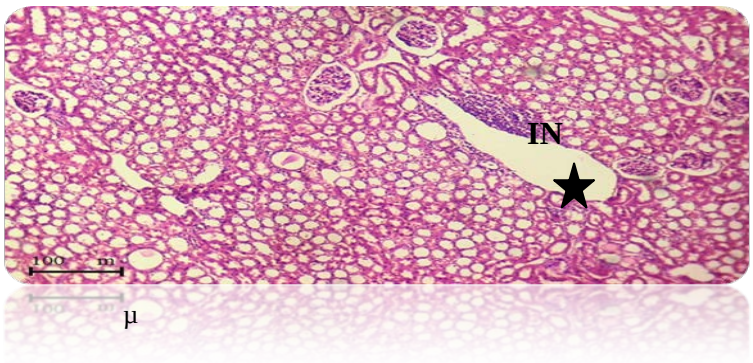

Figure {10}: Section in the kidney cortex of 20 mg/kg sodium nitrite + 50 mg/kg *Raphanus sativus* seeds extract treated group(T_2) shows no congestion ★ and infiltration IN (H & E).

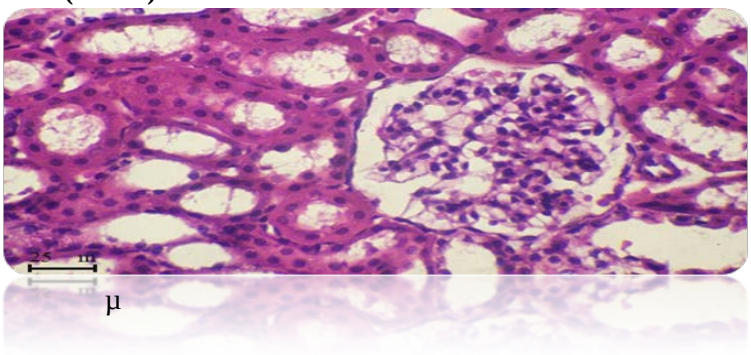

Figure {11}: Section in the kidney cortex of 20 mg/kg sodium nitrite + 100 mg/kg *Raphanus sativus* seeds extract treated group (T_3) shows normal structure of renal tissue (H &E).

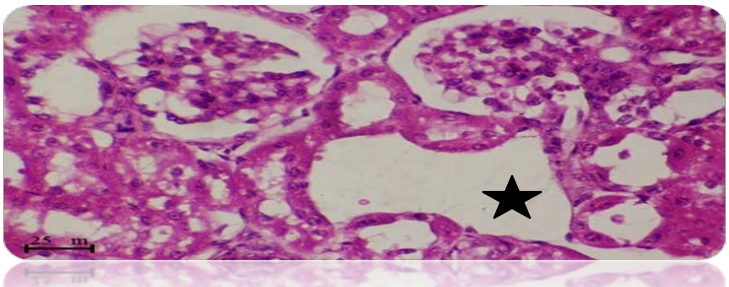

Figure {12}: Section in the kidney cortex of 20 mg/kg sodium nitrite + 200 mg/kg *Raphanus sativus* seeds extract treated group(T_4) shows normal renal tissue and no congestion ★ in blood vessel (H & E).

Disscussion

The results of the current study showed an increase in serum level of liver enzyme (AST, ALT, ALP) with daily intake of $NaNO_2$. These results agreed with [22],[23]. Higher elevation of the liver enzymes is due to liver damages. Increase of liver enzymes results from degeneration and damage of liver cells .Varieties of enzymes normally located in the cytosol and when the liver cell membrane is damaged, these enzymes are released into the blood stream. When liver enzymes level rises in plasma, it might be due to increasing permeability of plasma membrane or cellular necrosis [24]. Also increase the activity of AST, ALT and ALP enzymes in the serum of $NaNO_2$ treated rabbits may result from the toxic effect of nitroso-compounds which is formed in the acidic environment of the stomach as a result of combination of sodium nitrite with secondary amines in the food or the body , causing hepatic necrosis, and increased liver enzymes may be caused by methaemoglobinemia which induced hypoxic injury to centrilobular hepatocytes that consequently cause enzyme leakage [9],[25]. Increase in serum ALP enzyme activity in rabbits received sodium nitrite correlate with the damage of hepatocyte membranes and liver dysfunction which may be in part due to the effect of nitric oxide (NO) free radical production induced by nitrites [26].

The results of the current study showed an increase of serum level uric acid and creatinine in sodium nitrite treated group this result agreed with [24].Sodium nitrite affects kidney functions and causes an increase in serum uric acid and creatinine. These could be attributed to the changes in the glomerular filtration rate, renal blood flow and threshold of tubular reabsorption [9].

Various histological changes in liver and kidney were observed by sodium nitrites treated group

(T_1).Congestion caused by acute inflammation that results from vasodilation, is resulting in locally increased blood flow and engorgement of the down stream capillary bed. Infiltration of inflammatory cells is also caused by acute inflammation which is attributed by increase vascular permeability due to endothelial cell contraction leading to intercellular gaps. This process is elicited by chemical mediators, then adhesion of leukocytes to endothelium and migration to the interstitial tissues toward a chemotactic stimulus. Cell swelling is caused by fatty degeneration. Liver is the major organ involved in fat metabolism, so fatty change refers to any abnormal accumulation of triglyceride within hepatocytes. This may result from defects at any step from fatty acid entry to lipoprotein exit [27].

According to the results of the current study showed that there was decreasing in serum level GOT, GPT, ALP, uric acid, creatinine and also healing of liver and kidney tissue in animals treated with 20 mg/kg sodium nitrite +*Raphanus sativus* seeds extract 50, 100, 200 mg/kg (T_2, T_3, T_4) as compared with 20 mg/kg sodium nitrite treated group (T_1) These results explained that the radish decrease free radicals production in agreement with [28],[29], and radish seeds have antioxidant activity. It has several active compounds including alkaloid, flavonoids, saponins and anthocyanins which are important dietary antioxidants that protect living cells from harmful effect of oxidative stress resulting in the prevention of diseases [28],[30].

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Evaluation of the antimicrobial efficacy of hand gel sanitizer on hand hygiene

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Abstract: *In vitro* testing of antimicrobial agents is an important tool in the testing hierarchy, and may provide interesting insights into their potential clinical efficacy. *In vitro* testing of antimicrobial agents is benefit in screening antimicrobial agents in product formulations because such these agents which tested both *in vitro* and *in vivo* activity may have reduced antimicrobial effects when formulated into a hand clean prospective . Bactericidal activity of various hand gel sanitizer was performed against bacterial strains *Escherichia coli*, *Pseudomonas aeroginosa*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Staphylococcus epidermidis* ascertain the efficacy of four hand gel sanitizer (Dettol , Chicco ,Cleaner , HiGeen) by volunteers use. Also the effect of four hand senitizers on bacterial isolated study by agar well diffusion method .The result showed the Dettol and Chicco more effect than other type of soaps in all this methods.

Key words: Antibacterial Activity, Hand Hygiene**,** hand sanitizers, *in vitro*, *in vivo* , Skin

تقييم الفعالية المضادة للمايكروبات لمعقمات اليد على صحة اليد

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الخلاصة: ان اختبار فعالية العوامل المضادة للمايكروبات داخل المختبر هو أداة هامة في التسلسل الهرمي للاختبارات، ويمكن أن تقدم أفكارا مثيرة للاهتمام في الفعالية السريرية المحتملة .ان اختبار فعالية العوامل المضادة للمايكروبات داخل المختبر يفيد في اختبار فعالية العوامل المضادة للمايكروبات في تركيب المنتج لأن مثل هذه العوامل عندما تم اختبارها على حد سواء في التجارب المختبرية وداخل الجسم الحي قد خفضت من تاثير للميكروبات عند استعمالها في تنظيف اليد. تم اختبار فعالية اربع انواع من المطهرات (Dettol , Chicco ,Cleaner , HiGeen) في قتل السلالات البكتيرية التي تم عزلها من الايدي (*Escherichia coli, Pseudomonas aeroginosa, Bacillus subtilis, Klebsiella pneumoniae, Staphylococcus epidermidis* عن طريق استخدامه من قبل متطوعين . كذلك تم دراسة تاثير هذه المعقمات باستخدام طريقة الحفر على سطح الاكار .اظهرت النتائج ان المعقمين Dettol وChicco هما الاكثر تاثيرا مقارنة مع الانواع الأخرى.

الكلمات المفتاحية: فعالية مضادات البكتريا ، صحة اليد ، معقم اليد، خارج الكائن الحي ، داخل الكائن الحي ،الجلد.

Introduction

Bacteria transporting by hand is an important method for transmission of infection among people or from the patient to the health care worker. The most important tool in hospital infections control is hand hygiene. Failure to perform appropriate hand cleanliness caused hospital infections and rise of multi-resistant microorganisms (1). The microbial flora of hands skin consists of two types of microorganisms. Type1: microorganisms survive and multiply on the skin. Type2: microorganisms found by contaminants of the hands acquired from infected people or environment. These microorganisms (Type 2) are not easy isolated from most persons more than (Type1) microorganisms. Second Type microorganisms found on the hands of workers in hospitals which be source of almost infections in hospital (2).

Hospital infections caused by opportunistic microorganisms and some species such as *Staphylococcus aureus*, *Enterococcus spp.*, *Pseudomonas spp.*, and *Escherichia coli*. The most hospital infections occur in central nervous system urinary tract, respiratory tract, burns skin, blood, gastrointestinal tract, and surgical wounds (3).

When soap and water are not available have been used hand sanitizers which are personal care product. They contain component to decrease the number of bacteria on the hands. If hands have visible dirty, they cannot effective on them. Hand washing continuous is the best way to prevent sick and decrease disease. Hand sanitizer is a disinfectant that works without water(4) .

It helps to prevent bacterial transfer and reduces acquired hospital infections. Ethyl alcohol is one of components of hand sanitizers about (60%). Ethyl alcohol evaporates and leaves skin which is emollients such as natural refreshment derived from plant materials. Healthcare industry used hand sanitizer widely against spread of disease. They don't remove dirt from the surface so they are not cleaning agents, these used without water(5) .

The deliberate and intentional ingestion of hand sanitizer may be so dangerous, especially for children. Also, skin of children (less than three years age) can absorb enough alcohol which has effects on their health because of their small size. Isopropyl and ethyl alcohol are the most common active ingredients in hand sanitizers. Both of these substances can lead to heavy sickness and death if ingested (6) . Aim of this work; compare the activity of locally available market gel senitizers (Chicco, Dettol, Cleaner and HiGeen) against skin

infected some bacteria. As well as to provide data to clinician to decide for the selection of better and protective hand sterile against pathogenic microorganism.

Material &methods

1-Sample collection

A sum of 150 samples from unwashed hand were collected from different peoples (students, laboratory staff, sweepers , teachers and food vendors) by using sterile cotton wool swab sticks were wetted by soaking in normal saline . Then take the swab sample to the microbiology laboratory of biology department for further procedures.

2-Isolation of bacteria:

The swab was grown on nutrient agar then sub cultured on blood agar, MacConkey agar and EMB agar (HiMedia, India) to diagnosis various type of bacteria. The plates were incubated for 24 h at 37 ºC. Bacterial colonies that were then identified by colony morphology, microscopy of isolated bacteria and various biochemical tests (7).

3-Collection of hand sanitizer

To achieve this experimental study, different type of commen gel sanitizers were bought from shops to testing the activity of this sanitizers to inhibition the growth of bacteria. The hand sanitizers used were (Chicco , HiGeen , Cleaner and Dettol).

4-Sterility test:

Add 1ml of the undiluted gel sanitizers to (9ml) of peptone water (HiMedia, India) respectively and serial dilutions were made to 10^{-1},10^{-2},10^{-3}. About 1ml of each dilution was inoculated on to nutrient agar (HiMedia, India) via the pouring plate method. Incubation of plates was at 37˚C for 24h - 48h. After incubation examination the mount of growth (8,9).

5-Testing the bactericidal activity of hand sanitizer:

Choice (10) volunteers from positive result and sterile your hand with all type of sanitizers and then isolated bacteria after use hand sanitizers . Hand sanitizers used in a separate day about 7 days to collect samples concerning the different sanitizers (6) .

6-Testing the antimicrobial activity of hand sanitizer against bacteria:

In this step,we added(1ml, 2ml and 3ml) from all type of gel sanitizers in the petri dishes then added 30 ml of nutrient agar . when the media solidify they were inculation with all type of bacteria isolated finaly we incubated them over night at 37˚C and the results were observed after 24 h. and the growth of the organism will be registered (10).

7- Susceptibility test by agar well diffusion method:

Agar well-diffused method as described by (11) with slight modification was conducted. Mueller-Hinton agar was seeded uniformly by spread plate method with 1ml of standardized culture of each microbial isolate. The inoculated plates were allowed to set. A sterile cork borer was used to cut uniform wells of 5mm diameter on the surface of the agar and the wells were filled with neat and each dilution (10^{-1}, 10^{-2},10^{-3}) of the gel sanitizers using sterile Pasteur pipette. The plates were incubated at 37°C for 24h. The antibacterial susceptibility was indicated by measured the diameter of inhibition zone .

8-Statistical analysis:

Statistical analysis was performed using SPSS statistical computer software.

Result:

1-Sample collection:

It was observed that 41 (41 %) of the 100 cases, were positive, they included 11(11%) from laboratory staff , 9(9%) from sweepers , 5(5%) from students , 6(6%) from teachers and 10 (10%) from food venders, 59 (59 %) cases were negative.

Table(1) The number and percentage of people according to the nature of sample

Nature of samples	Positive NO %	negative NO %	Total
laboratory staff	11(11)	14(14)	25(25)
Sweepers	9(9)	7(7)	16(16)
Students	5(5)	15(15)	20(20)
Teachers	6(6)	14(14)	20(20)
food venders	10(10)	9(9)	19(19)
Total	41(41)	59(59)	100(100)

P= 0.717 , p>0.05 no significant difference

2-Isolation of bacteria:

Table (2) showed the type and the number of bacteria isolated from hand of different people.

Table (2) Number of isolated bacteria according to the nature of samples

Isolated Bacteria	Nature of samples					
	laboratory staff	sweepers	students	teachers	Food vendors	Total
Escherichia coli	4	7	4	9	7	31
Pseudomonas aeruginosa	4	0	2	3	3	12
Bacillus subtilis	5	4	0	0	2	10
Klebsiella pneumoniae	7	5	0	3	0	15
Staphylococcus epidermidis	10	6	7	3	1	27

P= 0.230 , p>0.05 no significant difference

3- Testing the bactericidal activity of hand sanitizer:

The study showed that Dettol sanitizers was more effect than another type by bactericidal of all type of bacteria

Table (3) Effect of using hand gel sanitizers on volunteers hand

Volunteers People	Bacterial number before using hand gel sanitizers	Bacterial number after using hand gel sanitizers			
		Chicco	Dettol	Cleaner	HiGeen
1	20	2	-	10	2
2	16	1	-	6	-
3	7	-	-	-	-
4	12	-	-	-	-
5	5	-	-	-	-
6	16	-	-	-	-
7	9	-	-	5	1
8	10	1	-	3	3
9	11	-	-	2	-
10	6	-	-	-	-

3-Sterility test:

All the hand sanitizer were sterile as none had growth of microbial colonies after 24 to 48h. incubation at 37˚C.

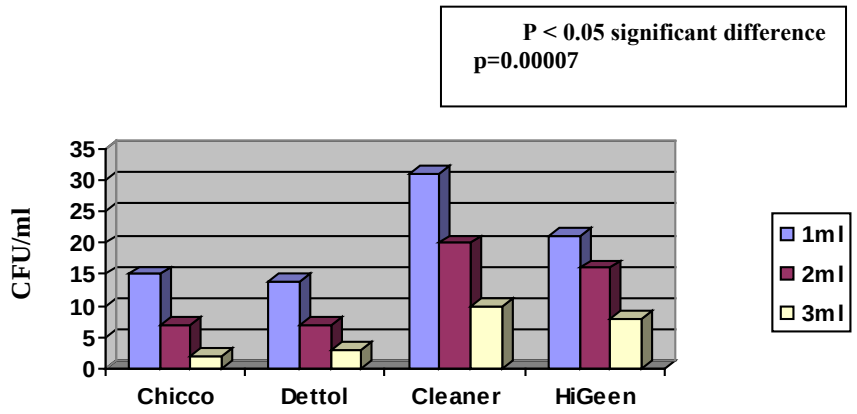
4-Testing the antimicrobial activity of hand sanitizer against bacteria:

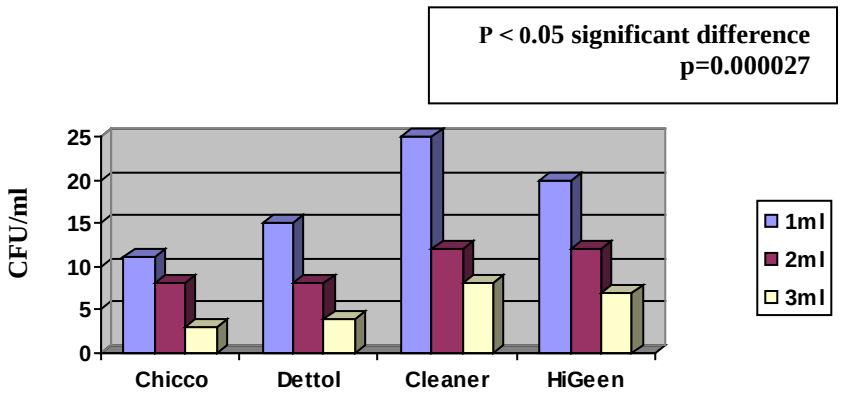
It was observed that Dettol and Chicco hand sanitizer more effect on all type of isolated bacteria compared with anther type of hand sanitizer (table (3)).

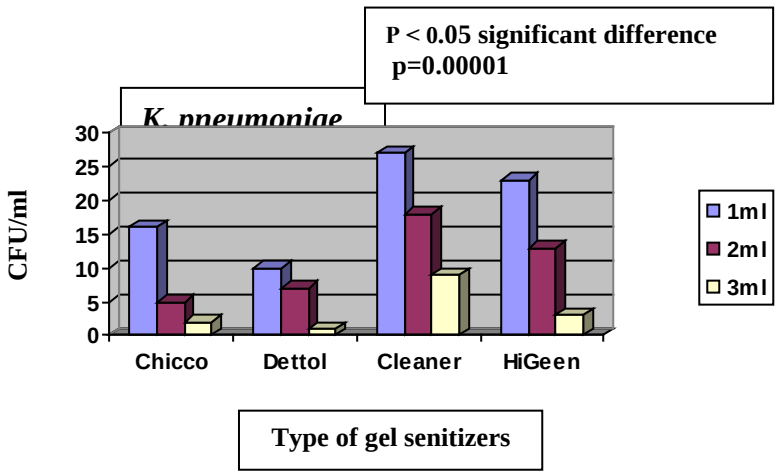
Table (4) Colony forming unit of different species of bacteria under different concentrations of hand sanitizer

hand sanitizer (ml)	Hand sanitizers/cfu			
	Chicoo	Dettol	Cleaner	HiGeen
Escherichia coli				
1	15	14	31	21
2	7	7	20	16
3	2	3	10	8
Bacillus subtilis				
1	11	15	25	20
2	8	8	12	12
3	3	4	8	7
Klebsiella pneumoniae				
1	16	10	27	23
2	5	7	18	13
3	2	1	9	3
Pseudomonas aeroginosa				
1	13	11	30	25
2	6	6	15	13
3	1	2	6	7
Staphylococcus epidermidis				
1	12	13	22	21
2	6	7	11	12
3	2	1	6	5

P= 0.000013 , P < 0.05 significant difference


P < 0.05 significant difference
p=0.00007
CFU/ml
35
30
25
20
15
10
5
0
Chicco
Dettol
Cleaner
HiGeen
1ml
2ml
3ml


P < 0.05 significant difference
p=0.000027
CFU/ml
25
20
15
10
5
0
Chicco
Dettol
Cleaner
HiGeen
1ml
2ml
3ml


P < 0.05 significant difference
p=0.00001
K. pneumoniae
CFU/ml
30
25
20
15
10
5
0
Chicco
Dettol
Cleaner
HiGeen
1ml
2ml
3ml
Type of gel senitizers

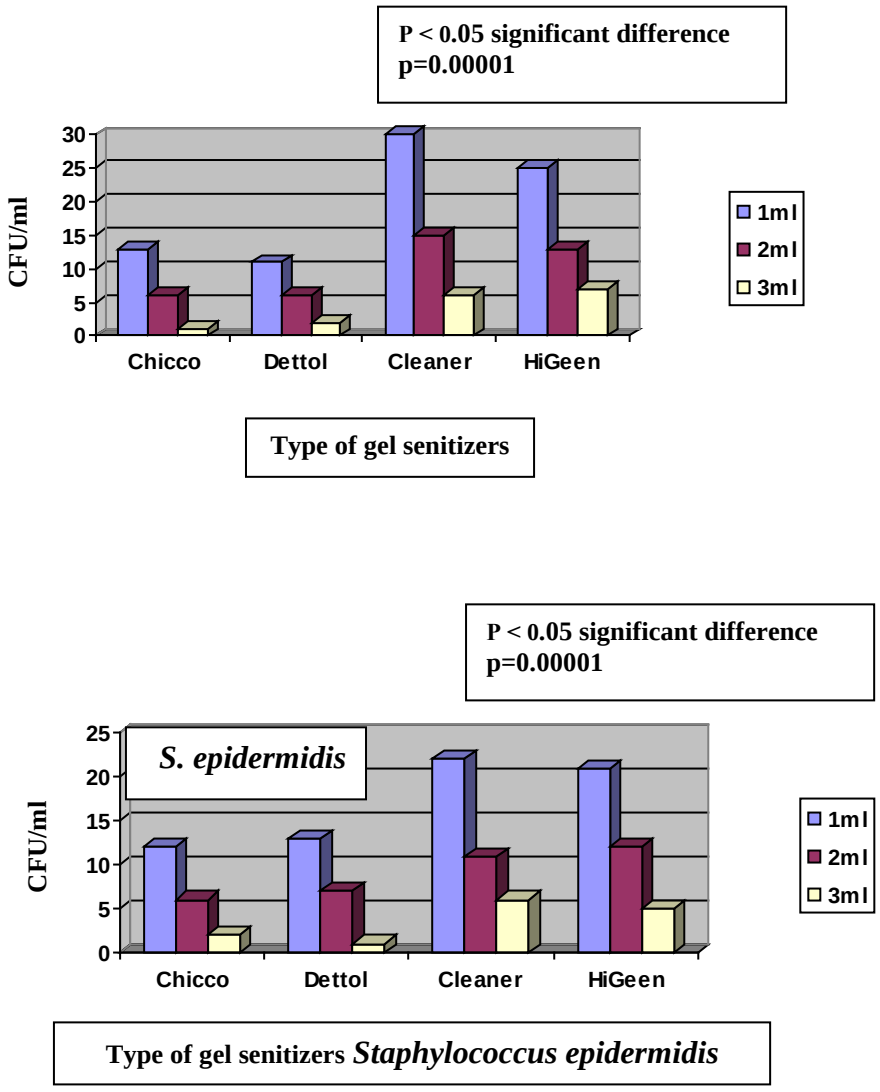


Figure (1) Colony forming unit of different species of bacteria under different concentrations of hand sanitizer

6- Susceptibility test by agar well diffusion method:

It was found Chicco and Dettol hand sanitizers has the greatest activity against all the microrganisms and at all dilutions. All the hand sanitizer was active on *E. coli*. Cleaner hand sanitizer had no effect on *B. subtilis* , *K. pneumonia and* and *S. epidermidis* in 10^{-2} concentration while HiGeen hand sanitizer had no effect on *P . aeroginosa* in10^{-2} concentration . Maximum inhibition concentrations in undiluted hand wash of all bacterial species.

Table(5) The diameter of inhibition zones (mm) in four hand sanitizers

Dilutions	Hand sanitizers diameter of Inhibition Zone (mm)			
	Chicoo	Dettol	Cleaner	HiGeen
Escherichia coli				
undiluted	22	20	11	16
10^{-1}	11	10	8	9
10^{-2}	4	7	2	3
Bacillus subtilis				
undiluted	23	20	9	11
10^{-1}	16	12	5	7
10^{-2}	10	8	-	2
Klebsiella pneumoniae				
undiluted	19	21	9	10
10^{-1}	12	12	3	6
10^{-2}	7	5	-	3
Pseudomonas aeroginosa				
undiluted	21	17	10	12
10^{-1}	16	11	6	7
10^{-2}	9	5	1	-
Staphylococcus epidermidis				
undiluted	20	17	9	10
10^{-1}	15	10	2	5
10^{-2}	9	6	-	2

- No inhibition

LSD(0.05) = 7.989

Discussion:

The goal of hand hygiene is a sufficient reduction of microbial counts on the skin to prevent cross-transmission of pathogens among patients. It is easier to keep hands clean than to make them clean. The critical density of microorganisms on the hands needed for the spread of pathogens remains unknown and it may depend on the type duration of contact, the type of microorganism and the patient's resident flora.

To determine the microbiological quality , the antibacterial property and dilution effects on activity of four type of hand sanitizers (Dettol , Chicco, Cleaner, HiGeen) were evaluated using susceptibility test by agar well diffusion. In study isolate *E. coli , P. aeruginosa, B. subtilis, K. pneumoniae, S. epidermidis* was isolated from laboratory staff ,

students , teachers , sweepers and food vendors used to test the efficacy of hand sanitizer. These result resemble the result obtain by (12) they isolated *Sta. aureus, P. aeroginosa* and *E. coli* from hands swabs of students and food vendors. and similar the result obtain from (13). where isolated *S. aureus , E. coli , P. aeruginosa, Enterobacter spp, B. subtilis, S. dysenteriae, Sta. epidermidis* from unwashed hand . Statistical analysis show there is $p>0.05$ no significant difference . The microbiological analysis of all the hand sanitizer samples recorded absence of growth meaning they are sterile and thus conform to the sterility standard required of such sanitary personal care products. Dettole showed an impeccable activity as compared to other hand washes, it was very effective in all the quality assessment methods used as a determinant of antibacterial activity it inhibited the growth of all the test organisms at different dilutions (14) Statistical analysis show there is $p>0.05$ no significant difference ..

If the hand sanitizer have diluted, its activity will be reduced. On this basis, decrease in zone of inhibition diameter and increase in microbial growth with increase in dilution. Microbes can degrade prolific ability a widely variety of substances due to its natural gift with plasmids, degradative enzymes, regeneration and mechanisms high protein repair. (15) Statistical analysis show LSD(0.05) = 7.989.

In this study, we suggest that hand sanitizer have high effective against Gram-negative and Gram-positive bacteria. The hand sanitizer and cleansing material useful for personnel health care. Patients who have problem in immunity system they are at high risk. Our result showed that hand sanitizer kills the bacteria at a specified concentration.

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Increase the readiness of phosphorus to *Rhaphanus sativus* & *Vigna unguiculata* by local isolates of bacteria *Pseudomonas spp*

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Abstract: In this research experiment, the action of the locally isolates of *Pseudomonas spp.***,** on the readiness and solubility of phosphorus (P) for plant was studied. This study included two experiments, a laboratory and field once. In the laboratory, diagnosis isolation of four isolates of *Pseudomonas spp.*(Ps1, Ps2, Ps3, and Ps4) from samples of the local Iraqi soil of different regions in Baghdad. Through bio-fertilization, four isolates have been added to the soil for increasing the readiness of P to the plants field. In the field experiment, *Rhaphanus sativus* and *Vigna unguiculata* were planted in plastic pots containing 5kg of soil and kept in a green house (Dept. of biology /College of Sciences). The soil in the pots were inoculated with broth of bacterial isolates. Agriculture process was performed in March, and after 50 days the plants were harvested and the morphological characteristics were studied such as: plant length, fresh weight, and phosphorus concentration in plants and soil before and after the addition of bacterial isolates. The results showed a significant increasing in growth parameters of plants, and in a percentage of P in plants and soils compared with the control plants. All the isolates lead to increase the P percentage in plants, and soil, except the isolate Ps2 which was not affected , and did not show any significant result in this experiment. Whereas, the isolate Ps4 was relatively the highest between the others in *Vigna unguiculata* , which the parameters of length, weight germination %, and P % were 31.27, 8.85, 90, and 5.37 respectively. Although, *Raphanus sativa* parameters of length, weight, germination %, and P % were 21.53, 10.57, 80, and 5.98 respectively, the isolate Ps2 was the lowest among them for each of the plant radishes and Cowpeas.

Key words: Phosphorus. Pseudomonas. Bio-fertilizer .

رفع جاهزية الفسفور لنباتي الفجل واللوبياء بواسطة عزلات محلية من بكتريا *Pseudomonas spp*

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الخلاصة: في هذه التجربة البحثية تم دراسة فعالية اربع عزلات محلية من بكتريا *Pseudomonas spp* على رفع جاهزية الفسفور للنباتي الفجل واللوبياء . شملت هذه الدراسة تجربتين المختبرية والحقلية . مختبريا تم عزل وتشخيص أربع عزلات من *Pseudomonas spp* وهي (PS1 ، PS2، PS3 و PS4) من عينات من التربة المحلية العراقية من مناطق مختلفة في بغداد. و من خلال التسميد الحيوي تم إضافة أربعة عزلات الى التربة لزيادة ذوبان الفسفور وبالتالي رفع جاهزيته للنباتات حقليا . زرعت بذور نبات الفجل *Rhaphanus sativus* واللوبياء *Vigna unguiculata* في سنادين بلاستيكية سعة (5كغم/تربة) تمت الزراعة في شهر مارس في البيت الزجاجي لقسم علوم الحياة / كلية العلوم / الجامعة المستنصرية ، بعد الانبات تم تلقيح التربة في السنادين البلاستيكية بالعزلات البكتيرية الاربعة وتم تعليم السنادين حسب العزلات الاربعة لكلا النباتين . وبعد 50 يوما تم حصاد النباتات و تمت دراسة الخصائص المظهرية منها طول النبات، الوزن الطازج، وتركيز الفوسفور في النباتات والتربة قبل وبعد إضافة العزلات البكتيرية. اكدت النتائج ظهور زيادة كبيرة في معدلات نمو النباتات، ونسبة الفسفور في النباتات والتربة مقارنة مع نباتات السيطرة. وكانت جميع العزلات فعالة في زيادة نسبة الفسفور في النباتات، والتربة، ماعدا العزلة PS2 لم يظهر أي فعالية ايجابية . حيث سجلت العزلة PS4 لنبات اللوبياء اعلى نسبة من طول النبات والوزن ونسبة الإنبات ، و نسبة الفسفور ٪ على التوالي : 31.27، 8.85، 90، و5.37 . في حين، سجلت العزلة PS2 أدنى نسبة بين العزلات الاربعة للنبات نفسه . في حين سجلت العزلة ps4 لنبات الفجل اعلى نسبة للصفات المظهرية من حيث الطول والوزن ونسبة الإنبات، وP٪ : 21.53، 10.57، 80 و 5.98 على التوالي . في حين، سجلت PS2 أدنى نسبة واقل فعالية بينهما ولكلا النباتين الفجل واللوبياء .

Introduction:

The phosphorus (P) is an essential macronutrient for plants, which is necessary for root growth, stem strength, flower, and seed formation. In addition, the resistance of plant against diseases is affected by nutrition of phosphorus [1]. In nature, the bio availability of (P) in soil is very poor or insufficient for plants, it's about (1.0mg (p)/ Kg) [2], and the phosphorus that added to the soil is as a phosphate fertilizer, however, large amounts of (P) in these chemical fertilizers are losing, and become unavailable to the plants [3]. For these reasons, modern studies reported that there are a special group of bacteria known as (phosphate solubilising bacteria), are capable to hydrolyse the (P) from insoluble compound or, convert it to soluble form, therefore the bacteria used the(P) in plant′s phosphorus nutrition when the soil is inoculated with bacteria [3,4]. One of the most important phosphate solubilising bacteria is *Pseudomonas spp*., which is used in agriculture as phosphates Bio fertilizer, and this will increase phosphorus (P) up taken by the plants, and improve plant growth and yield [5]. Some

studies presented that many of *Pseudomonas spp*., is considered as a phosphate-solubilising bacteria, and as important Bio-inoculants due to their bio fertilizing efficiency in improving soil nutrient, and secretion of plant′s growth promoters [6, 7].The purpose of this experiment was using an isolates of *Pseudomonas spp*. which is isolated locally as a(bio fertilizer) and added to the soil instead of chemical fertilizers to increase the (P) up taken by plants, and plant′s growth. Note worthy that using the bio-fertilizer is less expensive, and less polluted to the environment comparing with the chemical fertilizers. On the other hand, soil microorganisms have an important role in determination of plant productivity. For successful performance of introduced microbial bio-inoculants, intensive efforts have been made to explore the microbial diversity of indigenous community, their distribution, and behaviour in the soil habitats [8]. Therefore, soil microorganisms are directly affect the agriculture, which have principal action to enhance the replacement of plant nutrients in re-cycling operation and, as a result, reduce application of chemical fertilizers [9].

Materials and Methods:

Isolation and identification of bacteria: The soil samples were sieved well to remove the remains of plant roots and stones, then (10 gm.) were taken from each sample and D.W. were added to each one to a final volume of (100 ml)of soil suspension. Then, one (1) ml from each suspension was taken and cultured with chromo agar for *Pseudomonas*, and again with *Pseudomonas* base agar (selective for *P*. bacteria). The media were incubated in 28 ℃ for 2-3 days until bacterial colonies were appeared.

The resulted colonies were tested by Gram stain, and biochemical testes; which were (oxidase, catalase, citrate, in dole forming, methyl red, and Voges–Proskauer test [10,11].

Purification of bacteria: full loop of bacterial colonies was taken to make serial dilutions, and then one (1) ml was taken from the dilution (10^{-6}), and cultured on plates with pseudomonas agar base, then incubated in 28℃ for 1-2 days. This experiment was repeated many times to get a single pure colony.

Inoculation of soil culture by Pseudomonas broth: Pseudomonas isolates were grown in (10 ml) of nutrient broth, and incubated in 28℃For 2 days. The bacterial broth was centrifuged in (300 rpm/5 min.), and the bacterial suspension was precipitated by normal saline (0.85%). Bacterial

suspension was completed to a final volume of (100ml). and added to the pots after 2 days from planting.

Soil preparation & Agriculture: In this experiment, a loamy soil was used. The soil was sieved by sieve (2mm dia.), and autoclaved to kill any residual microorganisms. The soil was put in plastic pots (5kg), which filled with proper quantity (4kg) of soil. The seeds of two plants *Vigna unguiculata*, (Cowpea) & *Raphanus sativus* (Radish)were agriculture at a rate of 10 seeds per each pot. The pots were stored in the green house (dept. of biology / college of Sciences) under 27-30°C, and irrigated periodically.

The agriculture was done in March/2015, and after (50) days the plants were harvested. The harvested plants were washed by fresh water, and transported to the laboratory, where the following data were recorded: the length of shoot system, fresh weight of plant, the percentage of seed germination, and the concentration of phosphorus (P) in plant and soil.

The analyses for phosphorus:

The quantitative analyses for (P) were made in X-ray fluorescence (XRF) machine in University of Baghdad, College of Sciences, Dept. of Geology. The soil and plants samples were prepared, dried in closed and dry environment and crushed by specific mill into 70 µm. The crushing operation has done in dry and clean mill in order to avoid contamination .The sample entered to the XRF was 3 gm from the dried powder of each sample (soil and plants). The results are printed on a worksheet including the element, normal intensity, concentration, loss of ignition, and error. The loss of ignition ranged between 80 - 85 % of the whole plant, while 10 - 15 % in soil. Thereupon, the total percentage of plant and soil, which remained from the 5 gm of sample were 15 - 20 % and 85- 90 % respectively.

Statistical analysis: Least significant difference (L.S.D.)was used to appear the effect of bacterial isolates and concentrations in the study parameters. [12].

The Results

Identification of bacterial isolates: The morphological, microscopic, and biochemical testes present that the bacterial isolates were belong to *Pseudomonas spp*. The colonies were green and yellow-green they were (G^-). negative reaction for Voges-proskauer, and positive for the flowing testes: reaction for indole, catalase activity, oxidase activity, and citrate utilization, which these results agree with another studies [13,14].

Length, fresh weight, and germination of plant: The results in this experiment showed that the adding of *Pseudomonas* isolates to the soil increased the growth parameters of plants comparing with control. Three of bacterial isolates promoting the growth in all plants, and for two plants (radish) and (cowpea), except the isolate (Ps2) was unaffected, and showed sample increasing in the growth parameters in comparison with the control, but this increasing without significant different .

The results in table (1) & (2) showed a remarkable increase in length, and fresh weight of all plants under study compared to the control. Table (3) also showed an increasing in number of plants that germinated in pots comparing with control pots. These results were for all plants of both genus (cowpea) & (radish), except the plants that inoculated with isolate (Ps2). The enhancement in the growth of tested plants is due to the ability of *Pseudomonas* to produce some secondary metabolites compounds like siderophores, and indole acetic acid (IAA) [15, 16].In addition, many studies has been reported that the *Pseudomonas* bacteria can solubilise the in soluble phosphorus in soil, and be more available for plant growth [17, 18]. These results agree with other studies [19] which was present that inoculation by *Psedumonas* as (bio fertilizer) improving the growth , and grain yield of wheat , and barly . Also the results agree with [20] whict reported that inoculation the seeds of (*Pennisetum glacum*) by *Psedomonas striata* increasing the root ,and shoot length and biomass , also showed ahigth phosphorus uptaken by shoot and grains.

The percentage of phosphorus (P) in plants and soil:

The results in this experiment present that the inoculation of the soil with *Pseudomonas* isolates was lead to increase the ratio of the P element in plants and the soil in pots. Table (4) showed an increasing of P % present in all plants that inoculated with isolates Ps1, Ps3, and Ps4 compared to the control, whereas, the isolate (Ps2) did not show any role in plant growth . Table (5) also showed an increasing in (P)% in soils of the two plants under study after adding the bacterial isolates to them , in comparison with the control soil (without bacteria).

The results suggested that the *Pseudomonas* bacteria have a mechanism by which phosphorus could be solubilised in soil, and thus involved lowering the pH in soil by producing an organic acids, and mineralize the organic P by acids phosphatase. This operation will increase the solubilisation of P in soil, and, as a result, increase the

up taken of P by the plants, [21, 22]. Other study presented that Pseudomonas fluorescents released 62% of P, and P. putrid released 51% of P from FePO4 [23]. Another study showed that *P. fluorescents* solubilise about 92mg P / L containing (AlPO4), and about 100 mg P / L containing CaPO4 [24]. The results of this experiment are in agreement with other studies [18] which was reported that using the *Pseudomonas fluorescents* (RAF15) in agriculture incread the solubility of insoluble (P) in soil , so it will be more available to plants . Also the results agreement with [21] which present that inoculation by *Pseudomonas spp*. Improving nutrient availability and uptake of phosphorus in soybean.

Table (1): effects of *Pseudomonas* isolates on length (cm) of plants *Raphanus sativas and Vigna unguiculata*

Isolates	Length (cm) of *Raphanus sativas*	Length (cm) of *Vigna unguiculata*
Ps1	16.31*	25.25*
Ps2	10.65	16.77
Ps3	12.75	20.23*
Ps4	21.53**	31.27**
Control	8.23	13.18

Table (2): effects of *Pseudomonas* isolates on Fresh weight (gm) of plants *Raphanus sativas and Vigna unguiculata*

Isolates	Fresh weight (gm) of *Raphanussativas*	Fresh weight (gm) of *Vignaunguiculata*
Ps1	9.52*	7.61*
Ps2	5.62	4.69
Ps3	6.85*	5.71*
Ps4	10.57*	8.85*
Control	3.68	2.55

Table (3): effects of *Pseudomonas* isolates on Percentage of germination (%) of plants *Raphanus sativas* and *Vigna unguiculata*

Isolates	Percentage of germination (%) *Raphanus sativas*	Percentage of germination (%) *Vigna unguiculata*
Ps1	80%	80%
Ps2	50%	60%
Ps3	70%	60%
Ps4	80%	90%
Control	40%	50%

Table (4): percentage of Phosphorus to (100 %) of in *the Raphanus sativas* and *Vigna unguiculata*

Samples	(P) % in *Raphanus sativas*	(P) % in *Vigna unguiculata*
Ps1	6.07*	5.31*
Ps2	4.66	2.78
Ps3	5.67*	5.35*
Ps4	5.98*	5.37*
Control	3.53	1.90

concentration of phosphor in 3 gm of dry sample in the *Raphanus sativa and Vigna unguiculat

Table (5): comparison between the samples of soils containing Phosphorus 100% in the *Raphanus sativas* and *Vigna unguiculata*

Samples	(P) % in soil of *Raphanussativas*	(P) % In soil of *Vignaunguiculata*
Ps1	2.16	2.06
Ps2	0.88	1.77
Ps3	2.06	1.94
Ps4	2.30*	2.18*
Soil Control	0.80	0.89

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Extraction and identification of some active compounds from *Enteromorpha ralfsii* which isolated from Bahr Al-Najaf in Iraq

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Abstract: The present study included isolation and identification the active compounds of an Macro-algae from the Iraqi water ecosystem. The Macro-algae, *Enteromorpha ralfsii* first time was isolated from Baher Al-Najaf region in Holy Najaf city during summer 2012. Physico-chemical parameters were measured, such as air and water temperature, Turbidity , Electrical Conductivityand Salinity, pH, Ca^{+2}, Mg^{+2},Total Hardness and Total Alkalinity. Results indicated that the active chemical compounds in the hot extract to *E.ralfsii* were Tannins, Saponins and Flavonoids.

Results indicated that the hot extract of *E.ralfsii* were having a lot of active chemical compounds against micro-organisms by using the GC-Mass Spectrometry technology. These findings suggest the possibility of using the *E.ralfsii* as a novel source of natural antimicrobial agents in pharmaceutical industries.

Key words: *Enteromorpha ralfsii* , Macro-algae and Baher Al-Najaf .

استخلاص وتشخيص بعض المركبات الفعالة من *Enteromorpha ralfsii* المعزول من بحر النجف في العراق العراق

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الخلاصة: تضمنت هذه الدراسة عزل وتشخيص احد الطحالب الكبيرة من المسطحات المائية العراقية لأول مرة من منطقة بحر النجف الواقع في مدينة النجف المقدسة خلال صيف 2012 وهو طحلب الانتيرومورفا *Enteromorpha ralfsii*. درست بعض العوامل الفيزياوية والكيمياوية وشملت درجة حرارة كل من الهواء والماء و الكدرة والتوصيل الكهربائي والملوحة ودرجة حموضة المياه و تركيز كل من الصوديوم والبوتاسيوم والمجموع الكلي للمواد الصلبة الذائبة والعالقة والعسرة الكلية والقاعدية الكلية وكانت المياه قاعدية جيدة التهوية.

أشارت النتائج إلى أن المركبات الكيمياوية الفعالة في مستخلص طحلب الانتيرومورفا هي العفص، والصابونين والفلافونويد. وبينت النتائج أن المستخلص الحار للانتيرومورفا فيه الكثير من المركبات الكيميائية الفعالة ضد الكائنات الدقيقة ، ويمكن استخدامها كمصدر جديد من مضادات الجراثيم الطبيعية في الصناعات الدوائية .

Introduction

*Enteromorpha sp.*is floating green algae that belongs to Ulvaceae family[1]. It's a difficult genus to divide into species because the morphology of the different species tends to vary widely in nature. Sometimes the only way to tell different species apart is to use molecular sequence data. Because of this, it could be difficult to determine the species of a member of the genus *Enteromorpha* in the field [2, 3].

Enteromorpha is also similar to *Ulva* and it is sometimes difficult to differentiate between the two different genera [4]. *Enteromorpha* is rich in nutrients, such as essential amino acids, polysaccharides, fatty acids, vitamins, phenolic compounds and minerals, which have attracted considerable attention in the fields of biochemistry and pharmacology because of their biological activities [5, 3,6]. There for its very important to study and identify the active compound in the *E.ralfsii*.

Materials And Methods:

Collection and preparation of samples:

Samplings were carried out from Bahr Al-Najaf is situated southwest of Al-Najaf Al-Ashraf city.Which (located at longitude 31° 58′ 54.62 N and latitude 44° 12′ 28.27 E)during summer 2012. Samples of *E.ralfsii* were collected manually from the rock. The harvested macro-algae were stored in plastic bags for transported to the laboratory. Voucher specimen of species was pressed and stored in 5% formalin for identification according to [7] and [8].

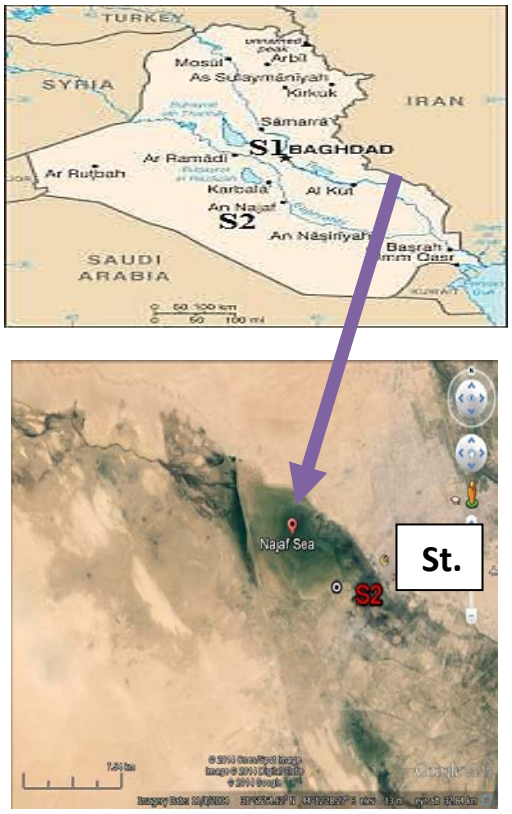

Figure (1): Sampling sites.

St: Poison in Bahr Al-Najaf (located on longitude 31° 58′ 54.62 N and latitude 44° 12′ 28.27 E).

Physio-Chemical Parameters:

Field Work:

Water samples were taken from both sides region in10L containers. On-metallic bucket was used to avoid metallic contamination. Water temperature, conductivity (µS/cm), pH, salinity and turbidity were measured immediately in the field. The electrical conductivity (EC), TDS and pH were measured by using pH -EC- meter (HANNA Instruments). Salinity was determined by depending on the electrical conductivity values (9).While, turbidity was determined by using a Turbidity meter (HACH Instruments) model 2100A after instrument calibration by knowing turbidity stander solution.

Laboratory Work:

The measure of total Alkalinity, calcium (Ca^{+2}) , magnesium (Mg^{+2}) , and total hardness studied of Bahr Al-Najaf

were determined by adopting standard protocol [10].

Preparation of alcoholic extract for active compound estimation and GC-Mass Technology:

The hot methanolic alcoholic extract was prepared by Soxhelet according to. [13] In this process the dried powder form of algae material extracted by using methanol alcohol.

After completing the process the extract from macro-algae were kept in sterilized test tubes stored in refrigerator till further use.

Qualitative estimation of active compounds:

The presence of active compounds of the studied macro algae as the hotmethanolic alcoholic extract were determined by adopting standard protocols as following :

1- Terpenoids indicators:

A – Foam test:

This reagent was used for the detection of saponines, bottle contained aqueous or ethanol extract was shacked and then the appearance of big foam for a long time as a result of stirring .The aqueous of macro-algae in test tube indicated Saponins existence [11].

B – HgCl2 reagent:

This reagent was used for the detection of Saponines by adding 1-2 ml from HgCl2 1% to 5 ml of aqueous extract then white precipitate was appeared which indicates that the test is positive[11].

C – Acetic anhydride reagent:

According to [11] 1 ml of the extract was added to 1-2 drops of chloroform, then 1 drop of anhydride acetic acid, and then 1 drop of concentrated H2SO4 .The appearance of brown colour means the presence of terpens, and the appearance after a period of time of black-blue means the presence of steroids.

2 - Alkaloids indicators:

A – Mayer reagent:

This reagent was used for the detection of alkaloids .The stock solution (1) was prepared by dissolving 13.5g HgCl2 in 60 ml water, stock solution (2) was prepared by dissolving 5g KI in 10 ml water, then combined with stock (1) and (2) and diluted with water to 100 ml, then added 1-2 ml of Mayer reagent to 5 ml of aqueous, or ethanol extract was added .A creamy or white precipitate indicates the presence of alkaloids[12].

B – Tannic acid reagent:

This acid was used to precipitate alkaloids [11] . 1% tannic acid was prepared, then 1-2ml of reagent was added to 5ml of the extract. The white turbidity was appeared.

3 – Phenolic indicators:

A – Lead acetate reagent:

It's aqueous or ethanol solution of lead acetate 1%.

Amount of reagent was added to an equal amount of aqueous or ethanol extract then white precipitate will appear which indicates the presence of phenols [11].

B – Ferric chloride and Potassium ferric cyanide reagent:

It was used for the detection of general phenols . Prepared by 2 equal amounts of aqueous solution of ferric chloride 1% and Potassium ferric cyanide 1% Blue-green colour appeared indicating that the test is positive [11].

Gas Chromatography - Mass Spectrometry:

For GC-MS analysis, a high-temperature column (Inert cap 1MS ; 30 m x 0.25 mm id x 0.25µm film thickness) was purchased from Agilent Technologies (SHIMADZU – Japan). By using a high- temperature column, to eliminate the need for derivatization of each sample. The injector and detector temperatures were set at 280°C while the initial column temperature was set at 100°C. A 5µl sample volume wasinjected into the column and ran using split (1:10) mode After 1 min, the oven temperature was raised to 225°C at a ramp rate of 12.5°C/min (hold time 4 min). The oven temperature was then raised to 300°C at a ramp rate of 7.5°C/min (hold time 5 min). The helium carrier gas was programmed to maintain a constant flow rate of 17.5 ml/min and the mass spectra were acquired and processed using both Agilent GC-Mass Solution (SHIMADZU – Japan) and Postrun software. The compounds were identified by comparison of their mass with NIST library search and authentic standards.

Results and Discussion :

Morphological Structure of *E.ralfsii*:

E.ralfsii was isolated from Baher Al-Najaf region, and this is the first time recorded in Bahr AL-Najaf ,while[14] record this species in south Iraqi water bodies .Those marine algae exposed to severe environmental stress and that agrees with [15] who mentioned that the water type of Bahr Al Najaf area is of marine origin preserved in semi-confined basin.

These isolated algae belong to Chlorophyta and they were easily obtained by hand and then washed with tap water.

E. raflsii macro-algae attached to rock and plant, fine, detached hair-like form cell detail of a cylindrical side branch, showing several lines of cells, branching pattern showing irregular side branches containing several lines of cells (multi seriate) figure (1), thesis findings agreed with Fish and Fish [16,17].

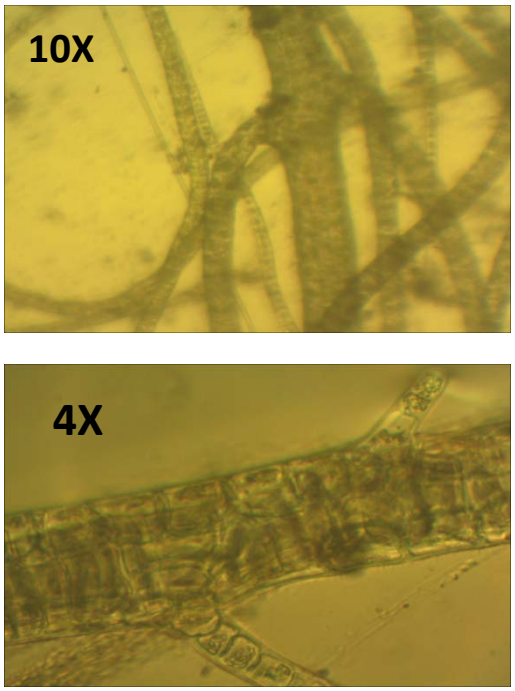

Figure (2): Filaments of *E. raflsii* showing the branch and multi seriate.

In this study, air temperature ranged between (35– 39) °C. While, water temperature ranged between(32–35)°C. Local variations in air and water temperature values might be due to the differences in sampling time in that day, which expose that the lower degrees in the beginning of the morning, then increase whenever reached the middle of the day [18,19] and (Table 1).Measuring turbidity in Bahr Al-Najaf is an important as an indicator of the concentration of suspended sediments in the water. The results showed that the water turbidity ranged between (32–41) NTU this agrees with [20].

The electrical conductivity ranged between (5040 –6000) µS/cm. Water conductivity was observed higher in Summer, might be due to the increase in temperatures causes evaporation of the water and so, concentrating the ions, conductivity was temperature sensitive and increases with increasing temperature[21,22].These results agreed with some studies in Iraq water bodies [23,24,25]. The increasing of the water salinity which ranged between (2.24-2.8) ‰ related to summer, this will lead to concentrate salts which increasing the salinity. These results agreed with some studies such as [20,26,27] . The pH values were low (5.8-6.2), because of the degradation of aquatic plants,

algae, organic materials and the high levels of salinity [23, 24].The increase the Ca^{+2} and Mg^{+2} concentrations in Bahr Al-Najaf , which ranged between (600-740) $mg.l^{-1}$and (800-820) $mg.l^{-1}$ respectively, due to the nature of the topography of the area [26, 28 , 29].

Table (1): The range and Mean ± Standard Errorof Physio-chemical water properties in the stations.

Properties	Summer's Season 2012
Air temp. °C	35-39 (37±2)
Water temp. °C	33-35 (34±1)
Turbidity NTU	32-41 (36.5±4.5)
E.CµS/cm	5040-6000 (5520±480)
Salinity‰	2.24-2.8 (2.52±0.39)
pH	5.8-6.2 (6±0.2)
$Ca^{+2}mg.l^{-1}$	600-740 (670±70)
$Mg^{+2}mg.l^{-1}$	800-820 (810±10)
Total Hardness$mg.l^{-1}$	1650-1795 (1722±72)
Total Alk.$mg.l^{-1}$	210-230 (215±5)

The results showed that total hardness values andtotal alkalinity ranged between (1650-1795) $mg.l^{-1}$ and (310-230) $mg.l^{-1}$ respectively, due to that Baher Al-Najaf near the places where the farmer, houses and industries were discharging their wastes directly to the Baher Al-Najaf water, and this waste will be decomposed by microorganisms leading to release of CO_2 gas which dissolved in water causing an increase of total alkalinity. These results agreed with those obtained by[29].

Qualitative estimation of active compounds from the macro-algae:

The results showed the presence of active compounds in the methanol extract of *E. rafsii*in the Table (2).

The results showed that methanolic extract of *E. rafsii*the Tannins, Flavonoids and Saponins were present , while Alkaloids, Glycosides, Terpenoid and

phenols, were absent .This result agreed with many studies such as [30,31 ,32] they screened the most active compounds in macro-algae , biochemical analysis were being undertaken to determine the structure and nature of compounds responsible of the bio-active compounds of the extracts with high antibacterial activity. Not only the presence of a particular compound which makes these organisms, interesting, but also their huge diversity and the possibility of not only harvesting them, but also of growing them at different conditions, leading to an enrichment of some bioactive compounds.

Table (2): Presence or absence of active compounds in *E. rafsii*hot methanolic extract.

Active compounds	Presence (+) Or Absence (-)
Alkaloids	-
Glycosides	-
Tannins	+
Terpenoids	-
Flavonoids	+
Phenols	-
Saponins	+

GC-MS Analysis:

Methanol extracts of the macro-algae *E.* ralfsii 8-major compounds were found in the hot ethanolic crud extract of E. ralfsii ,these were: Nonadecane (16.2%) is an alkane hydrocarbon , while Pentadecane represented (39.6%) from the crud hot extract of E. ralfsii . The alkane hydrocarbon is the generic name for the group of aliphatic hydrocarbons Cn-H2n+2 ,which represented reactive groups.

The materials in this group may be incompatible with strong oxidizing agents like nitric acid. Charring of the hydrocarbon may occur, followed by ignition of unreacted hydrocarbon and other nearby combustibles. In other settings, aliphatic saturated hydrocarbons are mostly unreactive, They are not affected by aqueous solutions of acids, alkalis, most oxidizing agents, and most reducing agents. When heated sufficiently or when ignited in the

presence of air, oxygen or strong oxidizing agents, they burn exothermically to produce carbon dioxide and water [33].The results are in accordance with the reported investigations [34,35] straight chain paraffins (n-alkanes), branched chain paraffins (alkyl-alkanes) and unsaturated hydrocarbons (alkenes) were already reported from many marine algae [36,35].Similar group of hydrocarbons Tetradecane, Octadecane and Hexadecane have been reported as common major volatile components in the crud extracts of macro-algae (*C. glomerata*(R) and *E.ralfsii*), and this results agreed with other studied such as [37].Recently, many papers have been published which discuss the methods of manufacture and the composition of algal extracts. The general conclusion is that the composition of extracts strongly depends on the raw material (geographical location of harvested algae and algal species) as well as on the extraction method. The biologically active compounds which are transferred from the biomass of algae to the liquid phase include polysaccharides, proteins, polyunsaturated fatty acids, pi agents, polyphenols, minerals, plant growth hormones and other. They have well documented beneficial effect on humans, animals and plants, mainly by protection of an organism from biotic and a biotic stress (antibacterial activity, scavenging of free radicals, host defence activity etc…) and can be valuable components of pharmaceuticals, feed additives and fertilizers [38,39,40].

Table (3): GC-MS analysis of major compounds of *E. ralfsii* hot methanolic extract

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Rt	Compound	Area%
12.98	Hexadecane –tetra methyl	10.2
13.78	Nonadecane	16.2
14.11	Pentadecane	39.6
16.61	Hexadecane- tetra methyl	3.6
16.75	Octadecane	5.3
18.94	Tetradecanedihydroxyl	2.1
21.72	Hexadecane 2-hydroxyl	4.8
23.63	Hexadecane 2-hydroxyl	3.8

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Isolation of Jasmimin from Jasmine (*Jasminum sambac)*

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Abstract: *Jasminum sambac* (oleaceae) cultivated in Iraq, it is an important medicinal plant, that is used as antimicrobial, anticancer and antioxidant. In previous study, Jasminin compound was extracted, isolated and identificated from chloroform, 100% methanol, 70% methanol and petroleum ether which extracted from whole dry plant (root, stem, leaf and flower). Quantitative and qualitative test, such as special reagent, TLC and HPLC tests were done. The result showed that chloroform and 70% methanol extract had the ability to extracted jasminin compound from plant parts than any other organic solvent. The qualitative data revealed that the retreadactin factor Rf value of jasminin was (0.8), quantitative test (HPLC) show that methanolic and chloroformic extract had (0.62696 mg) and (1.11386 mg) jasminin for one gram dry plant respectively. Jasminin purified from chloroformic and methanolic extract using preparative TLC (0.75 cm), the HPLC for the purified jasminin showed one peak respectively.

Key words: jasminin, *Jasminum sambac*, TLC and HPLC.

عزل مركب الياسمين من نبات الرازقي *(Jasminum sambac)*

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الخلاصة: نبات الرازقي *Jasminum sambac* (oleaceae) المزروع في العراق هو من النباتات الطبية المهمة، التي تستعمل كمضاد للميكروبات ومضاد للسرطان ومضاد للاكسدة. يتضمن البحث استخلاص مركب الياسمين وعزله والتعرف عليه . استخلص نبات الرازقي الكامل الجاف (جذور ، سيقان، اوراق، ازهار) استخلاص كلوروفرمي مطلق واستخلاص ميثانولي مطلق ومستخلص ميثانولي 70% و استخلاص البتروليوم ايثر. اجريت الفحوصات النوعية والكمية لهذه المستخلصات، مثل الكشف عن وجود بعض مركبات الايض الثانوي باستعامل كشوفات خاصة والكروماتوكرافي (TLC, HPLC). اظهرت النتائج ان المستخلص الكلوروفورمي والمستخلص الميثانولي 70% ، لهما القدرة الاستخلاص وسحب مركب الياسمين من الاجزاء النباتية اكثر من المذيبات العضوية الاخر، واوضحت الفحوصات النوعية ان retreadactin factor (Rf value) للياسمين هو (0.8)، في حين بينت الفحوصات الكمية HPLC ان المستخلص الميثانولي والكلوروفورمي يمتلكان كمية ياسمين قدرها (0.62696 mg) و (1.11386 mg) على التوالي لكل غرام واحد وزن نباتي جاف. نقي الياسمين من المستخلص الكلوروفورمي والميثانولي باستعمال TLC بسمك (0.75 cm)، بينما اظهرت نتائج HPLC للمادة المنقاة ظهور منحنى واحد فقط.

Introduction

Plants have been a rich source of natural compounds, that are synthesis naturally in plant, these phytochemical products are distinction and have marked every plant from the other. The secondary metabolite is accountable for the biologically medical activity [1],one of them is jasminin, that found in some species belong Oleaceae family. Jasminum*sambac*Linn. (Oleaceae) is one of flowering plant has many traditional name like Arabian Jasmine, Jasmine, Mogra, Motia, Lily ect..[2]. It has cultivated for their beautifulflowers. Moreover, all plant parts like root, stem, leaf and flower are used for the pharmacological contain [3]. Jasmine cultivated in tropical and subtropical parts of world, it is characterized by shrub evergreen plant, sub erect or ascendant, ovate opposite leave, fragrant white flower [4]. The plant used traditionally in cough, fever, lowering blood glucose level and ulcer healing. Sabharwal S. *et al*. showed that the plant have antidiabetic, antimicrobial, antioxidant and antitumor [5]. The plant contain many phytochemical compounds, such as alkaloids, flavonoids, tannins, saponins and terpenes[4].

Secoiridoid glucoside is one of the important activegroup of secondary metabolite in *Jasminum sambac.* The glycosides type presence in *Jasminum sambac*, which contain many compound, which are iridoidglycoside [6].

In this paper we examined the presence of some active compounds and purification of one of alkaloids which is Jasminin that is showed in figure1 [6].

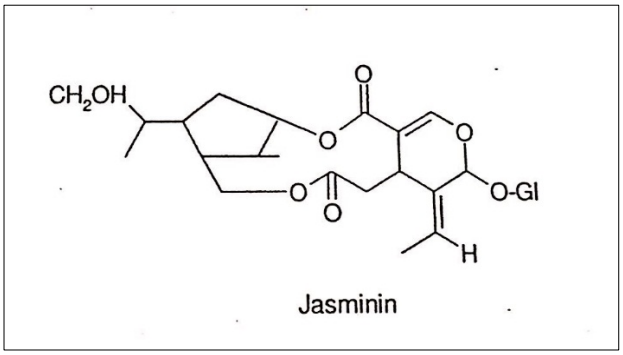

Figure 1: jasminin structure [1].

Materials and methods

Plant collection and extraction

Jasminum *sambac* Linn were collected from Baghdad nurseries. The whole plant washed with water to remove soil and other related particles, dried in shad at room temperature, then grinded into powder . The powder plant where extracted in sohxulate apparatus in (1:10) ratio of powder plant to organic solvent at 60 – 80 C ˚for 6 – 8 hour. Four extracted type was done which are:

1- 100% chloroform extract
2- 100% methanol extract
3- Petroleum ether for 4 hour, then 70% methanol extract
4- Petroleum ether extract

Phytochemical screening

1- Alkaloid detection
   - **a-** Mayer reagent
   - **b-** Wagner reagent
2- Terpenoid reagent
   - **a-** Chloroform and sulfuric acid reagent
   - **b-** Anace- aldehyde reagent
3- Saponin detection
   - **a-** Foam reagent
   - **b-** Mercury chloride reagent
4- Tannin detection
   - **a-** Ferric chloride reagent
   - **b-** Lead acetate reagent
5- Flavonoid detection
   - **a-** Magnesium crystals and hydrochloric acid reagent
   - **b-** sulfuric acid reagent

Qualitative analysis

Thin layer chromatography (TLC)

The TLC analysis for jasminin qualitative is done, the analysis done on 0.25 mm silica gel plates using specific mobile phase , we used many solvents system, the best solvent [ethyl acetate : methanol] (7:3) (v:v) [7].

Quantitative analysis

High performance liquid chromatography (HPLC)

The main compound of four extract were separated on fast liquid chromatography (FLC) column under optimum condition column: phenomenex c-18, 3 µm particle size (50 x 2.0Mm I.D) column. Mobile phase: acetonitrile: methanol: 0.1 % Formic acid (6:3:1) (v:v), flow rate 1.2 ml / min, detectionUV at 262nm [8].

Jasminin purification

Preparative TLC

Chloroformic and 70% methanolic extract were developed Preparative TLC 0.75mm plates, with mobile phase ethyl acetate: methanol (7:3) (v:v)[7], compared with stander jasminin, detection 254 nm UV light.

Results and discussion

Phytochemical screening

The results of phytochemical screening of the four whole plant extract types are presented in table 1. Qualitative taste for alkaloids, terpenoids, saponin, tannins, and flavonoids were carried out to know the presence of secondary compounds in these extracts.

Table 1: The phytochemical screening of four *Jasminum sambac* extract.

detection	100% chloroform		100% methanol		Petroleum ether and 70% methanol		Petroleum ether	
Reagent type	Reagent A	Reagent B	Reagent A	Reagent B	Reagent A	Reagent B	Reagent A	Reagent B
Alkaloids	+	+	+	+	+	+	+	+
terpenoids	+	+	+	+	+	+	+	+
saponin	+	+	+	+	+	+	+	+
tannins	+	+	+	+	+	+	+	+
flavonoids	+	+	+	+	+	+	+	+

This results we obtained from phytochemical analysis agree with the results by [2, 3], and [4] were showed the presence of alkaloids, terpenoids, saponin, tannins, and flavonoids secondary product in *Jasminum sambac* extracts. However, the results disagree with [5] and [9] where negative result for alkaloid and some other compound. The difference in results may be due to the environmental factors like (soil mineral compound , PH, temperature, light density and light period) that effect the type and the quantity of compound, which effect on the metabolic path way of active compound.

Qualitative analysis

Thin layer chromatography (TLC)

TLC analysisof Secoiridoidglucosidewas done. The jasminin analysis carried out by 0.25mm TLC, the four extracts of whole plant *Jasminumsambac* and stander jasminin were subjected on TLC and developed in the different mobile phase were tried for separation of jasminin secoiridoid glucosidefrom other compounds from *Jasminum sambac*extracts and good separation achieved by using ethyl acetate: methanol (7:3)(v:v)

solvent system. The extract sample showed the presence of jasminin as a dark spot under ultra -violet light 254nm. The identify of jasminin spot in samples was compared with stander jasminin, the RF value in the sample and reference stander was parallel 0.8.

Quantitative analysis

High performance liquid chromatography (HPLC)

HPLC analysis was carried out by providing the suitable condition for the four extracts. The results obtained by HPLC are showed in figures2,3,4,5 and 6 for stander, with (5.7) retention time. Jasminin presence in Chloroformic, 100% metnaolic, 70% methanolic, and petroleum ether extract according to stander retention time, and peak area (1.11386, 0.62696, 0.64614, and 0.01294) mg/ g dry wait respectively. The HPLC analysis ofjasminin showed higher presence of jasminin in chloroformic extract were the petroleum ether extract has the lowest percentages of jasminin.

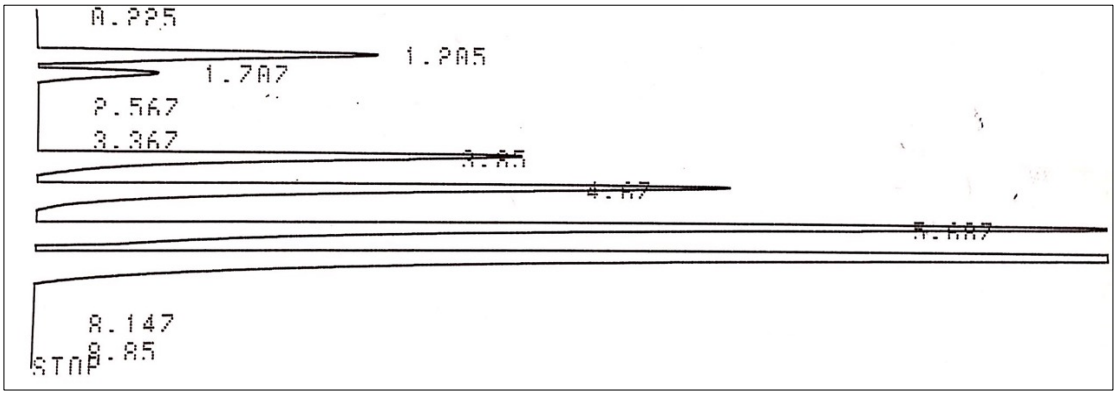

Figure2: HPLC chromatogram of crude chloroformic extract, the separation of jasminin peak at 5.687 retention time.

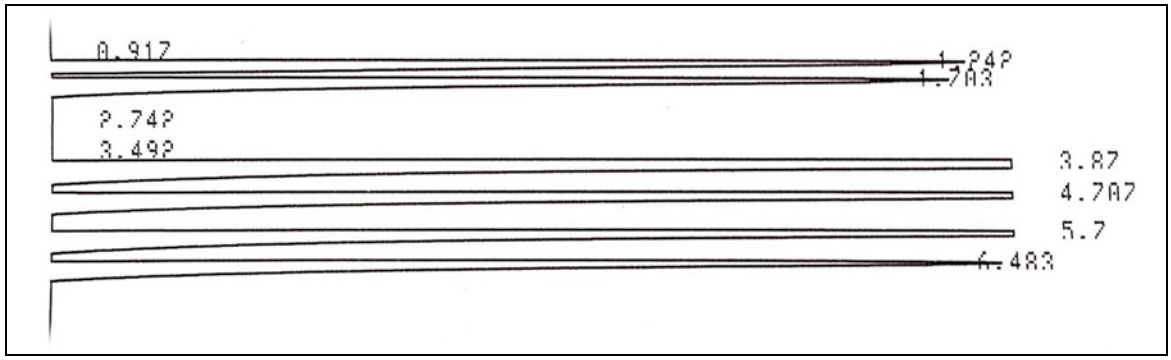

Figure 3:: HPLC chromatogram of crude 100% methanolic extract the separation of jasminin peak at 5.7 retention time.

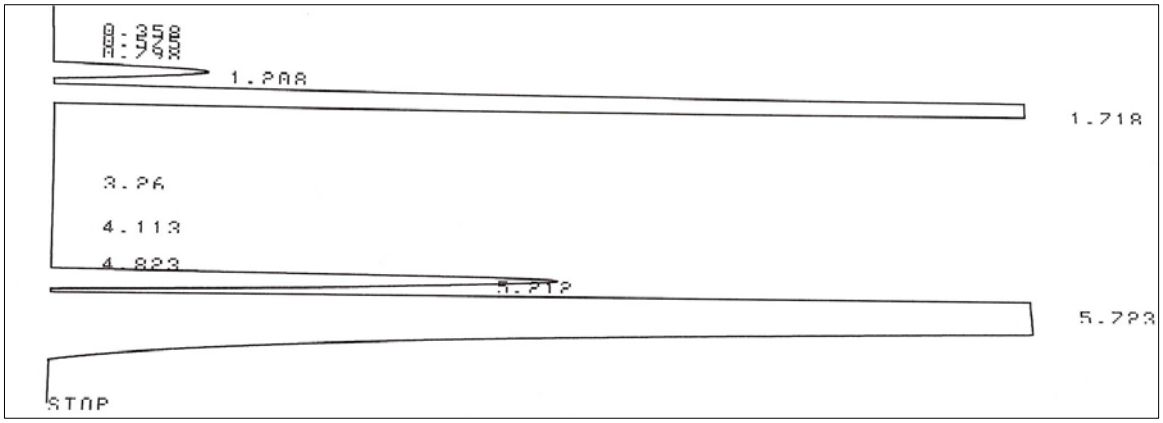

Figure 4:HPLC chromatogram of crude petroleum ether then 70% methanolic extract the separation ofjasminin peak at 5.723 retention time.

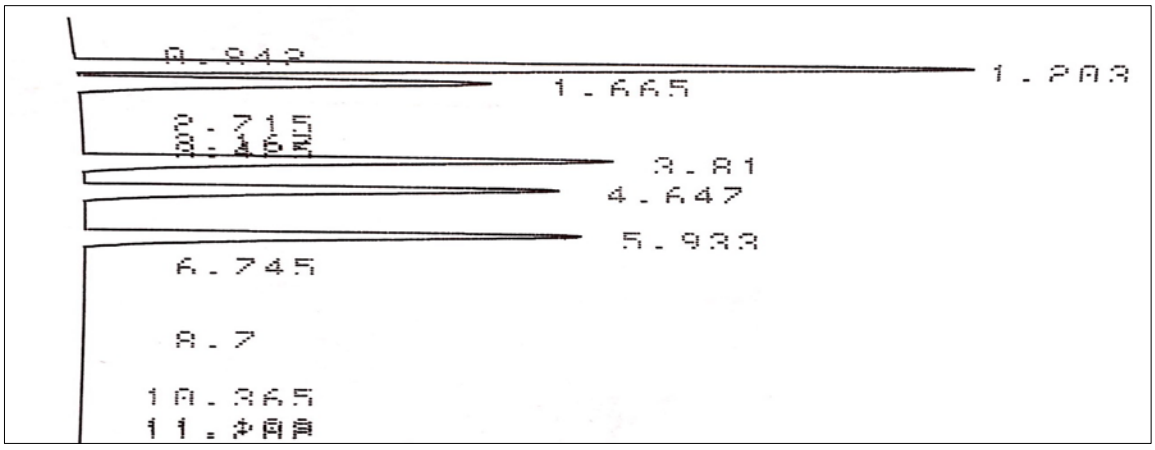

Figure 5: HPLC chromatogram of crude petroleum ether extract the separation of jasminin peak at 5.933 retention time.

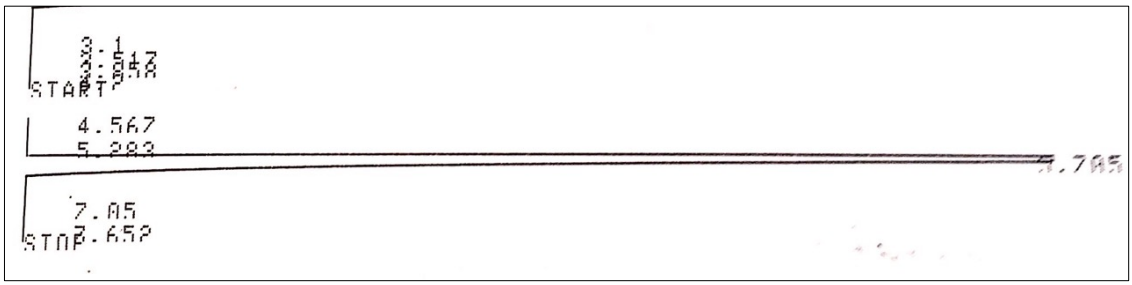

Figure 6: HPLC chromatogram of Jasminin of standard.

Jasminin purification

Jasminin Secoiridoidglucoside were purified from chloformic and 70% methanolic extract by using preparative TLC (0.75mm) as stationary phase and ethylacetate : methanol (7:3) (v:v) as a mobile phase and standerjasminin, the dark band under 254 nm ultra-violate light. The RF value was (0.8) as shown in figure6, the band were scraped and dissolved in chloroform. Then HPLC analysis for the purified compound were done, the result showed that the compound purified from chloroformic and 70% methanolic extract had one peak (Figure 7 , 9, and 8), for stander jasminin, which is jasminin compared with stander jasminn (figure 9) had the same result which purify jasminin from *Jasminumsambac*in 1984 but using column chromatography for purification, [10] .

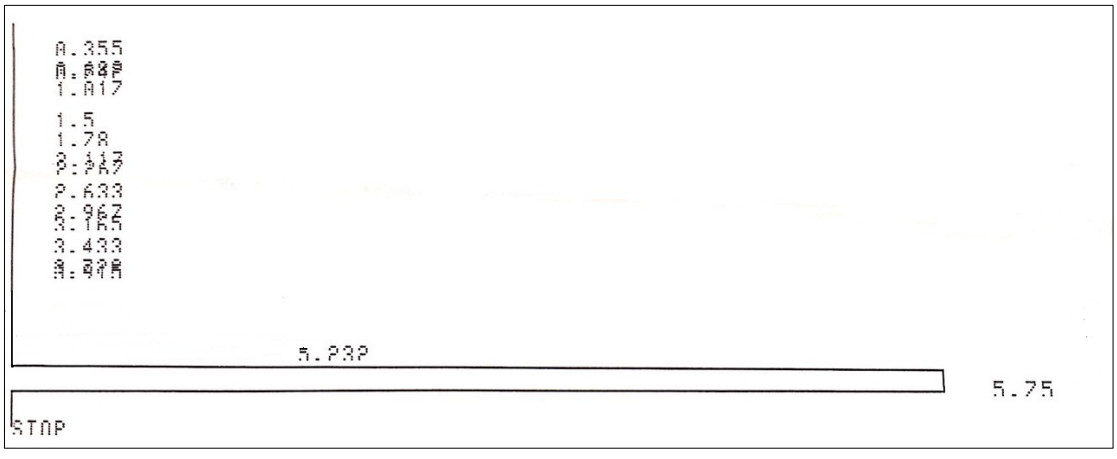

Figure 7:HPLC chromatogram of pure jasminin purified from chloroformic extract.

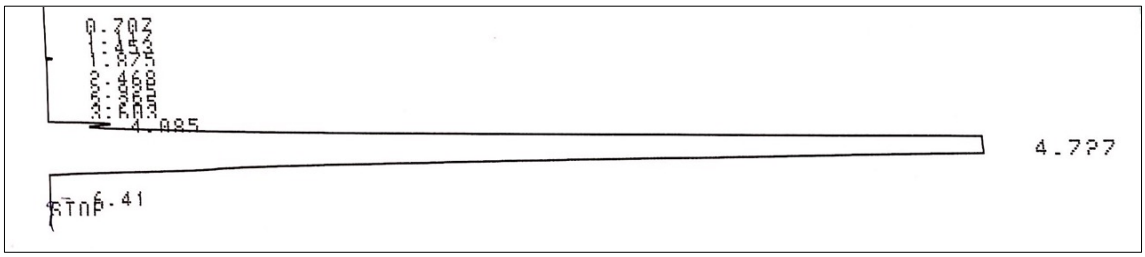

Figure 8:HPLC chromatogram of standerjasminin.

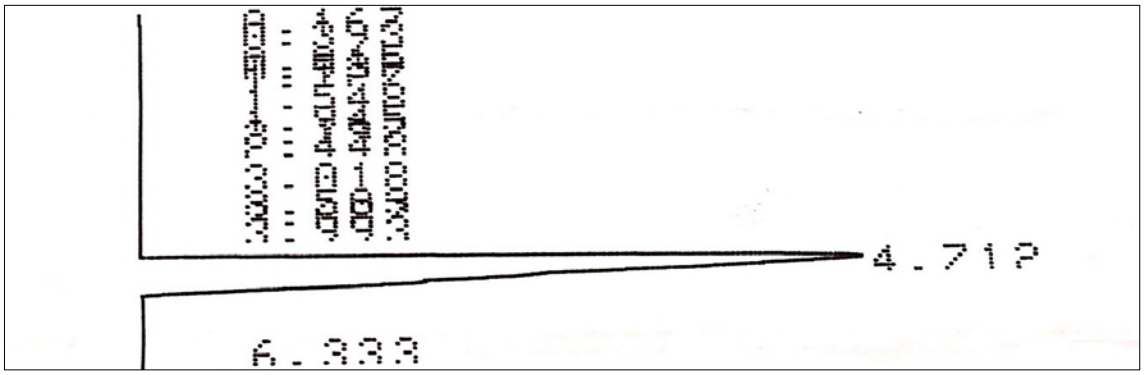

Figure 9: HPLC chromatogram ofjasminin purified from petroleum ether then 70% methanol extract.

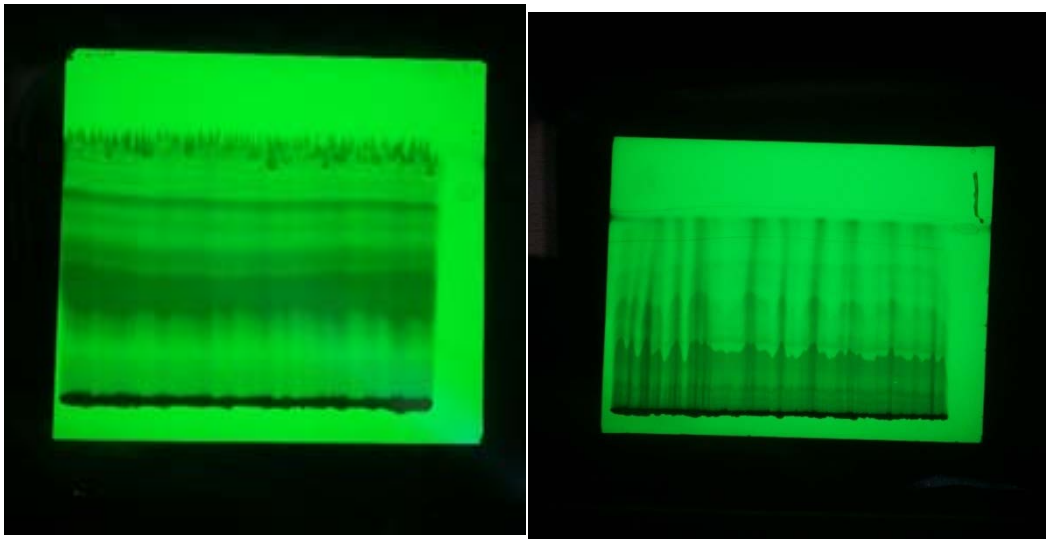

Figure10: Preparative TLC, right jasminin purification from petroleum ether then 70% methanol extaract, left jasminin purification from chloroformic extract.

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Isolation of dermatophytes species from patients with different types of leukemia in Baghdad Governorate

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Abstract: The study is concern on determine the type of dermatophytes species in leukemia patients that were infected with dermatophytoses as a result to their immune suppression (weekend immune system) due to their submission to radiation and chemotherapy treatment. The result showed that the most common isolates were *Microsporum canis* 7 isolates which represent (33.33 %) of cases, then followed by *Trichophyton rubrum* 5 isolates which represent (23.8 %), while the less common isolates were for *Microsporum ferrunginum*, *Trichophyton saudance* and *Trichophyton tonsurance* 2 isolates which represent (9.52 %), respectively.

Keywords: leukemia, immunocompromized patients, dermatophytes

عزل انواع الفطريات الجلدية من مرضى اللوكيميا بأنواعه المختلفة في محافظة بغداد

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الخلاصة: الهدف من هذه الدراسةهو تحديد أنوع الفطريات الجلدية في مرضى سرطان الدم الذين أصيبوا بداء المبيضات الفموية نتيجة لقمعها المناعي (ضعف الجهاز المناعي) نظراً لخضوعهم للعلاج الإشعاعي، والعلاج الكيميائي. أظهرت النتائج أن العزلات الأكثر شيوعاً كنت *Microsporum canis* 7 عزلة و التي تمثل (33.33%) من الحالات، ثم تليها *Trichophyton rubrum* 5 عزلة و التي تمثل (23.8%)، بينما كانت العزلات الأقل شيوعاً *Microsporum ferrunginum*, *Trichophyton saudance*و *Trichophyton tonsurance* 2 عزلات وتمثل (9.52%).

Introduction

Dermatophytes are a group of closely related fungi with the ability to colonize keratinized tissues. They are identified according to the features that produced when they grow on a suitable agar medium. Dermatophytes are a group of morphologically and physiologically related molds that cause well-defined infections in vertebrates. The incidence of dermatophytoses has increased over recent years, particularly in immunocompromised patients [1-4]. The fungi attack skin, nails and hair, where keratin is the major structural protein, leading to a wide variety of disease states [5], dermatophytes include fungi that involve keratinized tissue "hair, skin, nail and mucous membrane" .Dermatophytes may be described as anthropophilic, zoophilic or geophilic depending upon whether their normal habitat is on man, or an animal, or in the soil [6].

Leukemia is a type of cancer of the blood or bone marrow characterized by an abnormal increase of immature white blood cells called "blast". In turn, it is part of the even broader group of diseases affecting the blood, bone marrow, and lymphoid system. Patients with hematologic malignancies such as leukemia are susceptible to infectious complications, because the host is immunocompromised by both malignancy and cytotoxic treatment [7]. There for, immunodeficiency is defined as the failure of the immune system to protect against disease or malignancy, it includes two types, Primary Immunodeficiency which caused by genetic or developmental defects in the immune system. These defects are present at birth but may show up later on in life, and secondary or acquired immunodeficiency which means the loss of immune function as a result of exposure to disease agents, environmental factors, immunosuppression, or aging [8].

Materials and Methods

Sample collection: During the period that confined between November 2013 to April 2014, 100 specimens (nail clippings and skin fragments) were collected from 100 patients with different types of leukemia who diagnosed clinically by specialist doctors, nail clippings and skin fragments were examined directly by microscopic examination and then they were cultured on plates with SDA medium, from 10-14 days, at temperature 28-30 C°.

Detection of dermatophytes *spp*

The following identification tests were used to identify the dermatophytes *spp*.:

1. **Hair Perforation Test:**

This test was used to differentiate between *T. mentagrophyte* and *T. rubric*. Short strand of human hair (10mm in length) were placed in petri dish with 20 ml of distilled water and put in autoclave. Then 2-3 drops of 10% V/V sterilized yeast extract were added to the petri dishes. Then an inoculum of the test fungi which grown on (SDA) were added to the petri dishes. The plates were incubated at 28-30°C, the hair strands were examined periodically over a period of 4 weeks by putting them in lactophenol cotton blue for the presence of perforation under low power microscopy [9].

Urease Test:

This test was used to differentiate between *T. mentagrophyte* and *T. rubrum*. Slopes of urea's agar base were inoculated with the tested organism and incubated at 28-30 ^{0}C and they examined after 3-7 days. Positive result in this test was the change in media color from yellow (ve-) to pink (ve+).

2. **Growth on PDA Medium:**

This test was used to differentiate between *T. mentagrophyte* and *T. rubrum*, according to the ability of production red color on the reverse side of plates with PDA medium. Fungal inoculum was taken from colony edge and put in the center of plates, and then the plates were incubated at 28°C and they examined after (14) days for the observation of media color [10, 11].

3. **Growth at Temperature 37°C:**

This test was used to differentiate between some genera which related to the genus *Trichophytone* by their ability to grow at temperature 37 °C like *T.mentagrophyte* and *T. rubrum* which cannot grow at this temperature [12].

Isolation of fungi:

the total number of dermatophytes isolates was 21, table(1).

Statistical analysis

The statistical test which used in this study was the ANOVA test, p (<0.05). Statistical analysis showed a non- significant difference at level of probability p (<0.05) between dermatophytes species in leukemia patients.

Results and discussion

The number of dermatophytes isolates was 21 isolate. The most common isolates was *Microsporum canis* 7 isolates which represent (33.33%) of cases, then followed by *Trichophyton rubrum* 5 isolates which represent (23.8%) Table-1.

Table 1- The frequency of fungal isolates according to their species

dermatophytes *spp.*	Number of isolates	Percentage %
M. canis	7	33.333
M. rubrum	5	23.809
M. ferrunginum	2	9.523
T.mentagrophyts	3	14.285
T. saudance	2	9.523
T. tonsurance	2	9.523
Total	21	99.996

Superficial dermatophytosis, such as tinea and candidiasis, is quite common in ATLL (one type of leukemia of mature T-cell malignancy that related to ALL) patients, as approximately 50% of the patients develop cutaneous mycotic infections [13, 14]. These findings have suggested the defective immunity against dermatophytes in ATLL patients. The incidence of dermatophytoses has increased over recent years, particularly in immunocompromised patients [15, 16]. Since all patients with different types of leukemia were submitted to immunosuppressant, oral steroids, or chemotherapy which leads to decrease their immune response.

The increase in frequency of these infections has been attributed to several factors, including high-dose chemotherapy, oropharyngeal-mucositis and prophylactic therapy [17]. All the above findings were agreed with this study.

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Identification and transformation of *Lactobacillus acidophilus* bile salt hydrolase A gene (bshA) in *Escherichia coli*

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Abstract: The bile salt hydrolase gene (*bshA)*, encoding bile salt hydrolase enzyme (EC 3.5.1.24) from *Lactobacillus acidophilus Ar* probiotic isolate ,which is responsible for assimilation cholesterol were studied in the present work. This gene designed contain two restriction sites (PstI/SacI) were added to each end of fragment and amplified by PCR techniques use specific primer to genetic analysis sequencing for gene nucleotides which were 801 bp. Gene manipulation during cloning inserted into pJET1.2\blunt end vector respectively. pJET1.2\blunt end vector is overexpression plasmid for *E. coli* . The resulted constructs were named as pJET/bshA. The recombinant was transferred to *E. coli* MC1022 by chemical transformation. Obtained recombinants analyzed for expression and sequences. The results were confirmed that production of bile salt hydrolase from recombinant *E.coli* MC1022 pJET/bshA found to be higher expression while compared with *E. coli* MC1022 wild type strain from 100 to 383.3 (U\mg) . The recombinant plasmid also found to be stable in host organism after a few generations.

Key words: Probiotic strain *Lactobacillus acidophilus Ar, E. coli* MC1022, Bile salt hydrolase, *bshA* genes.

تشخيص وتحويل جين bile salt hydrolase (*bshA*) من البكتريا *Lactobacillus acidophilus Ar* في بكتريا *Escherichia coli*

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الملخص: شخص الجين bile salt hydrolase (*bshA*), المسوؤل عن التشفير للانزيم bile salt hydrolase (EC 3.5.1.24) من العزلة *Lactobacillus acidophilus Ar* probiotic المسؤول عن خفض الكولسترول الغير صحي في الانسان والحيوان ، وذلك ببلمرة الجين بواسطة تقنية PCR وتحديد التحليل التفاعل المتسلسل للقواعد النتروجينية لطوله البالغ 801 bp باستخدام البادئ الخاص لتسلسل الجين صمم يحوي على نوعين من من انزيمات التقييد (PstI/SacI) وتمت كلونته في احد البلازميدات لبكتريا *E. coli* MC1022 وهو pJET1.2\blunt end vector والنتاج المكلون اطلق عليه pJET/bshA . نقلت الاتحادات الجديدة الى بكتريا *E. coli* MC1022 باستخدام التحويل بطريقة الكالسيوم كلورايد ودرست ثباتية البلازميد المكلون في العائل ، وتم قياس التعبير الجيني من خلال قياس الفعالية النوعية للانزيم bile salt hydrolase للبكتريا *E. coli* MC1022 قبل وبعد التحويل وكانت النتائج هو زيادة في التعبير الجيني من 100 – 383.3(U\ mg) **.**

Introduction

In humans and other mammals, primary bile salts are produced de novo in the liver from cholesterol (1). Following manufacture, conjugated bile salts are stored in the gall bladder and secreted via the bile duct into the small intestine.

Here, these conjugates form spontaneous micelles that trap dietary cholesterol and fats, thus facilitating their absorption by the intestinal epithelium into the blood stream (2). While more than 95% of bile salts enter the enter hepatic circulation in humans , up to 650 mg of bile salts per day elude absorption through the intestinal epithelium.

Thus, high concentrations of these conjugates are present in the gastrointestinal tract. Certain species of the indigenous microflora, including a number of lactobacilli and bifidobacteria, have evolved the ability to deconjugate bile salts. This action is dependent on the presence of an enzyme known as bile salt hydrolase catalyzes the hydrolysis of glycine- and/or taurineconjugated bile salts into the amino acid residue and the bile acid (3).

However, studies on the impact of BSH producing organisms in the colonized host have produced much conflicting evidence. Observations that a reduction in the levels of serum cholesterol is associated with the presence of BSH-producing organisms has led to increased interest in the possibility of their use in hypercholesterolemia individuals or to prevent elevated cholesterol levels in individuals with normal cholesterol status (4).

Conversely, negative effects have also been reported including cases of contaminated small bowel syndrome, impaired lipid absorption, gallstone formation, and increased risk of colon cancer (5).

Lactobacillus acidophilus is a human isolate used commercially for over 25 years as a probiotic. The organism has the ability to survive in the gastrointestinal tract, adhere to human epithelial cells in vitro, utilize fructooligosaccharides, modulate the host immune response, and prevent microbial gastroenteritis (6). Analysis of the genome sequence revealed the presence of two putative bile salt hydrolase genes. The bile-hydrolyzing capability associated with Lb. acidophilus had been previously identified by phenotypic screen. Due to the implications of the presence of bile salt hydrolase in several probiotic strains, this study was designed to further characterize this activity in through targeted gene inactivation (7).

Probiotic *Lb. acidophilus* have bile salt hydrolysis gene responsible on the bile salt pool might be regarded

as a 'biological' alternative to common medical or surgical interventions to treat hypercholesterolemia (8).Thus , this study aims to:

Characterization to *Lb. acidophillus Ar* probiotic strain bile salt hydrolase *bshA* gene which is capable of the hydrolysis of some glycin-glycoconjugated bile salts, due to bile salt tolerance and treat hypercholesterolemia and bile salt hydrolase gene was sequencing analysis to identification . And cloning *Lb. acidophilus Ar* probiotic strain bile salt hydrolase gene *bshA* and overexpression of this gene in *E. coli* MC1022.

Material and Methods

Bacterial characterizations

1. Bacterialstrains , plasmids , and growth conditions:

Lactobacillus acidophilus Ar bacteria was growing in MRS broth medium at 37ºC for 42h ,activated bacteria was streaked on MRS agar to isolated single colony in same condition to prepare PCR single colony . *E. coli* MC1022 cells were propagated aerobically Luria Bertani (LB) media contain 0.1 mg/ ml con. of ampicillin or Erthromycin antibiotic for plasmid detection and for bacterial growth in broth medium, all incubation carried out at 37Cº for 18 hr . by shaking at 250 rpm (9). The bacterial strain and plasmids used in this work (table 1 and table 2).

Table 1. Bacterial strains were using.

Bacterial strains	Properties	Reference
Lb. acidophilus Ar	Probiotic strain	Commercial
E.coli MC1022	Plasmid free strain used for transformation	KSU university

2. Cholesterol tolerance

Lactobacillus acidophilus Ar growthing in MRS media containing cholesterol (4 mg/ml) at 37Cº for 42h.(10)

3. Bile salt tolerance

The *Lb. acidophillus Ar* strain was treated with using 0.5-0.2% (wt/vol) concentration from sodium salt of urodeoxycholic acid (SCA) and incubated at 37ºC aerobically for 42h. To test bacterial growth tolerance, *E. coli* MC1022 cells were incubated with bile salt at 37 ºC for 18hr. Than bacterial growth measured spectrometrically at 600

nm for 2 h intervals during the 8 h incubation period (11). For control MRS and LB media with / without bile salt and bacterial culture used as reference. (12).

4. Antibiotics

Lactobacillus acidophillus Ar bacteria was treated with 125mg/ml final concentration of different antibiotics (such as; gentamycin , streptomycin, erythromycin, tetracycline , vancomycin , rifampicin) by simply adding to 5 ml MRS broth (13).

5. Polymerase chain Reaction PCR

1. Colony PCR

Single colony selected from growing plate of *Lb. acidophilus Ar* to identified strain and prepare for gene encoding bshA into 10µl of sterile Milli Q water .(14)

A microliter of this suspension was added to PCR reaction mixture containing 32 µl of sterile Milli Q water and 7.5 µl of PCR mix contain primer for *Lb. acidophilus Ar* 16S DNA all in a 0.5 ml eppendrof tube. The PCR mix was composed of 4 µl PCR buffer which are; 1 µl of each of forward and reveres primers, 0.5 µl of Ampli taq. (4 µl buffer,0.5 µl taq DNA polymerase, 1 µl dNTP). The amplification was performed on a DNA thermal cycler using following program; initial denaturation 3min at 94 ^{0}C , 1min denaturation at 94 ^{0}C, Annealing 30 sec at 55 ^{0}C, 30 sec at 75 ^{0}C .. Final extension was at 72 ^{0}C for 1 min 4 for 35 cycles .

2.Electrophoresis of DNA Molecules and Cloning Plasmids

For electrophoresis of DNA molecule, 1% (w/v) agarose gel and for cloning fragments electrophoresis which digested with restriction enzymes, 0.7 % (w/v) agarose gel in 1× TBE electrophoresis buffer (0.1 mM Tris/HCl, 0.1 mM boric acid, 0.002 mM EDTA, pH 8.3)(A=60,V=70-120) were used (15). The gel was stained in 0.5 µg ethidium bromide ml−1and after distaining the gel was photographed (16).

3. Molecular cloning Techniques, reagents and Enzymes

Throughout this work standard molecular cloning techniques were used (17). All restriction endonuclaces,T4 DNA ligase and Taq polymerase supplied from sigma .

4. Primers

Primers were designed by using a clone manager (demo 9.2 designer). These primers were stored at -20ºC. Primers utilized in (table 2).

Table 2. Primers used in Research .

Primers	Primer sequence 5´ to 3´
***Lb. acidophilus.* 16SF**	5´ACTACCAGGGTATAATCC3´
***Lb. acidophilus.* 16SR**	5´AGCTGAACCAACAGATTCAC3´
***bshA* F**	5´AAGAGCTCATGTGACATCAATTATATT3´ *SacI*
***bshA* R**	5´TTCTGCAGTTAGTTTTGATGGTTAAATTTAG3´ *PstI*
pJET 1.2 vector F	5´CGACTCACTATAGGGAGAGCGGC 3´
pJET 1.2 vector R	5´AAGAACATCATTTTCCATGGCAG 3´

4. DNA sequencing of *bshA* gene

Sequencing of the gene was performed according to manufacturer protocol. A sequencing reaction was set up as follows; for purification 7µl *bshA* gene PCR product were used in 3 µl exo sap solution. Thermocycle were run at 37ºC for 30 min then at 80 ºC for 15 min. 2 µl of sterile Milli Q water and 2 µl of bshA gene primers (table 2.3) with 6 µl of ABI PRISM Big Dye TM terminator Cycle ready reaction were prepared (supplied by Applied Biosystems). The sequencing reaction was than performed at 96 ºC for 2 min, than 10sec at 96 ºC, Annealing (5 sec at 50ºC), (4min at 60 ^{0}C) for 25 cycles in a Hybrid Omni gene Thermal Reactor. Once the sequencing reaction was completed the product was purified according to specifications provided with the ABI PRISM BigDye ^{TM} Kit.

5. Conformation and Cloning *bshA* gene

a. Restriction enzymes

For digestion of (5 µl) p JET 1.2 vector (fig. 1) and bshA gene PCR product, 9 µl sterile Milli Q water,used 2 µl *SacI* and *PstI* restriction enzyme and 4 µl enzymes buffer were mixed gently than incubated at 37ºC in water bath for 2 hr. (18). pJET 1.2/blunt vector were also digested with same way to use Thermo scientific clone JET PCR cloning Kit (Table 3).

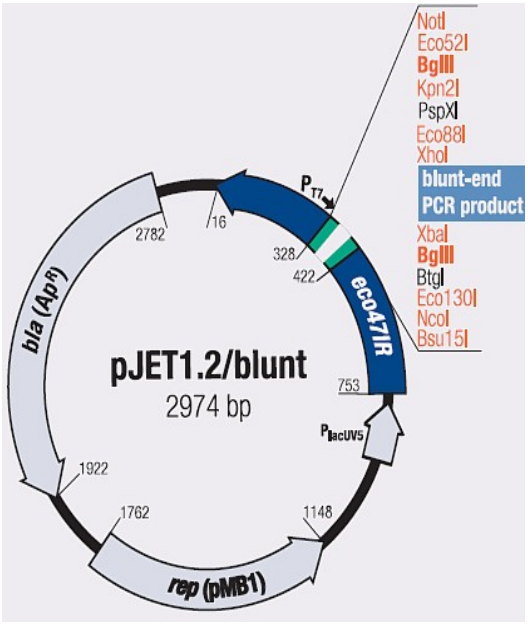
b. Ligation plasmids with *bshA* gene

Ligation of (2 µl) digested plasmids with (3 µl) digested *bshA* gene were performed by using (1 µl) T4 DNA ligase, (2 µl) ligase buffer , (2 µl) sterile Milli Q water. Thermo cycle was used for incubation at 22ºC for 20 min (table 3) . (19)

Table 3. Plasmids and constructs were using

Plasmids	Properties	Reference
pJET 1.2 \ blunt vector	Cloning and expression plasmid ,Replicon (rep),T7promoter,Amp^R	Commercial
pJET/*bshA*	pJET vector carry ≅ 1kb fragment of *Lb. acidophillus Ar bshA* gene	

Figure 1. Physical map of pJET 1.2 \ blunt end vector.



c. $CaCl_2$ transformation

1. Calcium Chloride competent cell protocol for pJET/*bshA* vector transformed in *E. coli* MC 2210

LB broth was inoculated with overnight culture of *E. coli* MC 1022 into 100mL falcon tube and incubated at 37°C to reach appropriate cell density (O.D. 0.350-0.600) or $1*10^{-6}$ cell/ml. cells were colded on ice for 10 mins. cells were collected by centrifugation at 3500 rpm for 10 min. Then supernatant was removed and pellet gently resuspended with 10 mL cold 0.1M $CaCl_2$. Leaved on ice for 5 mins, centrifuged at 3500 rpm for 10 min. Again supernatant discarded and pellets resuspended on 1mL cold 0.1M $CaCl_2$. Then about ~200µL of competent cells, 5µL of circular plasmid or all of a ligation reaction of plasmid DNA added into a microtube (pJET /*bshA* vector) was used for ligation (20) .

left on ice for 30 mins. The mixture was heat shocked at 42 C˚ for 30 sec, replaced on ice for 10 min and than 1ml LB broth added, incubated (water bath) shaken at 37 C˚ for 1-2 hours. Then centrifuged at 5000 rpm for 5 min, then 500 µL LB broth added to competed cell pellets , vortexes, Transformed cells 100 µl were plated out in LB agar plate containing appropriate antibiotic (Amp 5 µg/ml or LB Emr 100µg/ml). (21)

6.Cloning strategies

Cloning *E.coli* MC 1022 with pJET/*bshA* vector was carried bshA gene cultured in serial growth to plasmids extraction for high cloning efficiency and constructional measurement of plasmid. (22).

7. *BshA* enzyme assay

1- The best wild and cloning *E. coli* MC1022 isolate was grown in the nutrient broth medium for bile salt hydrolase production which contained 0.5% Na-urohydroxycholic acid bile salt (substrate), 2.5% glucose (pH 5.8). To examine the effect of carbon sources on the enzyme production. A 250-ml flask containing 50 ml of the medium was inoculated with shaker (120 rpm) for 18 hr at 37 Cº. Control bacteria was prepared from wild type of *E. coli* MC1022 without bile salt. (23)

2- cells culture were concentrated from 50 ml liquid medium by centrifugation at 8,000 x g for 20 min. The cell pellets were resuspended in 1 ml of ice-cold condition containing 0.01M Na-Phosphate buffer pH 6,7. Cells were washed twice and resuspended with 0.01M Na-Phosphate buffer containing 0.1M cysteine and 1M

EDTA to take $O.D_{600}$ for cells in (0.30-0.40)nm.

The cell lysis process used sonication with a Soniprep 150 (USA) in ice-cold condition. The sonicator was set to 16 micron amplitude for 5 minutes (1\2 min turn on and 1\2 min turn off). Cells suspension was centrifuged at 8,000 x g for 30 min , supernatant took for enzymetic activity proteins quantity measurement to calculate enzymtic specific activity in ninhydrin method (fig.2) (28) and proteins concentration measurement in Bradford method (fig 3) (24).

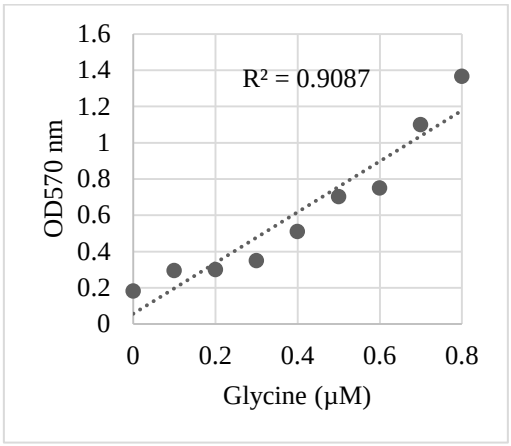


Figure 2. Glycine standard curve (ninhydrin method)

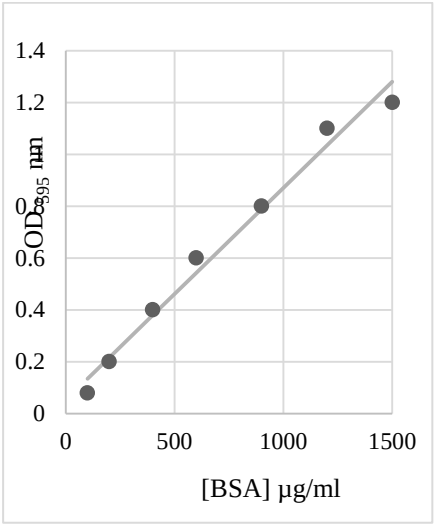


Figure 3. Bovine Serum Albumin standard curve. (ninhydrin method)

Results

1. Bacterial strain characterization

Lb. acidophilus Ar probiotic strain was isolated from capsule treatment for woman candida diseases which was identified morphologically as LAB, its gram positive bacteria and has ability to remove cholesterol. Its tolerance to cholesterol was observed when growth bacteria in MRS media containing 4mg/ml cholesterol at 37 ^{o}C for 42 hr incubation (fig.4). This strain has ability to resistance different antibiotics as well as the showing distinct biochemical characteristics (Table 4).

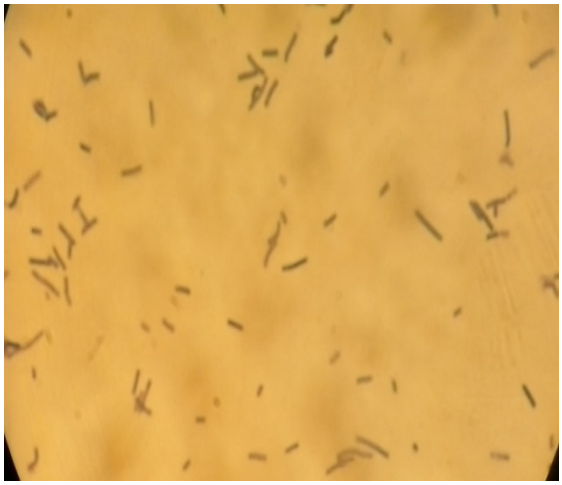


Figure 4. Single and group cells of *Lactobacillus acidophilus Ar* strain.

Table 4. Antibiotics sensitively of *Lb. acidophilus Ar* strain

Antibiotic types	Antibiotics con.	Resistance test
Refambicine	125 mg\ml	R
Erthromycin	125 mg\ml	R
Tetracyclin	125 mg\ml	R
Vancomycin	125 mg\ml	S
Gentamycin	125 mg\ml	S
Setrptomycin	125 mg\ml	S

2. Bile salt tolerance

Tolerance to bile allows lactic acid bacteria to survive in the small intestine. At the beginning of the performed experiments *L. acidophilus Ar* culture O.D $_{600}$ different when the substrates with the addition of 0.5% bile salt was the increase in the number of live cells from (O.D $_{600}$= 1.25 to 1.85) cfu\ml.

3. Molecular characterization of *Lb. acidophilus Ar* probiotic strain

The probiotic strain *Lb. acidophilus Ar* was amplified in PCR reaction using the specific primers of partial DNA of 16S rRNA gene that results of strain from MRS media were detected as *Lb. acidophilus Ar* (Fig. 5 a) .

4. Screening *BshA* Gene *Lb. Acidophilus Ar* Probiotic Strain And Sequencing Analysis

The *bshA* gene of *Lb. acidophilus Ar* was amplified using primers that mentioned (Fig 5 b), derived from the nucleotide sequencing of *Lb. acidophillus Ar bshA* gene. The length fragment was 801 bp used Master cycle PCR, big dye Terminator Cycle Sequencing standard Kit (Fig. 6).

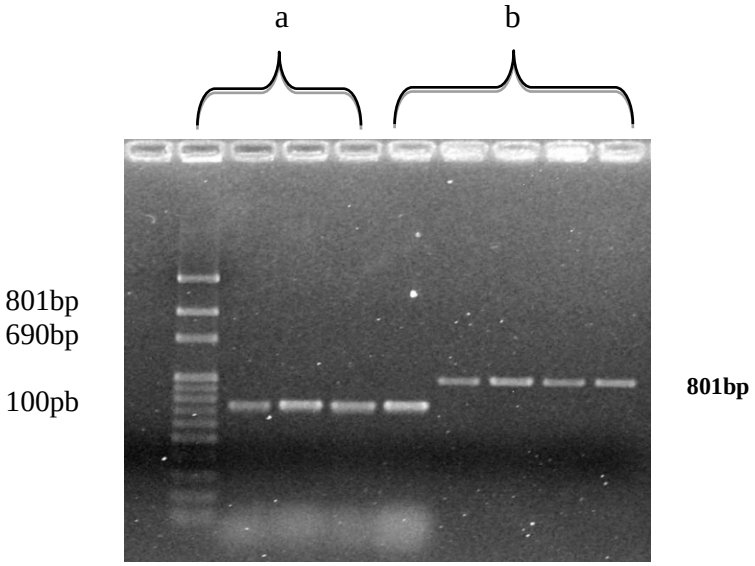


Figure 5. a) Lanes of PCR products of amplified 16S rRNA gene to identified *Lb. acidophilus Ar* strain using specific primers. b) Lanes PCR products of amplified *bshA* gene of *Lb. acidophilus Ar* strain using specific primers lanes: 100 bp DNA marker.

P*st*I revers primer

CYKATTKRTWRGGARWYCTTCTAGAAGATCTGCAGTTAGTTTTGATGGTTAAA
TTTAGTTTTATCAAGCATATTATAAGWGATCAAATTGCTGCTATCTAGATCTTC
TTTATTCATATCAACAACGTTAATTTGTTTGTTTGAATAAGTGGTGTAGTAGAA
AATACCTTTGTCTAAGTTAGTTCCATCAGAATAAATTGTATATTCAAATGAGTT
TGGACCAACTTCATCCAGTCCCTTTTGTTGTTCAACCGAATGTAAAATGTGGAA
GTAAGTATCAATATTTTCTTCTTCGGTTTCAGCAATTGGAGCATTAAATTTATT
GAAAGCTACTCTGACAAAACGTGATTCAGAATCCATTCCACCTGGTAAGTTGT
GAGACCCTAATCCACGGCTGTAGCCAGCCATATTTACTTTATCTGAGAAGTTAT
TTTTAGGCATTTTTGGAGATACGTCAGCATAGTTATTTAAATTGAATAATTGCT
TTGGAAATTGTGGATTATTAGTTAAGCAGCCAACTGGATTATCATAAATATGC
ATTCCATCTTTGTCTGTTTCAACAACTAATGATGTACCTGTTTTATCTGCAATA
AGCCAGTGAAGAGAGGAGGTTTGCATTTTTTCGCTGAAATTTAAATCGGCGAT
GTTGATTCTGCTAAGTAAATCCTTTACTTCGCTAATAGTGCTACACTGTCCTAA
AATCCAAGGGATGAATTCAAAGGAAGCAATATTATCTTTATTTTCTTTTTCTTC
GTAATATGTAGCATTTCCTGGATAGTTGAGTCCGGCCATACCTAAACCT

***Sac*I**

Figure 6 .*bshA* gene sequence data is 801 bp only revers strand

5. Analysis of Sequence Data

Sequencing analysis of the clone obtained from PCR reaction showed high similarity (44%) from *L. acidophilus Ar* probiotic strain (fig.6) When sequencing results were obtained in a Genetic analyzer system and they were analyzed using BLAST programmer in NCBI nucleotide database confirmed that the strain belong to *Lb. acidophillus* species .

6. The primers

Sequencing analysis results for the primers *bshA* F ,*bshA* R (table 2) ,that *bshA* R can homology matching with revers strand of *L. acidophilus Ar bshA* gene , but *bshA* F do not contain homology matching only 5 bp from GC can homology matching occurred with forward strand of *bshA* gene appear in clone managing designer because of *L. acidophilus Ar* bshA gene was different from *L. acidophilus* strain which obtained for primer designing in BLAST program (demo 9.2) (Fig6).

7. *E.coli* MC1022 charecterazion

E. coli MC1022 is a Gram-negative artificial, plasmid free bacteria and can be able to express foreign gene. This bacteria can grow in LB-broth at 37 ºC and sensitive to erythromycin and ampicillin antibiotics.

8. Plasmid conformation and gene fusion analysis

i. Constriction of *bshA* gene reporter gene fusion

To clone bshA gene from chromosomal *DNA of Lb. acidophilus*, primers *bshA* F and *bshA* R (table 2) were designed, the primers were engineered to contain a *PstI\SacI* restriction sites for both *bshA* F and *bshA* R respectively. After PCR amplification, resulted fragment was inserted into pJET1.2\blunt vector and this new recombinants named as pJET/*bshA* respectively. Obtained recombinants then transformed into *E.coli* MC1022 by chmical method. After transformantion 4-5 cell/100µl single colonies obtained (20), and each plate containig transforment colonies were screened by PCR amplification (Fig.7) using *bshA* F and *bshA* R primers and restriction digest analysis was performed to ensure that the *bshA* fregment present. Purified gene fragment was then sequenced to check no erroreous base pair changes occurred during the PCR amplification process.

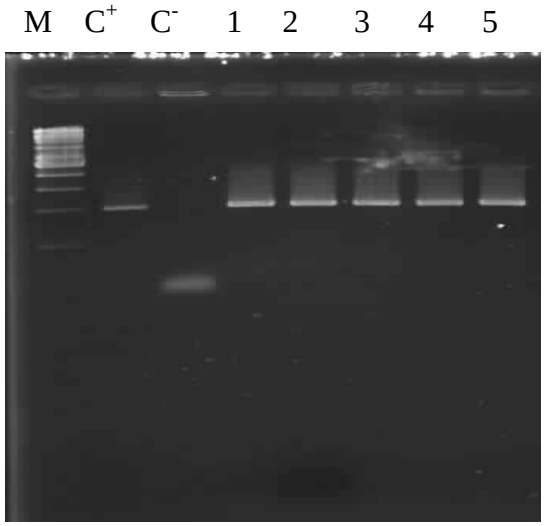


Figure 7. C+) *bshA* gene control all lane (1-5) for cloning single colony carry *bshA* gene after CaCl transformation of pJET/*bshA* M) used 1000bp ladder.

ii. Conformation of cloning plasmids with *bshA* gene

Recombinant *E.coli* MC1022 which carriy recombinant pJET/*bshA* vector was growth in the appropriate conditions, after that recombinant plasmid were isolated and pJET/*bshA* was digesed with *PstI\SacI* to to confirme presence of bshA gene by gel electrophoresis separation (Fig.8,9) . It has been confirmed that after gel electrophoresis separation two plasmid fragments and bshA gene with control appeared. (17)

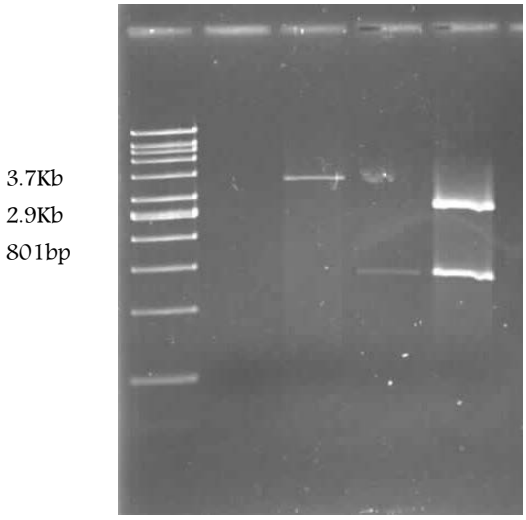


Figure 8) Lane 1, pJET/*bshA* vector 3.7Kb, Lane C+, PCR reaction of *bshA* gene 801bp (control) Lane 2) pJET/*bshA* vector digested with *PstI/SacI* restriction enzyme to get tow fragments (2.9 Kb+ 801bp),M) 1000bp ladder.

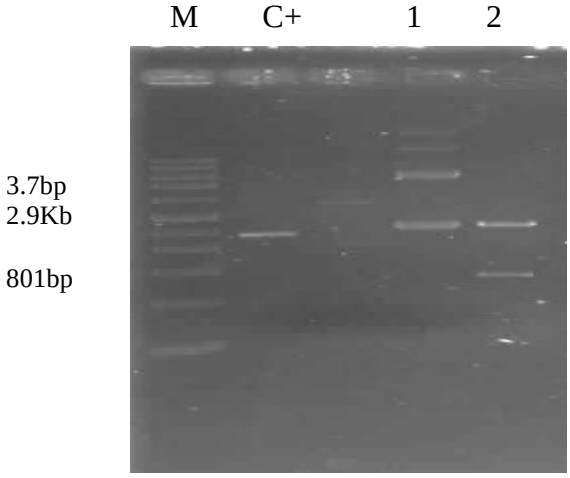


Figure 9 M) 1000 bp ladder lane C+) pJET1.2 vector 2.9Kb (control) , lane 1) pJET/*bshA* vector 3.7 bp and pJET1.2 vector 2.9Kb (control) lane 2, pJET/*bshA* vector digestived use *PstI* *SacI* restriction enzymes to get tow fragments (2.9 Kb+ *bshA* gene 801bp).

9. Analysis of pJET/*bshA* vector stability in *E. coli* MC1022

A bacterial cloning system for mapping and analysis of complex genomes has been developed. It is capable of maintaining bacterial *bshA* fragment of greater than 300 kilobase pairs. Individual clones of this gene appear to be maintained with a high degree of structural stability in the host, even after many generations of serial growth arrived (30 – 40) times of plasmids extractional concentration . Because of high cloning efficiency, easy manipulation of the cloned DNA, and stable maintenance of inserted DNA, pJET/*bshA* system may facilitate construction of DNA libraries of complex genomes with fuller representation and subsequent rapid analysis of complex genomic structure (Fig. 8,9) . (22)

10. Bile salt hydrolase enzyme assay and over expression

For assaying most bile salt hydrolase activity was measured by using ninhydrin detect with glycine curve test and proteins concentration by Bradford method (coomasia blue detect), to preserve the enzymatic activity, enzymes must retain their native form extraction capability.

The bile salt (Na-urohydroxycholic acid) concentration and fermentation substrate was used. The substrate specificity of BSH from revealed several interesting results. For instance, the former authors have demonstrated that the position of the amide bond, changes in shape and chiral nature of the amide bond, and introduction of various amino acids at or around the amide bond influence the rate of hydrolysis. indicated that glycine and taurine conjugates of cholic and deoxycholic acids were hydrolyzed; however, readily as compared to glycine conjugated with ninhydrine detect and glycine standard curve (fig. 2, table 4) for bshA enzyme activity assay.

Table 4. Absorbance of enzyme extract samples at A_{595} (ninhydrin method)

Enzyme Samples	**Absorbance $O.D_{570}$**
E.coli MC1022 growth without bile salt	0
E.coli MC1022 growth with 0.5% bile salt	0.096
E.coli pJET/*bshA* growth with 0.5% bile salt	0.345

These studies are in agreement with previous studies **(**25**).** It is noteworthy that unlike previous studies where partially purified enzyme *E. coli* MC1022 pJET/*bshA* from culture were used as the source for BSH activity, can be used for more detailed studies on its substrate specificity using various strategies employed by (26**) .**Upon bile salt hydrolysis, glycine or taurine is liberated from the steroid moiety of the molecule, resulting in the formation of free (deconjugated) bile salts. Free bile salts are more easily precipitated at low pH. They are less efficiently reabsorbed than their conjugated counterparts. . Since the steady state requires that the amount of bile salts extract was measured in bradford method used BSA standard curve (Fig.3, table 5) to calculate bile salt hydrolase specific activity.(27)

Table 5 . Absorbance of Protein Purification Samples (ninhydrin method)

Proteins Sample	Average A_{595}
E.coli MC1022 growthing without bile salt	0.09
E.coli MC1022 growthing with 0.5% bile salt	0.32
E.coli pJETAr growthing with 0.5% bile salt	0.30

The benefits s of bile salt hydrolase specific activity for comparative between recombinant *E .coli* MC1022 with wild type bile salt overexpression and bile salt effect on *bshA* gene function when bacteria was cultured with and without bile salts (table 6) (Fig.10).(25)

The result after took spectrophotometer absorbance (O.D 570,595 nm) and enforcement enzyme specific activity formula (28) ,these data indicate that the *bshA* gene overproducing in *E. coli* MC1022 pJET/*bshA* strain while same bacteria growth in broth media from non-bile salt substrate no produced enzyme for *bshA* gene function for because bile salt led to simulate gene and as enzyme substrate same time (Table 6).

Table 6. The results of overexpression data compression between cloning *E. coli* MC1022 and wild type.

Samples	Specific Activity (U\mg)
E.coli MC1022 growth without bile salt	0
E.coli MC1022 growth in 0.5% bile salt	100
E.coli pJET/*bshA* growth in 0.5% bile salt	383.3

These results appeared that pJET/*bshA* vector construct active . Because its specific for *E. coli* strains and there is evidence that *E. coli* MC1022 recognizes it as original host organism. This has been shown for a *bshA* gene from *Lb. acidophilus Ar*, but may also apply to genes from other species. The expression occurs in *E. coli* ribosome binding sites are generally observed in the expected position for genes, but internal translational start points and proteolysis can complicate interpretation of cloned gene products were expressed in *E. coli* MC1022.(fig. 10) However, the importance of confirming promoter identity empirically has been demonstrated by studies in which transcriptional sites operating within *E. coli* MC1022 differed significantly from those that were active within the native bacterium (29).

The importance of probiotic bacteria in the assimilation cholesterol processes of human fecal bacteria make them logical target for genetic manipulation to improve cholesterol balance efficiency. Thus, introducing bile salt hydrolysis functions into non- bile salt hydrolase bacteria such as *leuccuccuse mesenteroides* or over expression occurrence for bacteria use as hypercholesterolemia treatment (30).

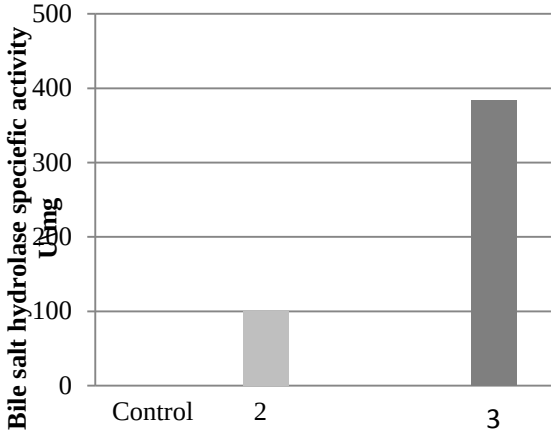


Figure 10. Bile salt hydrolase spesific activity and Overexoression values (U\mg). 1: (Control) *E. coli* MC1022 wild type growthing without 0.5% bile salt. 2: *E. coli* MC1022 wild type growthing in 0.5 bile salt. 3: Recombinant *E. coli MC1022* with pJET/*bshA*.

11. Analysis of bile salt enzyme amino acid Sequence Data

Amino acids Sequence analysis of *bshA* enzyme encoding gene from *L. acidophilus Ar* probiotic strain was done. Sequencing results were obtained by using BLAST programmer in NCBI nucleotide database. Each of the sequence results showed after at least 50 amino acids found stop codon. The amino acids sequence results of gene encoding bshA enzyme of *Lb. acidophilus Ar*, confirmed that the strains belong to *Lb. acidophilus* species (fig.11).

Figure 11. Amino acids sequencing Bile salt hydrolase isolated in the present work.

Discussion

1 .Identification of *Lactobacillus acidophilus Ar bshA* gene and cloning in *E. coli* MC1022

The bacterial cholesterol assimilation in an important character for many organism because of its chemical and healthy benefits .The hypercholesterolemia problem is solve for human healthy and other organism energy sours by the removal cholesterol in bacterial environment by microflora, It is well Known that probiotic lactic acid bacteria like *Lb. acidophilus* is

one of the major cholesterol removal organism that have (bile salt hydrolase genes) *bsh* genes responsible for this process .

The anti- candida woman disease probiotic strain *Lb. acidophilus Ar* was examined to bile salt resistance , Colonies were identified according to their morphological, cultural, physiological and biochemical characteristics , the result appeared its consist of bile salt genes resistance .

The experiments described here demonstrated that simplified in vitro mimicking of complex environmental niches can result in the identification of genes that are relevant in situ in these niches. Moreover, this approach potentially provides clues to the environmental trigger involved in the in situ regulation of specific genes to play an important role to resistance at low pH when the presence of bile salts, which should enable future unraveling of the genetic behavior of during passage through (specific parts of) the GI tract in vivo. The observed gradual decrease in the growth rate coincided with the gradually increasing severity of changes in morphology of *Lb. acidophilus Ar* .(31).

Moreover, the observed formation in vitro the increasing growth *Lb. acidophilus Ar* after addition of bile, The optical density at 600 nm change from 1.25 to 1.85 cfu /ml possibly because leakage of intracellular material from the cells and a disturbed energy balance(32)

Identification of *Lb. acidophilus Ar* is more accurate if the whole gene is sequenced . In the early 1990s, many microbiologists have demonstrated that phylogenetic relationships among living organisms can be traced by comparing sequences of their genes and gene regions, encoding ribosomal RNAs (33 , 34). Ribosomal genes are more conservative than most of the genomic genes. The gene sequences of small (16S) rRNA subunit is particularly widely used in taxonomic studies of bacteria. The method of comparing 16S rRNA gene sequences, along with DNA typing with the use of various PCR-based techniques, is also often used for species identification in microflora of probiotic lactic acid bacteria .For instance, to identify microorganisms.

The *bshA* gene was selected from *Lb. acidophilus Ar* strain genes used PCR purification and comparing gene sequencing analysis from the corresponding sequences of strains from the NCBI database that studied it , the sequence results showed little different homology.

1. Expression of *bshA* in *E. coli MC1022*

To create the recombinant plasmids with pJET1.2\ blunt end vector specific for *E. coli* to clone *bshA* gene, that *bshA* gene was amplified with the primers *bshA*-F and *bshA*-R (Table 2). And *SacI* site was designed in primer *bshA*-F and a *PstI* site was created in primer *bshA*-R to include the start codon sequence and the stop codon (TGA) sequence.

Cloning into the *PstI* -*SacI* sites of vectors resulted in the translational fusion of the *bshA* gene to the T7 promoter of pJET/*bshA* vector , a ribosome binding site and the start of an open reading frame are present and *E. coli* MC1022 ribosome binding site.(35)

The previously constructed *bshA* complementation library in *E. coli* MC1022, was exploited for identification of clones containing *Lactobacillus acidophilus Ar* chromosomal fragment that harbor promoter elements conditionally activated by bile salt, Which play a role in the pJET/*bshA* vector when created in *E. coli* MC1022 and higher stability after serial sub-culturing, (25).

2. BshA Enzyme activity

The nucleotide sequence of the bshA gene of *L. acidophilus Ar* was analyzed, which revealed its location and showed that it was surrounded by 801 nucleotides in a single open reading frame (ORF) and encoding a 50 amino acid in protein. BSH promoter was located upstream of the start codon. The expressed protein exhibited high homology with BSHs from other source organisms , located around the active site, were highly conserved.

The *bshA* gene was cloned in pJET1.2\ blunt end expression vector . The produced recombinant *bshA* enzyme exhibited hydrolase activity against sodium salt of uroconjugated bile salts .The *bshA* gene, has been identified encoding bsh enzyme in the genome sequence of *L. acidophilus Ar*, where substrate specificities of enzyme was observed. Indeed, the bsh enzyme has substrate specificity, depending on the gastrointestinal ecosystem. glycine or taurine amino acid moiety (26).

This principal due to enforce glycine standard curve and Bradford method in calculate differentiation overexpression levels for cloning *E. coli* MC1022 and wild type by know the bile salt hydrolase specific activity.

The results were confirmed that production of bile salt hydrolase from recombinant *E. coli* MC1022

pJET/*bshA* found to be higher compared with *E. coli* MC1022 wild type e. It could be thought that pJET vector based constructs specific for *E. coli* strains and there is evidence that *E. coli* MC1022 recognizes promoters different from those used in the original host organism.(30)

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Direct Detection of 5′-Non Coding Region of All Enterovirus Serotypes Genomes in Stool Samples

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Abstract: Cell culture of RD (Rhabdomyosaroma) and L20B (L-cell) are a gold test advised by the World Health Organization for enterovirus (EV) detection. Otherwise, some EV serotypes no or slowly growing in common cell lines used for diagnosis, lack of cell sensitivity to low viral load in clinical samples, cost and long time required for final identification. Hence, this study aimed to investigate conserved regions of all enterovirus serotypes genomes in the stool among acute flaccid paralysis (AFP) cases. A total 68 samples were selected for RNA extraction directly from stool which were positive, weak positive, and negative results in cell culture among 130 stool samples, from children aged 5 months-7 years. Reverse transcriptase-PCR amplification relied on 5′non-coding region (5′-NCR) of EV genome with 144-149 bp nucleotide visualized on 1% agarose gel. Results showed RT-PCR more accurate for positive cases (100%) with a short time diagnosis than cell culture, in addition to 3.85% detection of viral 5′-NCR in negative sample. Furthermore, 5′-NCR RT-PCR is the best for overcoming cell culture problems and medical practice reduction that carry out AFP containment.

Key words: Enterovirus, Acute flaccid paralysis, Reverse transcriptase-PCR, Cell culture of RD and L20B.

الكشف المباشر للمنطقة غير المشفرة بالطرف 5′ في جينوم كافة الأنماط المصلية للفايروس المعوي في عينات البراز

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الخلاصة: تمثل الزراعه الخلوية بأستخدام خلايا RD وL20B الأختبار الذهبي للكشف عن الفيروسات المعوية والتي تنصح بها منظمة الصحة العالمية. في خلاف ذلك، توجد بعض الأنماط المصلية للفيروسات المعوية لا تنمو أوتنمو ببطء في الخلايا المستخدمة بالتشخيص، عدم حساسية الخلايا للمحتوى الفيروسي القليل في العينة السريرية و الكلفة وخسارة الوقت اللازم للوصول الى التشخيص النهائي. لذا هدفت هذه الدراسة الى التحري عن مناطق ذات تتابع نيوكلوتيدي ثابت في جينوم كافة الأنماط المصلية للفيروسات المعوية في البرازلحالات الشلل الرخو الحاد (AFP). تم اختيار 68 عينة لاستخلاص الحمض النووي الريبي (RNA) بشكل مباشرة من البراز حيث تميزت بنتائج موجبة، ضعيفة الأيجابية ، وسالبة عند أختبارها بالزراعة الخلوية من بين 130 عينة خروج جمعت من الاطفال بعمر تراوح من 5 اشهر الى 7 سنوات. أعتمدت عملية تضخيم أنزيم عكس الاستنساخ-لتفاعل سلسلة البلمرة (RT-PCR) على منطقة غير مشفرة بالطرف 5' (5'-NCR) لجينوم الفايروسات المعوية بحجم 144-149 نيوكليوتيدة شخصت على الهلام بتركيز1٪. أظهرت نتائج أختبار RT-PCR بأنه الأكثر دقة لتشخيص الحالات الموجبة للفيروس (100%) وبوقت قصير مقارنة مع زراعة الخلايا، بالإضافة إلى الكشف عن 3.85٪ من المنطقة الفايروسية غير المشفرة بالطرف 5' في العينات السالبة .وعلاوة على ذلك، يفضل تطبيق أختبار RT-PCR NCR-5' للتغلب على مشاكل زراعة الخلايا والحد من الاجراءات الطبية التي تتخذ لاحتواء الأصابات بالشلل الرخو الحاد.

Introduction

Enterovirus (EV) including the polioviruses and non-polioenterovirus (NPEV) as like coxsackieviruses, echoviruses and other new EVs, which are small, non-enveloped viruses that have a spherical shape of about 30nm in diameter with a single-stranded, positive-sense RNA genome comprises a 5' non-coding region (5'-NCR), a long open reading frame that encodes a protein of approximately 2,100 amino acid residues, a short 3' NCR, and a polyadenylated tail (1, 2). Enteroviruses affect millions of people worldwide each year, and are often found in the respiratory secretions (e.g., saliva, sputum, or nasal mucus) and stool of an infected person (3).

Poliovirus and NPEV are the major viral cause of neurologic disease with a known etiology in

humans, including meningitis, encephalitis and acute flaccid paralysis (AFP) (4). The AFP is a rapid and sudden onset of weakness of muscles in different parts of the body, including foot, hand and rarely muscles of respiration tracts and swallowing system, that begins anytime between after birth and under 15 years of age and does not progress further after its increase through 4-5 days of symptoms (5).

Virus isolation in cell culture is still being the most common laboratory method for EV detection prior to neutralization or molecular typing of EV. But, other EV serotypes no growing or slowly in common cell lines used for diagnosis, lack of cell sensitivity to low viral load in clinical sample, cost and long time required for final identification (6). On the other hand, the amplification of the conserved 5'NCR allows fast, specific and sensitive detection of EV directly from the sample (7, 8). Hence, this study aimed to investigate conserved regions of all enterovirus serotypes genome in the stool among acute flaccid paralysis cases.

Materials and Methods

Sample collection and preparation

A total of 130 stool samples were tested in tissue culture technique to diagnosis of enterovirus. They were collected from children aged less than 15 years (5 months-7 years old) and accompanied by an AFP notification during January-May 2015. All stool samples were processed with chloroform before inoculation into RD cells and L20B cell lines from National Polio Laboratory (NPL/Iraq) stock held in liquid nitrogen at low passage (passage up to 10). Briefly, 10 ml PBS buffer was added to 1g glass beads and 1 ml chloroform, then transferred to 2g of fecal sample and shaked vigorously for 20 min. Centrifugation at 1500g in cooled centrifuge for 20 min to transfer supernatant from each sample into new tube and stored at 4C^{o} to be inoculated in the same day or kept at -20 C^{o} to 3 months (9).

Virus detection

Each stool suspension was injected in healthy monolayer of RD cells were grown in minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) and then it providing with maintenance medium supplemented with 2% fetal

bovine serum. The inoculated tubes were placed to incubator at 36°C for 7 days and examined daily for the specific enterovirus cytopathic effects (CPE) of rounded, refractile cells detaching from the surface of tubes. The cells with CPE up to 75% were harvested and stored at -20 C°, whereas those negative results were repassaged on to another RD cells. Positive isolates on RD cells were passaged on L20B cells to identify polioviruses (10, 11). Samples that showed CPE on L20B were confirmed with international laboratory for WHO to differentiate between wild and vaccine polioviruses, while the sample grown only RD cell detected as NPEV.

RNA extraction

RNA genome was extracted directly from 68 original stools tested in tissue culture included positive (37), weak positive (3) and negative (28) samples to detect the EVs gene comparison with cell culture efficiency. The QIAamp Viral RNA Mini kit used with some modification to appropriate stool sample including: 3 ml of 0.9% Nacl saline was added to 1g fecal sample and shaked vigorously for 20 min. Centrifugation at 1500g in cooled centrifuge for 20 min. The supernatant was filtered with 0.22 µm filter paper to concentrate the viral load in the sample. RNA of the filtrate was extracted according to kit instructions with the RNA elution in 40µl (12).

Gene amplification

One step RT-PCR was performed for EV detection based on 5'NTR amplification. A forward primer (5′-CCCTGAATGCGGCTAATCC-′3, positions 456 to 474), and reverse primer (5′-ATTGTCACCATAAGCAGCCA-′3, positions 582 to 601) were used (13). After optimization of the RT-PCR condition, all samples assayed in a 25 µl reaction mixture containing 8µl of template RNA, 1 µl of enzyme mix and 5 µl of buffer 5x (QIAGEN one step RT-PCR), 0.6 µM conc. of each forward and reverse primers for EV, 1 µl of $Mgcl_2$, 400 µM conc. of each dNTP (1 µl) and DEPC(Diethylpyrocarbonate). The RT-PCR amplification protocol was done as follows: 30 min at 50 C° for reverse transcription reaction, followed by 15 min at 95C° for initial Taq NA polymerase activation, and 35 cycles of 30 s at 95C°, 1 min at 48C° and 1 min at 70 C° then final extension 5 min at 70

C°. The amplicon visualized with ethidium bromide staining on 1% agarose to detect the specific product size 144-149 base pair.

Statistical analysis

All data were tabulated and analyzed using the SPSS IBM version 20. The Chi-Square test was done to find the significant role between cell line and RT-PCR assays for enterovirus diagnosis. Values were considered statistically significant $P \leq 0.05$.

Results and Discussion

According to the cell line technique, 30.77% (40/130) of AFP were positive for enterovirus detection that compromised of 92.5% (37/40) at cytopathic effect (CPE) during the first passage with +3 to +4 CPE and 7.5% (3/40) had difficulty with two passages (10 days) for final identification as a weak positive in +1 CPE shown in figure 1. This explanation that some EVs serotypes slow growing or/ and the sample had few viral loads, and cell line has low sensitivity to all the serotypes. Hence, recombination more than two cell lines make a good chance for most EVs detection (14-16).

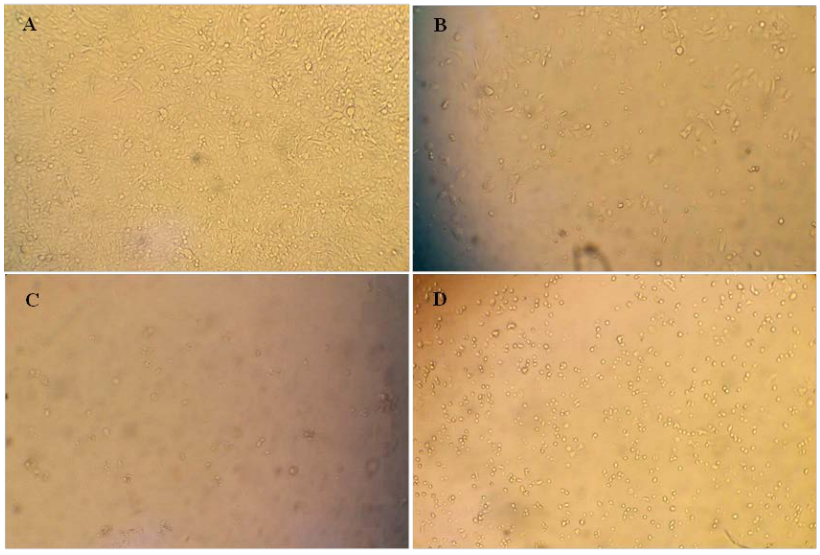

Figure 1: Cell line growth of RD cells, filed A represent of control and non-infected cell with enterovirus, while B and C observe the CPE due to infected cell with EVs in +3 and +4, respectively. The result of weak positive appears in field D with +1 CPE after 10 days of incubation.

Regarding other studies that reported variable of enterovirus detection among AFP between 14-50% (17-19), but the current results agreed with previous local study (20).

On the other hand, cell line assay have some troubles with EVs diagnosis include: no one cell line support all serotypes, time consuming, the presence of fecal inhibitors, cost and long incubation exposure the cell line to contamination (21, 22).

Furthermore, the molecular method performed as a rapid and sensitive for EVs detection in stool sample in this study. Results of 5'NCR amplification showed that high ability for positive cases (100%) diagnosis in a stool similar of cell line technique with 144-149 bp of RT-PCR product (Fig. 2). Moreover, it detected the weak positive cases quickly like other positive sample, and 3.85% (1/28) detection of viral 5′-NCR in negative sample. However, RT-PCR of 5'NCR amplification pointed out that 60.29% (41/68) EV positive (Fig. 3). Although the RT-PCR method increased the percent of enterovirus diagnosis, there is no statistical significant differences (P>0.05). Some investigators using two these methods in EV detection and they displayed the results of RT-PCR more accurately (23, 24). Abbasian et al, (2011) observed in 24% of stool samples using 5'NCR RT-PCR while cell lines isolated in just 14.4% of the samples (25).

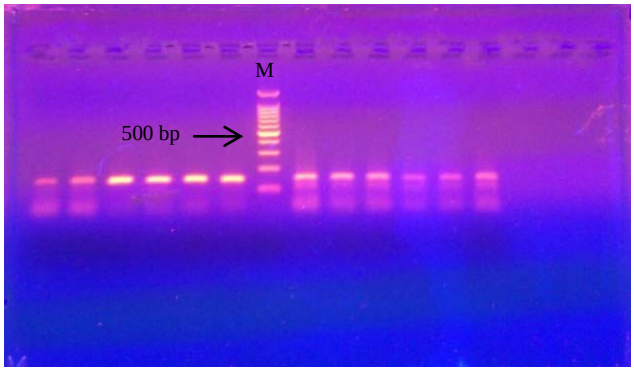

Figure 2: Amplification of partial enterovirus genome at 5'NCR with 144-149 bp. Agarose gel 1%, 5 V/cm for 45 min and visualized under U.V after staining with ethidium bromide. M: Marker ranged 1000-100 bp.

Amplification of the conserved 5'NCR makes the reaction for EV diagnosis directly from the sample fast, specific and sensitive. Nevertheless, 5'NCR PCR is not appropriate for molecular typing because of low sequence diversity in this region and frequent recombination events resulting in EVs with divergent sequences between their 5'NCR and the regions coding for neutralizing epitopes. Therefore, samples positive in 5'NCR PCRs need to be examined by less sensitive methods in order to specify the subtype: either by VP1 RT-PCR and sequencing, or by virus isolation and then subtyping by neutralization with specific antisera or VP1 sequencing (26, 27).

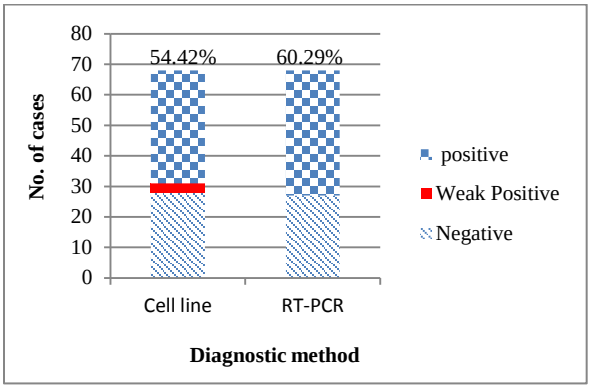

Figure 3: Observation of enterovirus cases among AFP identification in both cell line and RT-PCR assays.

Using primer of this study gave away revealed that the incidence in the all serotypes genomes of positive samples which noticed previous studies (13, 28, 29).

Since RNA extraction direct from stool proved high efficiency to obtain genome enough for 5'NCR amplification of all serotypes, this study concludes 5′-NCR RT-PCR is the best for overcoming cell culture problems and medical practice reduction that carries out AFP containment.

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Detection the Co-Infection of Human Respiratory Syncytial Virus and Influenza virus in Iraqi patients

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Abstract: This study was aimed to investigate the co-infection of Respiratory Syncytial Virus (hRSV) with Influenza viral infection A and B. Two hundred clinical samples of nasopharyngeal and throat swabs were collected between first of January until the end of March of 2014 from Iraqi patients of different ages and from both males and females hospitalized with influenza like illness. The samples were subjected to RT-qPCR for Flu virus infections A and B. 22 positive samples for flu A and 22 positive samples for flu B were subjected to RT-PCR test for hRSV infection detection. The results showed that 7 out of 22 (31%) positive samples for flu B were positive for hRSV infection while 2 out of 22 (9%) positive samples for flu A were positive for hRSV viral infection. The results reveal that there is co-infection of respiratory viruses, flu A or flu B with hRSV in hospitalized patient suffering from respiratory distress.

Keywards: hRSV, FluA, Flu B, Co-infection.

الكشف عن العدوى الفيروسية الناتجة عن الاصابة بفيروسي المخلوي التنفسي البشري وفيروس الانفلونزا لمرضى عراقيين

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الخلاصة: هدفت هذه الدراسة إلى الكشف عن الإصابات التقسية الناتجة عن الإصابة لفيروس المخلوي التنفسي البشري (hRSV) مع فيروس الأنفلونزا نوع A وB. تم جمع مائتي عينة سريريه من مسحات البلعوم والحنجرة من بداية شهر كانون الثاني وحتى نهاية شهر آذار من عام 2014 من مرضى عراقيين بمختلف الأعمار و من الجنسين ذكور وإناث يعانون من أعراض مرضية مشابهة للأنفلونزا من مختلف الأعمار. خضعت العينات لفحص RT-qPCR للتحري عن الإصابة بفيروس الأنفلونزا A و B. تم فحص 22عينة موجبة لأنفلونزا A و22 عينة موجبة لأنفلونزا B إلى اختبار RT-PCR للكشف عن الإصابة بفيروس المخلوي التنفسي البشري. وأظهرت النتائج أن 7 من أصل 22 (31%) عينة إيجابية لأنفلونزا B كانت إيجابية للإصابة hRSV بينما كانت 2 من أصل 22 (9%) عينة موجبة لأنفلونزا A كانت موجبة للإصابة بفيروس المخلوي التنفسي البشري. بينت الدراسة أن هناك إصابات بفيروس المخلوي التنفسي البشري في مرضى راقدين في المستشفى والذين يعانون من الإصابة بفيروس الأنفلونزا.

الكلمات المفتاحية: فيروس المخلوي التنفسي البشري، أنفلونزا A، أنفلونزا B

Introduction

Respiratory tract disease is result in morbidity and mortality throughout the world accounting in approximately 4 million deaths worldwide (1). Most infections of the lower respiratory tract in infants are resulted from viruses. Respiratory syncytial virus (RSV) infection is the major cause of hospitalization among infants. Other viruses are also important etiological factors of respiratory infections like, human metapneumovirus (hMPV); adenovirus (ADV); parainfluenza (PIV) 1, 2 and 3; influenza (Flu) A and B; rhinovirus; bocavirus; and coronavirus (2).

Viral coinfections had greater attention after the introduction of the molecular biology techniques, such as polymerase chain reaction (PCR) that can detect not only a larger number of viruses, but also more than one virus using the same respiratory secretion specimen. These techniques have been used to estimate the variable prevalence of coinfections by respiratory viruses (3).

The presence of more than one viral agent generates uncertainties about the prognosis of the infections. Some authors suggested that, in infants with bronchiolitis coinfection may increase the severity of the disease (4).

Influenza viruses are members of orthomyxoviridae family which include influenza virus types A, B, and C. Influenza virus types A, B have negative sense RNA genome composed of eight separate segments. Influenza A virus is zoonotic pathogen which capable of infecting birds, swine, horse, felines and other species (5). In human influenza A, is mostly responsible for seasonal epidemics and global pandemic (6).

Respiratory syncytial virus (RSV) is a member of the paramyxoviridae family (7). It infects the epithelium airway and cause acute respiratory infection especially in infants, young children, asthmatics, and adults (8). RSV is one of the most common etiologies in bronchiolitis (9). The acute infection of RSV-induced bronchiolitis results in hospitalization of 1–2% of infants and children (10). RSV can be divided into two major antigenic groups, A and B (11). There are very few reports on the coinfection of RSV and influenza virus among the patients (12).

The majority of practitioners approach respiratory viral infections is assuming a single-agent etiology. However, other groups and others have realized that a many patients with respiratory tract disease have more than one viral pathogen (13). The frequency of respiratory virus co-infections varies widely but is often

reported between 10–20% (14). This is understandable as many respiratory viruses circulate at similar times often with wintertime predominance in temperate climates. Brunstein *et al*. provided statistical evidence that co-infection with certain pathogens occur more frequently than expected if co-infection was random (15). Influenza has been identified with co-infecting viruses (16).

There is increasing importance of polymicrobial infections (17). The clinical relevance of respiratory virus copathogens in association with the disease is unclear. Several studies have reported viral co-infections are associated with increased morbidity. These studies reveal greater hospitalization rates and admission to intensive care associated with co-infection (18). However, other reports describe that co-infection having no increase in patient morbidity (19).

This study was done to estimate RSV coinfection with influenza virus in hospitalized patients suffering from acute lower respiratory tract disease. Molecular biology techniques were used.

Material and Methods

Sample collection

A total of 200 samples of nasopharyngeal secretions and throat swabs were screened for the presence of Influenza virus. The samples were collected from different hospitals and they were sent to Central Public Health Laboratory (CPHL)/National Influenza center (NIC) /Baghdad/Iraq between January and April of 2014 from patients of different ages hospitalized with respiratory distress symptoms. Ten samples were collected from healthy individuals with negative results for Influenza and hRSV viruses.

The samples were collected in 5 ml of transport media (phosphate-buffered saline containing 10% glycerol, 1 mg/ml gentamicin together with 8 IU/ml of penicillin, 8 µg/ml of streptomycin, 0.02 IU/ml of amphotericin B (Invitrogen, Carlsbad, CA) and stored at 4°C.

RNA extraction, Reverse Transcription

The Nucleic acid from respiratory samples were extracted using QIAcube (QIAamp Viral RNA Kit, QIAGEN, Valencia, CA) according to the manufacturer's protocol. Spesific primers for influenza A ,influenza B and hRSV (Sigma,USA) were used. The positive samples were investigated for hRSV. Screening for influenza A and B was done by RT-qPCR using the primers, InfA forward: GACCRATCCTGTCACCTCTGAC, InfA reverse:AGGGCATTYTGGACAAA

KCGTCTA, InfA probe TGCAGTCCTCGCTCAC T GGGCACG. InfB forward: TCCTCAAYT CACTCTTCGAGCG, InfB reverse: CGGTG CTCTTGACCAAATTGG, InfB probe: CCAATTCGAGCAGCTGAAACTG CGGTG. For hRSV conventiol RT-PCR was done and the N forward primer: CATCCAGCAA A TACACCATCCA, N reverse primer: GCA TCTCTGAGTATTTTTATGG.

Detection the Infection

Quantitative analysis was performed on a StepOne Plus Taqman Real Time PCR for detection the infection with influenza virus A and B using Directigen Flu A and B kits (Becton Dickinson, Cockeysville, Md.) and the test was done according to the instructions. RT-PCR was done to detect the infection with hRSV using Onestep RT-PCR Kit (Qiagene /USA) and the reaction was done according to the kit instructions.

Result and Disscussion

Among 200 clinical respiratory secretions, 67 samples gave positive results for influenza virus; 45 of them were positive for flu A and 22 were positive for flu B. The investigation for hRSV shows that there were 7 (31%) positive samples within the 22 flu B positive and 2 (9%) samples were positive for hRSV within 22 flu A positive specimens. These results reveal the co-infection of respiratory viruses, flu A or flu B with hRSV in hospitalized patient suffering from respiratory distress. Figue 1reveals the gel-electrophoresis of the amplified samples of RSV N gene to detect the infection with hRSV.

Evidence in many literatures indicates that the clinical meaning of the identification of more than one virus in the same respiratory secretions is controversial. Cilla *et al*. also did not find any differences in the prognosis of children infected by one or more viruses according to hospitalization time (5).

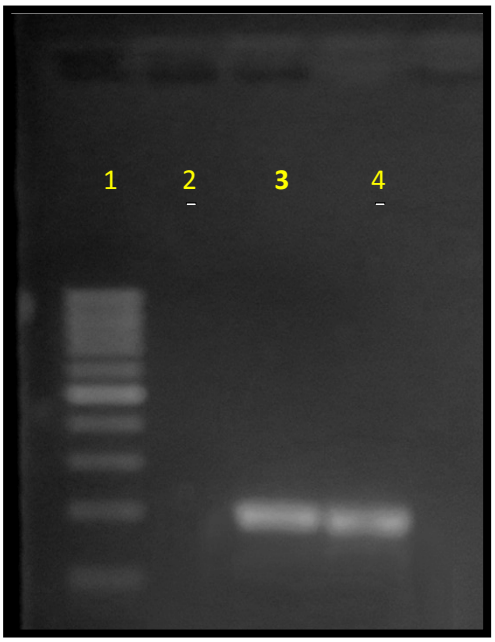

Figure 1: Gel-electrophoresis of the amplified samples of RSV N gene using 1.5% Agarose.
No. 1: 100 bp Marker, 2: negative control, 3 and 4: positive sample for RSV.

In a study, from total 153 samples, 35 samples (22.9%) were positive for influenza A viruses. From the 35 positive samples for IAV, 14 were positive for swine H1N1 subtype. All the positive samples for influenza showed negative for RSV infection which revealed no coinfection with RSV (20). The absence of specific co-infections may be as important. In a study both RSV and HPIV1 were significantly absent from influenza samples greater than expected if co-infection is occurred by chance alone. This pattern is noted with RSV, influenza and parainfluenza: when one of these epidemic respiratory viruses reached a peak the others were relatively inactive (21).

Other studies found different and conflicting results. Some suggest that severity is greater in viral co-infections, while others found greater severity in infections by a single pathogen.

Greensill *et al*. the evaluated children with bronchiolitis caused by RSV which received mechanical ventilation; 70% had coinfection by hMPV, thus suggested greater severity in these cases (5). On the other hand, Canducci found lower

severity in cases of coinfection by RSV + hMPV than in infections with a single virus in hospitalized children (22). Conflicting results may be explained by many factors, such as the fact that different pathogenic mechanisms could be triggered by different viruses that mutually potentialize or mitigate each other's effects. Moreover, the actual pathogenic role of each virus may be unclear. The simultaneous detection of one or more pathogenic virus, such as those investigated in this study, is classified as coinfection. However, the presence of viral genome detected using molecular biology techniques may indicate viral persistence, without significant pathogenic effect at the time of detection (23). Our results provide data on the considerable ratio of patients who receive outpatient care for influenza and RSV illnesses and serve to inform analyses of prevention programs and treatments for both influenza and RSV disease (20). Also the results of this study stress the importance of the correlation between laboratory findings and clinical Severity of coinfection with RSV. Furthermore the results of this study also highlight the impact of these viruses on the health care system and on caregivers and provide data necessary for cost/benefit analyses of prevention programs and treatments for influenza and RSV infection.

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Dictyostelium discoideum paracaspase functions in secretion of lysosomal enzymes

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Abstract: *Dictyostelium discoideum* has proven as useful tool in studying lysosomal membrane trafficking. In *Dictyostelium*, lysosomes connect two main membrane trafficking pathways including the endocytic and the phagocytic pathway. Paracaspase, a caspases- like protein discovered in *Dictyostelium*, has been implemented in trafficking roles along these pathways. In this study, the cellular role of *D. discoideum* paracaspase was investigated. Cell line was generated that over-expressing paracaspase protein. In addition, cell line with knockout pcp was used. Both cell lines show over-secreted lysosomal enzymes, especially β-glucosidase, galactosidase and acid phosphatase. These data support a role for paracaspase in trafficking along the lysosomal enzyme secretion which in turn effect on the endocytic, and phagocytic pathways.

Key words: *Dictyostelium*, Paracaspase, lysosomal enzymes

بروتين الدكتيستليم براكاسباس وتاثيره على عمل الانزيمات الحالة

انتصار جبار صاحب

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الخلاصة: قد اثبت مؤخرا ان اميبا *Dictyostelium discoideum* مثال مفيد في دراسة مسار عمل الانزيمات الحالة. الانزيمات الحالة تربط اثنين من أهم مسارات عملية البلعمة والشرب الخلوي. بروتين البراكاسباس اكتشف حديثا في *Dictyostelium*، وقد تبين ان له دورفي عملية البلعمة والشرب الخلوي. في هذه الدراسة، تم البحث في الدورالخلوي للبراكاسباس حيث تم ستخدام نوعين من الخلايا المطفرة، احداهما تفرط في انتاج البراكاسباس والاخرى ليس لها القدرة على انتاج البراكاسباس, ومن ثم تم اختبار قابلية هذه الاميبا على افراز الانزيمات الحالة. وقد بينت النتائج انه كان هناك زيادة ذات دلالة احصائية في افراز الإنزيمات الحالة، خصوصا β جلوكوزيد، غالاكتوزيداز والفوسفاتيز الحمضي. وتدعم هذه البيانات ان بروتين البراكاسباس له دور مهم في افراز الانزيمات الحالة والذي بالمقابل سوف يؤثر على مسار عمليات الشرب الخلوي والبلعمة في الخلية.

Introduction

Dictyostlium discoideum is a unicellular eukaryotic organism that has proven to be a useful system in which to investigate endosomal and lysosomal membrane trafficking (1, 2). In *Dictyostelium*, lysosomes connect three membrane trafficking pathways including; the endocytic pathway, the biosynthetic pathway, and the phagocytic pathway (1, 2, 3). The biosynthetic pathway consists of the biosynthesis of lysosomal enzymes. Lysosomal enzymes are synthesized as membrane-bound, N-glycosylated precursoe proteins in the endoplasmis reticulum (ER) and then transported to the Golgi.

Lysosomal enzymes are then targeted to lysosomes however; machinery is poorly characterized in *Dictyostelium*. In mammalian cells, lysosomal enzymes are targeted to lysosomes through the recognition of mannose 6-phosphate sugars by mannose 6-phosphate receptors (MPRs). MPR requires Rab9 for retrieval and recycling back to the Golgi. MPRs have yet to be identified in *Dictyostleium* suggesting the existence of another lysosomal enzyme receptor (1, 2, 4). Subsequent enzyme secretion from lysosomes is regulated, with significant secretion of processed enzymes occurring following the onset of starvation (3).

Paracaspase (Pcp) has been discovered in metazoans and in the protozoan, *D. discoideum* (5). Paracaspases belong to clan CD, family C14 cysteine proteases. The active site of paracaspase contains the His/Cys catalytic dyad, a structure conserved in all caspases that is necessary for substrate recognition and catalysis (6). Paracaspases have immunoglobulin Ig within a death pro-domain (5, 7).

Little has been known about the molecular function of paracaspase proteins. However, some studies have reported some information about its function in humans. It has been determined that the paracaspase expressing mucosa-associated lymphoid tissue 1 (MALT1) protease inhibition might provide a pharmacological treatment for lymphoma (8). It has been reported that there is a functional relation between the caspase and paracaspase families to facilitate non-apoptotic phenomena within these cells. Data from another study demonstrated that MALT1 could be a possible drug target to treat aggressive B cell lymphomas (9).

Dictyostlium paracaspase associates with the CV as well as playing a role in its structure and function, interacts with the vacuolar V-ATPase. Additionally, cells over-expressing Pcp had higher rates of

growth, ATP level, endocytosis and phagocytosis (10). Pinocytosis and phagocytosis are trafficking between compartments to coordinate the membrane recycling. They have also been implemented in the control of cell-type-specific functions, such as regulated secretion (11). In this study, our analysis of these Pcp proteins indicated that pcp plays a role in the secretion of lysosomal enzymes.

Materials and Methods

Cells and culture conditions

For all experiments, *D. discoideum*, wild-type strain AX4, *D. discoideum* overexpressing Pcp (pcp-OE), *D. discoideum* cells with the knockout pcp (*pcp*-) were grown axenically at 21^0C in shaking culture at 150 rpm in HL5 medium:1% oxoid proteose peptone, 1% glucose, 0.5% yeast extract, 2.4 mM Na2HPO4, and 8.8 mM KH2PO4, pH 6.5. This media was supplemented with 300 mg/ml of streptomycin sulfate and 100 mg/ml of ampicillin (Sigma). Additionally, for the transfected cells, HL5 medium was supplemented with 10 mg/ml of G418 (Invitrogen) (www.dictybase.org).

Creation of *D. discoideum* cell lines with Pcp over-expressing and *pcp* knockout mutant

D. discoideum cells with Pcp over-expressing (Pcp-OE) was briefly prepared as following: a full length of *D. discoideum* paracaspase (pcp) was cloned into the pDneo2a-GFP vector. These vectors' construct (pDneo2a-GFP-pcp) was sequenced for errors and reading frame confirmation. Primer design, PCR sequencing of the DNA, cloning, and Western blots analysis were performed. On the other hand, the mutant *D. discoideum* with *pcp*- mutant cells were maintain in HL5 media supplemented with 10 mg/ml blasticidine (12).

Assay of secretion the lysosomal enzymes under starvation conditions

The standard secretion assay was performed as described by (13). Log phase cells were harvested by centrifugation and re-suspended at a concentration of 5×10^7 cells/ml in starvation buffer (10 mM phosphate buffer, pH 6.0). Cells were incubated at 21^oC and samples removed at times indicated. Enzyme assays were performed on the supernatant (extracellular) or pellets (intracellular). The α-mannosidase, N-acetylglucosaminidase, β-glucosidase, β-galactosidase, acid phosphatase,

and β-glucosidase activity was measured as previously described (14, 15, 16, 13). All enzyme assays are performed at 35^0C as flowing: for α-mannosidase: cells were incubated with 5 X 10^{-3} M ρ-nitrophenyl α-mannosidase in 5 X 10^{-3} 5 mM acetate buffer (pH 5.0). For N-acetylglucosaminidase: cells were incubated with 4 mM 4-nitrophenyl β-N -acetylglucosamine in 10 mM sodium acetate (pH5.0). For β-glucosidase: cells were incubated with 10^{-2} M ρ-nitrophenyl-β-D-glucopyranoside in 10 mM acetate buffer (pH 5.0). While for β-galactosidase: cells were incubated with 4 mM 4-nitrophenylα-galactose in 10 mM acetate buffer (pH 5.0). For acid phosphatase: cells were incubated with 37mM acetate, (pH 5.0). The reaction was stopped by addition of an equal volume of 1M Na_2CO_3.

Standard Secretion Conditions

Cells were harvested at 2.0×10^7 cells/ml, washed once with cold Sorensen Buffer and re-suspend in 25 ml cold buffer into an Erlenmeyer flask. 100µl was removed of cell suspension, spine 10s at maximum speed, supernatant was removed, and freeze at -20 ^{o}C for protein determination and incubated at 24^{o}C throughout the experiment. 2ml samples at selected time points (15, 30, 60, 90, 120, 180, and 280) were removed. Cells were centrifuge for 3 min at 4,000 rpm to separate cells from supernatant. The supernatant was collected and store at -20^{o}C until assayed. Cell pellet was re-suspended in 1ml cold distilled water and store at -20^{0}C until assayed or precede with lysing. Cells were lysed in 1 ml of water containing 0.1% Triton X-100. The enzyme formation was estimated by measuring absorbance at 420nm with units of activity defined as nanomoles released per minute. Protein concentrations are measured using BSA protein kit.

The amount of extracellular activity was given as a percentage of the total activity at that time point. The amount of enzyme secreted into the culture supernatant was calculated by subtracting the amount of enzyme in the supernatant from the other supernatant values. At each time point, the cellular activity and the secreted activity were summed to give the total amount of enzyme in the culture at that time and the secreted enzyme was expressed as a percent of this total. The amount of extracellular activity is given as a percentage of the total activity at that time point.

Data Analysis

The Statistical Analysis System-SAS (2012) was used to effect of different factors in study parameters.

Least significant difference –LSD test was used to significant compare between means in this study and a probability (P) value of <0.05 was considered as statistically significant.

Results

Paracaspase effects the secretion of lysosomal enzymes

Starvation initiates the developmental cycle in *Dictyostelium*, which is, in part, characterized by induced secretion of lysosomal hydrolases relative to secretion during growth (2). The role of paracaspase in the secretion of lysosomal enzymes was examined by performing standard secretion assays, as described above. Using cells that had been re-suspended in starvation buffer, log phase cells were collected by centrifugation and the enzyme activity was measured in the cells as well as in the supernatants (extracellular). The extracellular (secreted) enzyme activity was expressed as a percent of total enzyme activity. AX4, Pcp-OE cell line, and *pcp*- mutant cells were assayed for secretion of α-mannosidase, N-acetylglucosaminidase, β-glucosidase, β-galactosidase, and acid phosphatase activity.

AX4 cells secreted all enzymes at a roughly equal amount as determined by the analysis of Figure 1. Interestingly, the secretion of all of the lysosomal enzymes assayed was affected in paracaspase over-expressing (Pcp-OE) and knockout Pcp (*pcp*-) mutant cells. The secretion of α-mannosidase and N-acetylglucosaminidase by Pcp-OE and *pcp*- cells was similar to AX4 however; after 180 minutes Pcp-OE cells secrete approximately 20% more enzyme than AX4 cells (Figure 1).

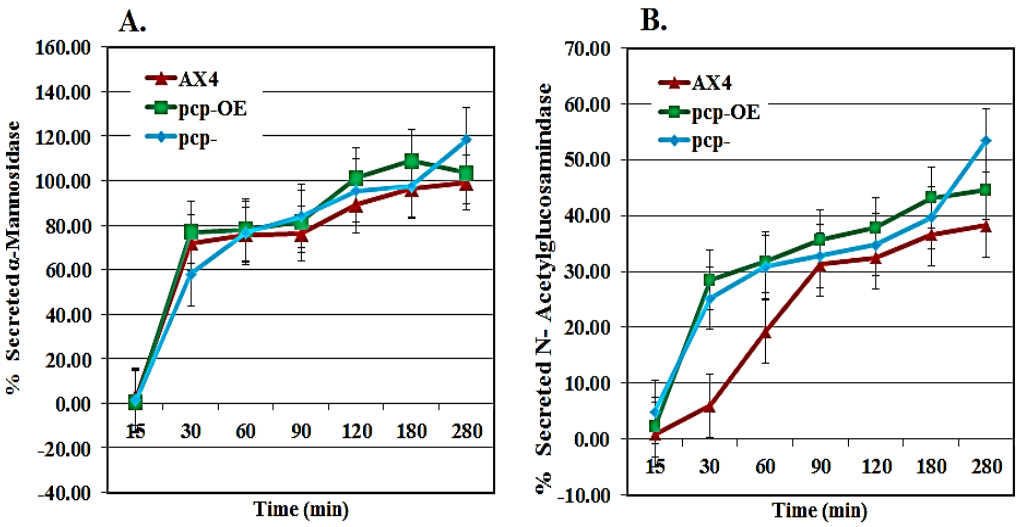

Figure1. Paracaspase effects on the secretion of lysosomal enzymes (α-mannosidase and N-acetylglucosaminidase). The data correspond to the average of 3 independent experiments for all cell lines. Logarithmically growing cells were grown under standard secretion conditions, separated into cellular and media fractions by centrifugation, lysed with Triton X-100. Samples were removed at the times indicated. Both the cell samples and supernatants were assayed for enzyme activity. Pcp-OE and *pcp*- cells show slightly abnormal secretion of the enzymes assayed compare with the control wild type Ax4.

The secretion of β-glucosidase enzyme was equal to that of the AX4 cells in *pcp*- cells. Interestingly, cells over-expressing paracaspase at 120-minute time point secreted around 40% of β-glucosidase more than the wild type Ax4 cells. While after 280 minutes, Pcp-OE cells were secreting 80% more β-glucosidase than the wild type Ax4 cells (Figure 2). Initially, the secretion of β-galactosidase by Pcp-OE, and and *pcp*- cells is similar to Ax4 however, after 180 minutes Pcp-OE and *pcp*- cells secrete approximately 50%, 90% more β-galactosidase enzyme than the wild type AX4 cells respectively.

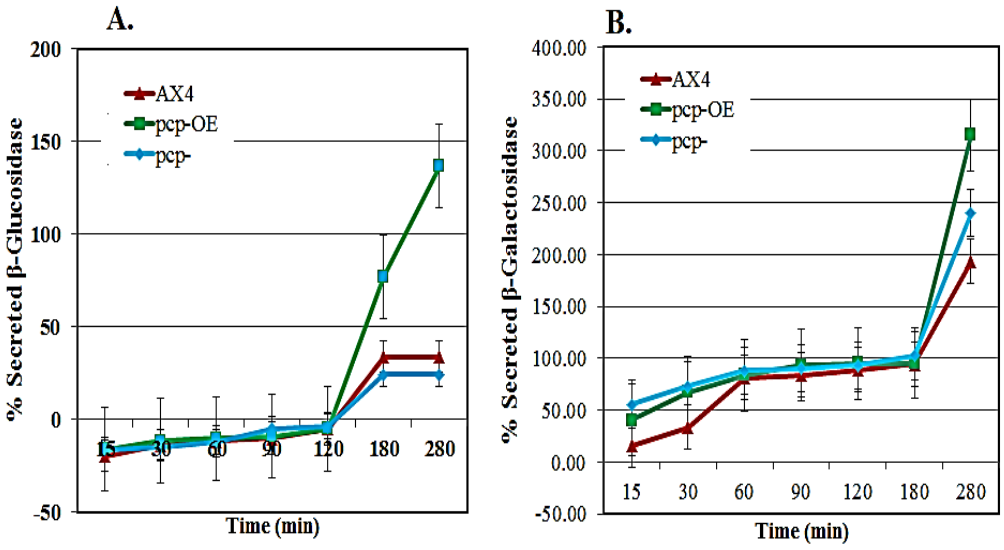

Figure 2. Secretion of the lysosomal enzyme (β-glucosidase and β-galactosidase) was affected in Pcp-OE and *pcp*-. The data corresponds to the average of 3 independent experiments. After 120 minutes, cells over-expressing the Pcp protein had a significantly higher rate of secretion of β-glucosidase compared with the other cell lines. After 180 minutes, cells over-expressing the Pcp protein had a significantly higher rate of secretion of β-galactosidase compared to the *pcp*- mutant cell line and the control WT-AX4 cells.

The secretion of acid phosphatase by Pcp-OE and *pcp*- cells was most affected. Until 120 minutes, the enzyme acid phosphatase secretion was almost equal to that of the AX4 cells, Pcp-OE, and *pcp*- cells however, after 120 minutes, statically analysis showed that there was a significant increase of acid phosphatase secretion in Pcp-OE and *pcp*- cells compare with the AX4 cells. At 180 minutes, acid phosphatase secretion was 40% increase in Pcp-OE cells. Interestingly, *pcp*- mutant cells had increased secretion activity after 30 minutes and reached to its maximum values at 280 minutes compared to the control WT-AX4 cells (Figure 3). These results showed significant difference when analyzed statically by using LDS value at probability $P \leq 0.05$.

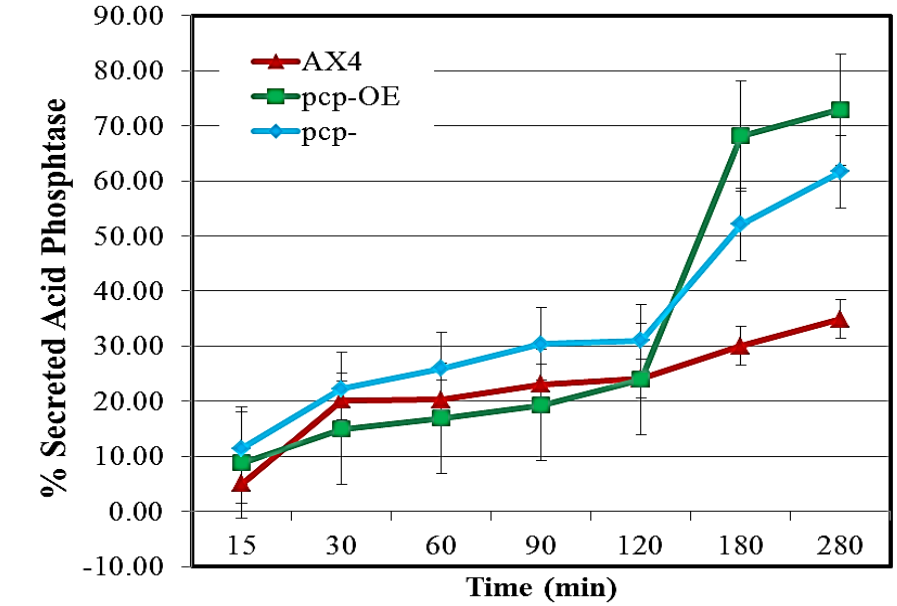

Figure 3. Secretion of the lysosomal enzyme acid phosphatase was increased in Pcp-OE and *pcp*- cell lines. The data corresponds to the average of 3 independent experiments. Symbols are as follows: (▲) AX4, (■) Pcp-OE, and (♦) *pcp*-. After 120 minutes, cells over-expressing the Pcp protein had a significantly higher rate of secretion of acid phosphatase while *pcp*- mutant cells had increased secretion activity after 30 minutes compared to the control WT-AX4 cells.

Discussion

In this study we have analyzed *D. discoideum* Pcp to gain insight into its role in lysozyme secretion. It was demonstrated that the Pcp protein localizes with lysosomes and the contractile vacuole (17). This result is agreed with (10) who found that over-expressing Pcp causes a significant defect in the rates of phagocytosis and pinocytosis. Microscopic analysis also shows differential localization of Pcp cells to endosomes and lysosomes. This study demonstrate that both *Dictystelium* cells over-expressing paracaspase and *pcp*- cells exhibit defects in the secretion of lysosomal enzymes with the greatest defect being observed in the over-secretion of β-glucosidase, β-galactosidase and acid phosphatase. This data suggests that the Pcp protein functions in the

transport of proteins from the ER-Golgi-CV, as well as along the endo-lysosomal pathway. These results could be due to Pcp may down-regulating during signaling pathways accounting for the increased rate of pinocytosis.

The endo-lysosomal system of *Dictyostelium* consists of multiple compartments that rapidly process endocytosed materials and excrete indigestible substances. Endocytic and phagocytic vesicles are quickly acidified and receive lysosomal enzymes to digest their contents. The acidic lysosomal vesicles subsequently mature into post-lysosomes, neutral secretory vesicles that are destined for exocytosis (18, 19, 20). Consequently, the *Dictyostelium* lysosome is not a terminal organelle as in most mammalian cells, but is most similar to the secretory lysosomes of specialized mammalian cells (21, 20). We followed standard secretion conditions (13) in which greater than 50% of the total cellular activity of several lysosomal glycosidases are secreted within a few hours. These conditions are particularly useful for the study of secretion because the lysosomal enzymes are secreted in the absence of any appreciable enzyme synthesis or degradation. Thus, by monitoring the release of lysosomal enzymes, the functioning of the lysosomal vesicles involved in secretion can be observed (13). We observed that Pcp mutants significantly over-secrete β-glucosidase, β-galactosidase and acid phosphatase. The secretion of α-mannosidase and N-acetylglucosaminidase appeared unaffected. It has been proposed that there are three functional classes of lysosomal vesicles in *Dictyostelium*, one class contains α-mannosidase, N-acetylglucosaminidase, β-galactosidase, and β-glucosidase. A second class contains acid phosphatase (22, 23). Another possibility presented by Bush and Cardelli 1989 is that acid phosphatase secretion is differentially regulated compared to the glycosidases. Our results indicate that Pcp is involved with the class of lysosomal vesicles contains, β-galactosidase, and β-glucosidase as the secretion of α-mannosidase, N-acetylglucosaminidase appeared unaltered. Interestingly, the secretion of acid phosphatase was most altered in Pcp over-expressing cells. These results are consistent with a role for Pcp in the secretion of lysosomal enzymes. There are several lines of evidence that lysosomal enzymes reside with fluid phase markers in a secondary lysosomal compartment

that is functionally connected to the endosomal system and that both lysosomal enzymes and fluid phase markers are released from this compartment (3). We hypothesize that Pcp has a role in regulation the rates of phagocytosis and pinocytosis (10). These proton pumps originate in the ER, are modified in the Golgi, and are transported to mainly the CV, but also along the endo-lysosomal pathway. The over-secretion defect observed in Pcp over-expressing, and *pcp*- cell lines together with the increased rate of endocytosis suggests that Pcp play a role in the recycling of lysosomal enzymes.

Conclusion

The results of this work show that Pcp-OE and *pcp*- cells cause over-secretion of lysosomal enzymes, specifically, the over-secretion of acid phosphatase. Our current model for Pcp is that this protein functions along the secretory pathway, trafficking proteins or possibly vacuolar proton pumps from the ER-Golgi to the CV as well as along the endo-lysosomal pathway.

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Adhesion Capacity of *Bifidobacterium* to Abiotic Surfaces

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Abstract: *Bifidobacterium* is a genus of Gram-positive, nonmotile, often branched anaerobic bacteria. They are ubiquitous, endosymbiotic inhabitants of the gastrointestinal tract, some bifidobacteria are used as probiotics.The adhesion of *Bifidobacterium on* abiotic surfaces(stainless steel , polystyrene and galvaniz iron) in cultures with different temperatures (5,37 and 40◦C) and different pHs (4, 7, and 9) were studied in recent study .This isolate gave positive results with congored agar and Christensen method .The results indicated that the type of material had effect on the attachment capacity of *Bifidobacterium* in other word , the galvaniz iron was significant highest attractive surface to bacterial cells and biofilm formation($P < 0.05$) ,then follows by the stainless steel while polystyrene was the least .On other hand ,adhesion to all solid surfaces was influenced significantly by temperatures, the best adhesion occured at 40◦C($P<0.05$),while the best studied pH was 9($P<0.05$). The results showed that time factor also had positive effect on biofilm formation , the best biofilm formation occurred after 72 hr.
Key words: adhesion, *Bifidobacterium*, abiotic surfaces

قابلية التصاق بكتريا البفيدو على الالتصاق على السطوح غير الحية

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الخلاصة: بكتريا البفيدو هي جنس موجب لصبغة كرام غير متحركة تعود غالبا للبكتريا لاهوائية .هذه البكتريا موجودة بصورة تعايشية في الجهاز الهضمي بعضها تستعمل معززات الحياة .تناولت هذه الدراسة التصاق بكتريا البفيدو على السطوح غير الحية (الحديد المقاوم للصدأ والبولي ستايرين واالحديد المغلون) بدرجات حرارة C◦ (5و37و40) وقيم اس هيدروجيني مختلفة (4و7و9).

اعطت هذه البكتريا نتيجة موجبة على وسط احمر الكونغو وكذلك بطريقة كرستينسن .اشارت نتائج الدراسة الحالية الى ان نوع مادة السطح تؤثر على قابلية التصاق البكتريا بعبارة اخرى ان الحديد المغلون اكثر السطوح جذبا للبكتريا وتكوين الغشاء الحياتي ($P < 0.05$) ويليه الحديد المقاوم للصدا وثم البوليستيرين .من جهة اخرى ان الالتصاق قد تأثر معنويا بدرجة الحرارة اذ كان احسن التصاق حدث بدرجة C◦ 40 ($P < 0.05$)بينما احسن اس هيدروجيني كان 9 ($P < 0.05$). اشارت النتائج الى ان عامل الوقت ايضا له تأثير ايجابي على تكوين الغشاء ا الحياتي وكان افضل تكوين للغشاء الحيوي عند 72 ساعة .

Introduction

Bifidobacteria are Gram-positive bacteria that represent one of the major genera of the intestinal tract of humans and animals[1]. They are strictly anaerobic, although some species tolerate low oxygen concentrations[2] . They do not form spores, have an irregular rod-shaped form .[2].

Several beneficial effects on the health status of the human host have been claimed to be related to the presence of bifidobacteria in the colon [3-5], thus they become increasingly interesting for probiotic applications in pharmaceutical and dairy products. Several criteria are used for the selection of probiotic strains. Besides manufacturing criteria and shelf life, species- and strain-specific properties related to the probiotic effects are of major importance [6,7].

One of the most commonly tested parameters is the ability of a probiotic to colonise the gastrointestinal tract (GIT) of the host. This is associated with resistance to the conditions of the GIT (low pH, high concentration of bile salts) and adhesion to mucus . There are a number of studies assessing the adhesion of probiotic bacteria quantitatively [8-11].

The aim of this study was demonstration the use of abiotic surface (stainless steel , polystyrene and galvaniz iron) as a tool to study *Bifidobacterium* bacterial biofilms and study factors involved in adhesion such as (temperature and pH) to these surfaces that may have potential applications .

Material and methods

Isolation of Bacteria

Bifidobacterium was isolated from probiotic yogurt(Activia) available in local markets. Redetected according to

Wood, andHolzapfel [12] by using the cultural, microscopical and biochemical examinations .The Isolate was grown in De Man Rogosa sharpe (MRS) broth for 24 h. at 37 ºC under anaerobiosis.

Detection of bacterial ability to produce slime layer

A- Congo red agar method

Congo red agar was inoculated with single colony of tested bacterial isolates by streaking, incubated at 37º C for 24 hr.; a positive result was indicated by black colonies. Non slime producers usually remained pink [13].

B- Christensen's method

Glass tubes containing 10 ml of tryptic soya broth were inoculated with single colony of test bacteria by sterile loop, negative control was made by adding 10 ml of tryptic soy broth to a glass culture tube. The tubes were incubated at 37ºC for 24-48 hr. After that the tubes content was decanted and 10 ml of 0.1%

safranin stain solution was added to all tubes. Each tube was then gently rotated to ensure uniform staining of any adherent material on the inner surface and the contents was gently decanted. The tubes were then placed upside down to drain. A positive result was indicated by the formation of an adherent layer of stained material to the inner surface of the tube [14].

Selection of test surfaces

Polystyrene (disposable food dishes were used), galvanized iron , stainless steel were selected for the adhesion tests, were cut into uniform size of 1Cm2 coupons. Prior to adhesion tests, galvanized iron and stainless steel coupons were soaked for 24 hr in absolute ethanol,while polystyrene were swabbed by absolute ethanol then washed and rinsed thoroughly eight times with deionized water . Galvanized iron and stainless steel coupons were directly autoclaved at 121◦C for 15min [15].

Study the effect of temperature and p*H* on *Bifidobacterium* biofilm production on different abiotic surfaces.

This study investigate the biofilm formation by *Bifidobacterium* isolate on three different surfaces: galvanized iron , stainless steel and polystyrene surfaces under different temperature and pH values by crystal violet binding assay under anaerobiosis.

A single, isolated colony was grown in 50 ml of BHI-broth that incubated at 37°C for 18 h. these used in adherence study.Working culture was containing 2 ml of the bacterial suspension in BHI-broth and 18 ml of low nutrient medium BHI-broth (Brain Heart Infusion Broth was prepared according to the instructions of Manufacturer Company , and then diluted ten times . [16].

The effect of pH was investigated by allowing biofilm production in low nutrient broth BHI-broth adjusted, before the autoclaving, to pH values of 4 , 7 and 9 with NaOH or HCl, while the effect of temperature was investigated by incubated of the broth under different temperature 5 °C, 37 °C and 40 °C at pH 7.

For inoculation, 2 ml of the *Bifidobacterium* suspension was inoculated on different coupons.

For the negative controls, 2 ml of sterile low nutrient medium solution was used to substitute the 2 ml of *Bifidobacterium* suspension, then these coupons incubated for (24, 48, 72) hr.

Biofilm quantification.

Crystal violet assay was used to quantify the biofilm formation which measures the total biofilm biomass, including bacterial cells and extracellular matrix. [17,18.19]

.At the end of each incubation periods, a set of coupons were aseptically removed, these coupons were rinsed three time using 1 ml distilled water. This step was used to rinse off loosely attached bacterial cells. Then they were air dried and adherent bacteria were stained with 2 ml of crystal violet stain 0.1% (w/v) for each coupon at 37°C for 20 min. The exceed stain solutions were removed from the coupons, rinsed with 1 ml distilled water thrice and coupons were kept for drying at room temperature. After drying, the attached crystal violet was solubilized with 2 ml of 99.9% (v/v) ethanol for 20 min., finally the concentration of crystal violet was determined by measuring the optical density of de-staining solution at 620 nm OD620 value- C value(control). This experiment conducted twice with triplicates in each time.

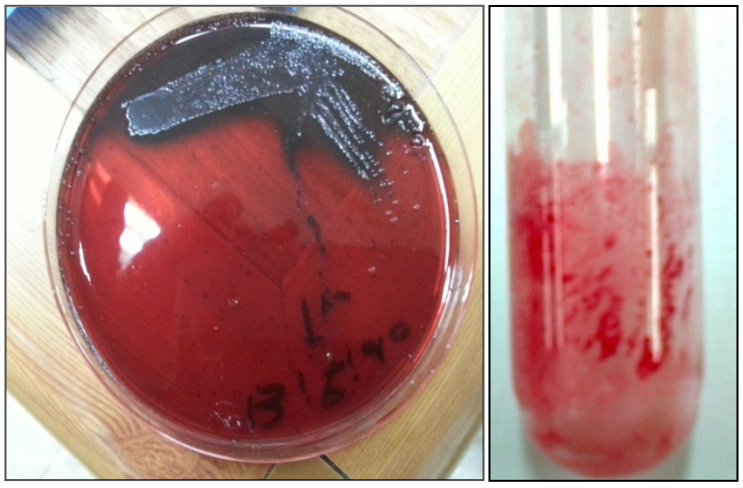
Statistical Analysis

The Statistical Analysis System-SAS (2012) was used to effect of different factors in study parameters. Least significant difference –LSD test was used to significant compare between means and in this study [20].

Results

Detection of bacterial ability to produce slime layer

Bifidobacterium isolates were cultured on Congo-red agar plates. The results showed that bacteria were slime producer by forming black colonies also gave positive results in Christensen's method (adherent layer of stained material to the inner surface of the tube) figure1.



A **B**

Figure 1: A- Slime layer producer isolates on congo red agar **B-** adherent layer of stained material to the inner surface of the tube(Christensen's method)

Adherence of *Bifidobacterium* on the solid coupons and effect of temperature

The first phases of biofilms formation are influenced by a series of environmental factors like temperature [21]. The dye bounded to adherent cells as well as matrix was resolubilized and measured their optical density (OD) at 620nm.

According to the obtained data,the galvanized iron coupons had a higher capability to attract bacterial cell and biofilm formation(in three temperature values) followed by stainless steel surfaces while polystyrene was regard as a less attractive surface to bacterial adhesion and biofilm progression(figures2,3, and 4).

In general the statistical analysis shows significant differences ($P<0.05$) in biofilm densities by *Bifidobacterium* isolate with variation of incubation temperature values that formed on three surface (table 1,2,3) .

This pattern of adhesion can be explained by the composition of the materials that were tested .The physical properties of the surface, such as roughness, rougher is the surface, the more deep crevices or polish lines present on the surface. The high retention of the bacterial cells during rinsing process may be due to the possible entrapment of microbial cells in crevices of the surface, because these crevices provide refuge to the adherent bacterial cells from shear force [26].

Hydrophobicity, surface energy, and electrostatic charge, also affect this process,

especially in metallic materials [22],this results came in agreement with[23 , 24].

Galvanized iron has more roughness than stainless steel and polystyrene that explain recent results of adhesion on these surface ,this results came in agreement with other research who found that bacterial adhesion to metals more easily than polymer such as polystyrene [22 ,25].

Surface roughness of the attachment surface is an important factor which can affect the removal of bacterial cells. The

From obtained data and under different conditions, the OD value which represent biofilm density, increased with the increased incubation time ,the increase reached a maximum at time 72 h. Incubation time here can be known as contact time which is the time for the bacterial cells to contact with the fresh coupons surface [23,19].

Other studies were conducted on adhesion of probiotic and dairy *Lactobacillus* strains such as[8,9] .

According to the recent data, *Bifidobacterium* formed biofilm at 40 ◦C on all three coupons and it

was greater than other degrees. This may be attributed to the stressful conditions on bacterial growth that forms more slime layer and biofilm to protect cells against this physical stress such phenomenon was explained in detail by[27] the tendency of bacteria to regulate gene expression in response to the environmental signals, such as temperature and pH for instance.

On the other side, biofilm was less formed on the coupons at 5 ◦C despite of the unfavorable conditions. This might be due to the stable bacterial count as compared with the other temperatures as shown in figures 2,3, and 4.

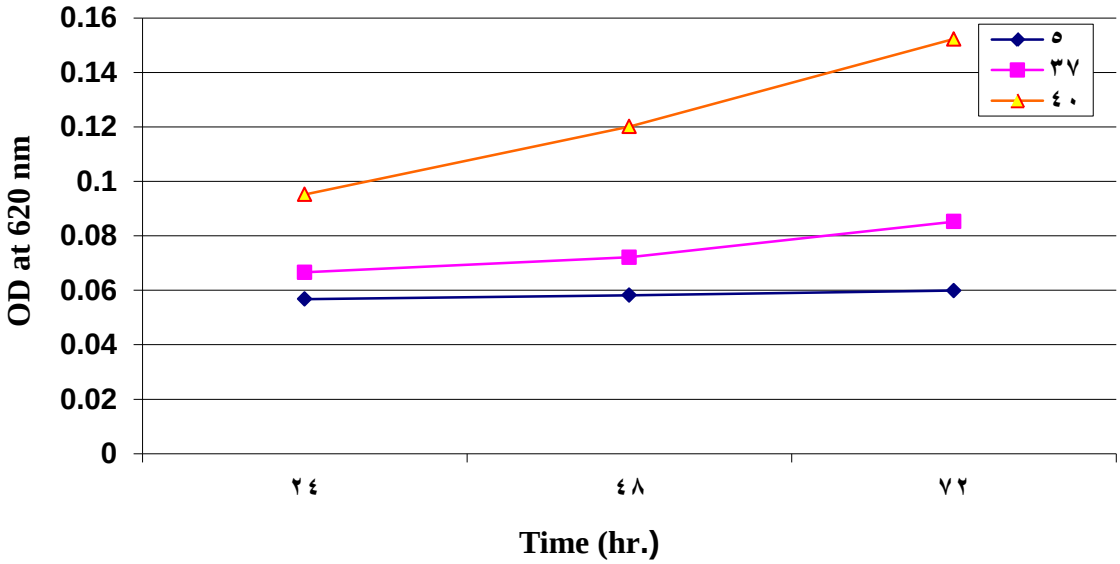


Figure 2: Relationship between Polystyrene surfaces and mean values of biofilm formation by *Bifidobacterium* at 5˚C, 37˚C and 40˚C at pH7 represented by OD 620nm.

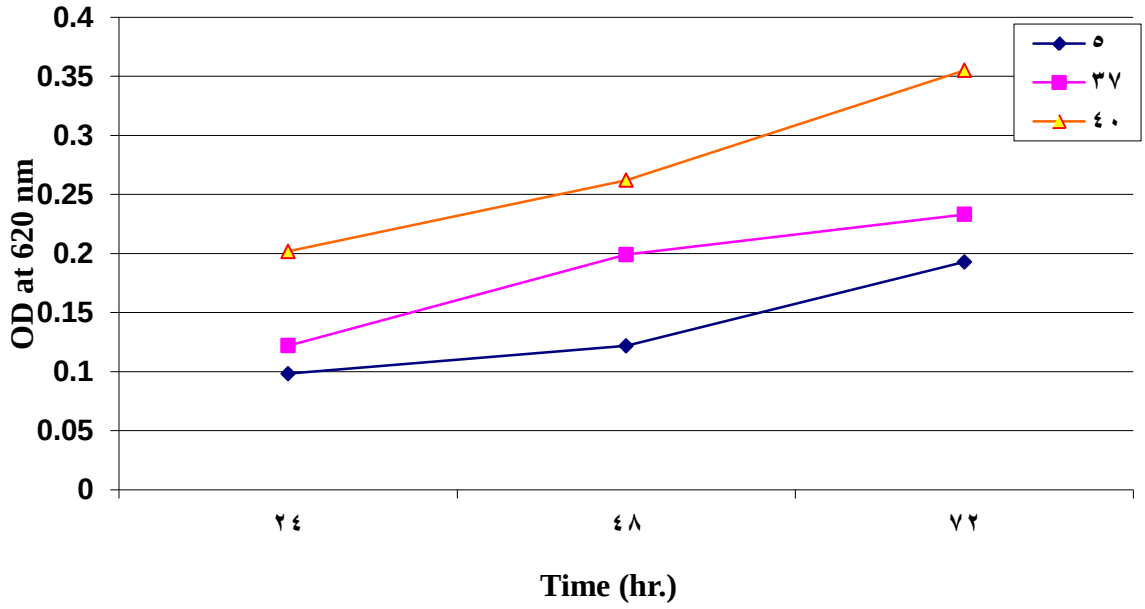


Figure: 3 Relationship between stainless steel surfaces and mean values of biofilm formation by *Bifidobacterium* at 5˚C, 37˚C and 40˚C at pH7 represented by OD 620nm.

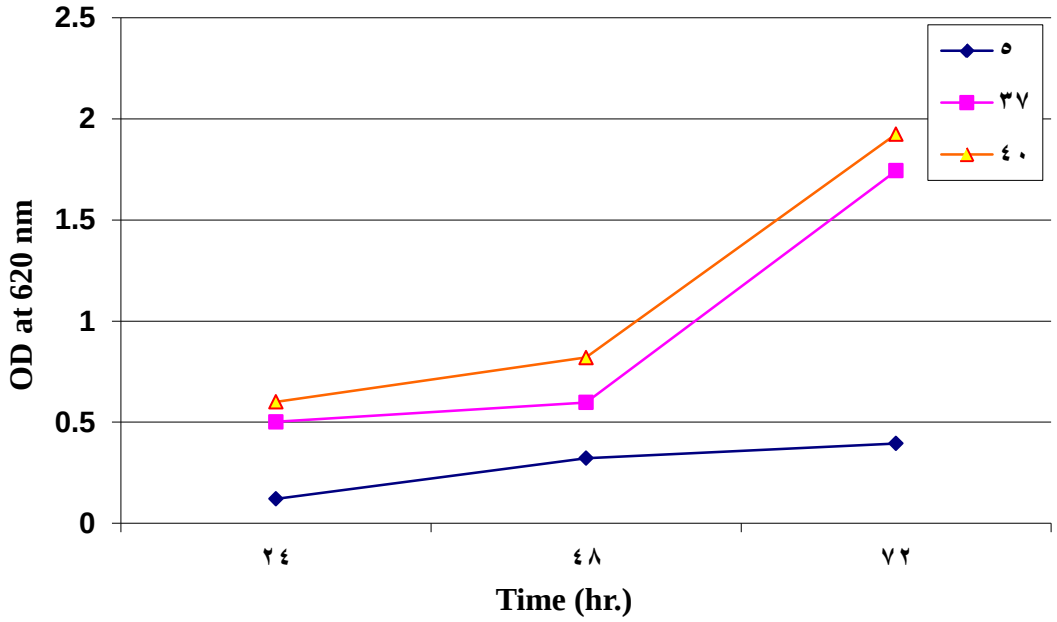


Figure 4: Relationship between galvanized iron surfaces and mean values of biofilm formation by *Bifidobacterium* at 5˚C, 37˚C and 40˚C at pH7 represented by OD 620nm.

Adherence of *Bifidobacterium* on the solid coupons and effect of pH

The impact of cultivation pH was investigated by allowing biofilm production in a culture media adjusted, before autoclaving, to pH values (4, 7 and 9) .The results were summarized in figures 5, 6, and 7 which showed the ability of *Bifidobacterium* to adherence to all three coupons surfaces under all selected pH values, but in different degrees,in which increased pH (pH9) lead to a higher biofilm production in galvanized iron coupons surfaces ($P<0.05$), followed with neutral pH (pH7), while at the acidic environment (pH5) a less biofilm production was observed, but in case of polystyrene and stainless steel the increasing in biofilm production occurred but non significantly table (4,5,6).

These results were similar to results obtained by[28,29] who demonstrated that bacterial attachment to surfaces and the rate of cell deposition were pH-dependent, also found that The adhesion forces at pH 9 were higher than at pH 7 due to the increase in theattraction between Fe ions and negative carboxylate groups.

In addition Wood found that the adhesion of a specific organism can be manipulated by changing the pH and temperature [30].

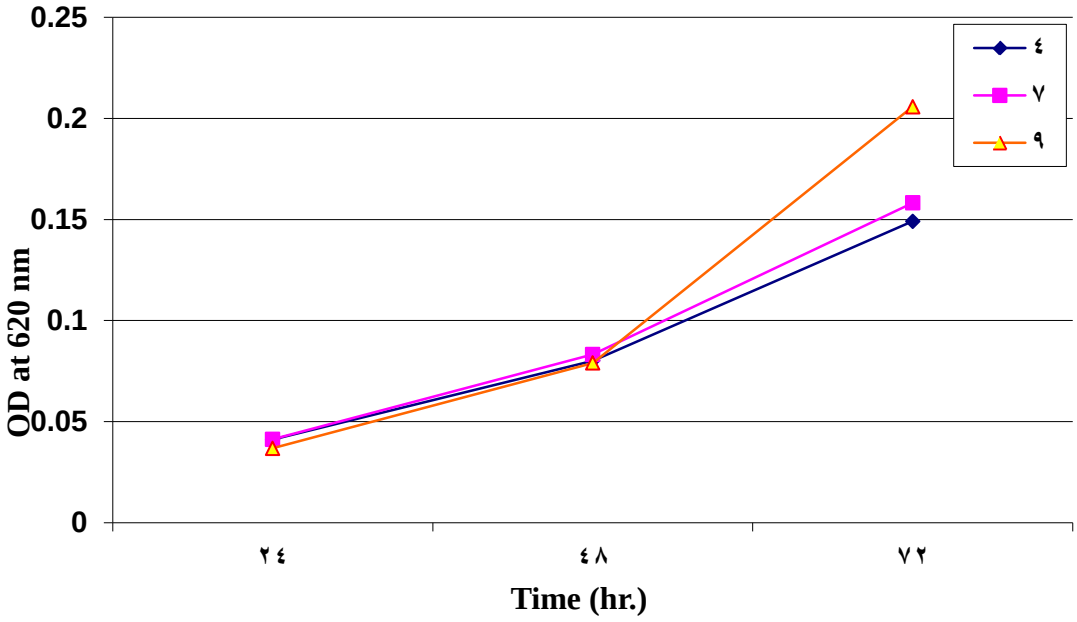


Figure 5: Relationship between Polystyrene surfaces and mean values of biofilm formation by *Bifidobacterium* at pH5m pH7 and pH9 and at 37˚C represented by OD 620nm.

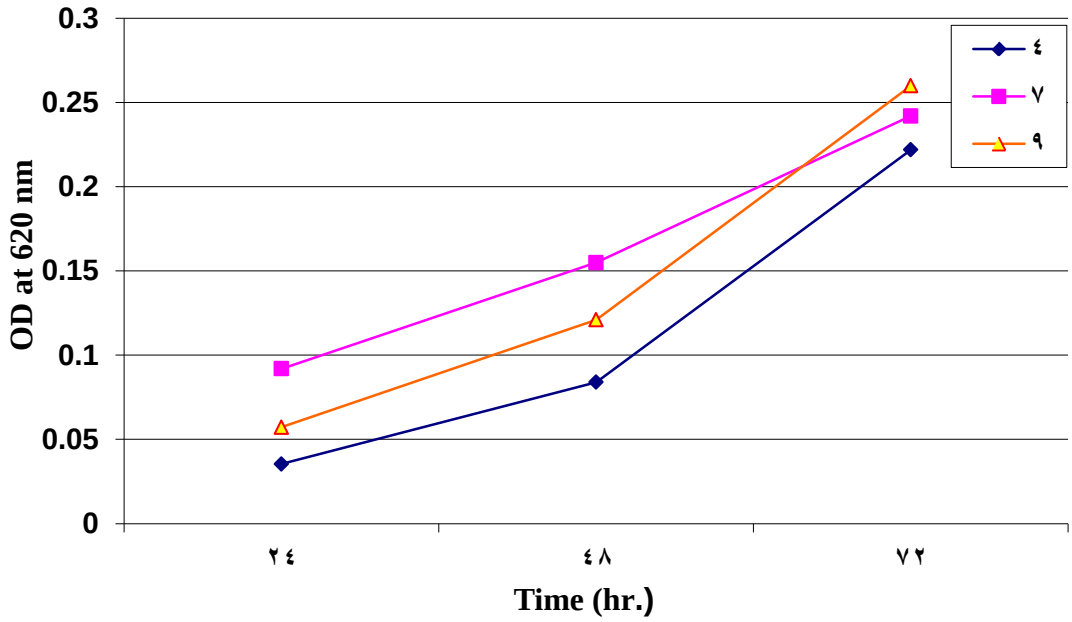


Figure 6: Relationship between stainless steel surfaces and mean values of biofilm formation by *Bifidobacterium* at pH5m pH7 and pH9 and at 37˚C represented by OD 620nm.

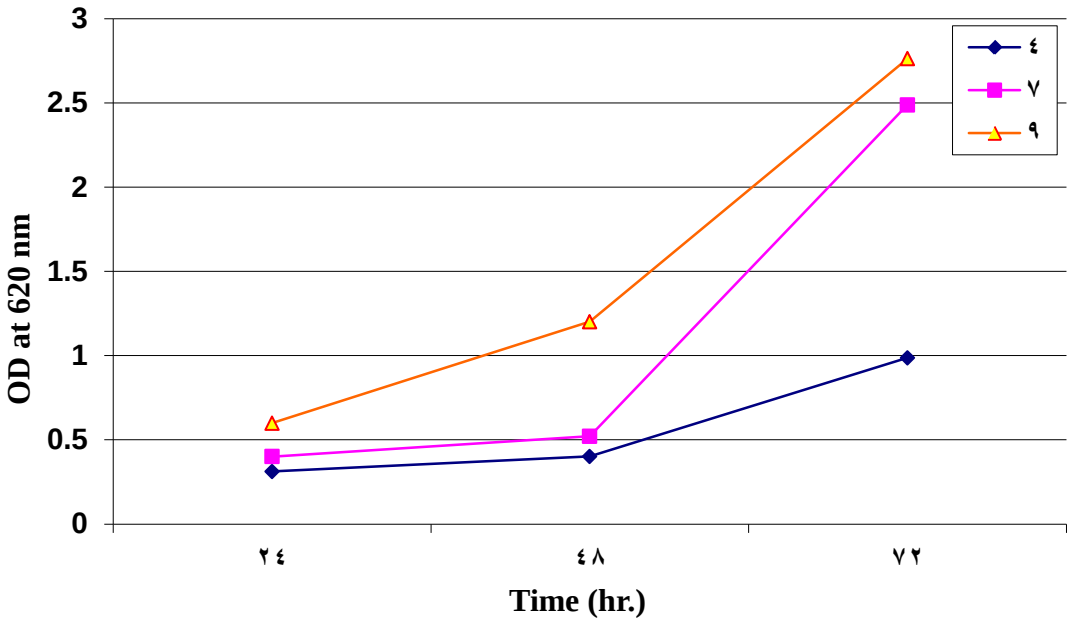


Figure 7: Relationship between galvanized iron surfaces and mean values of biofilm formation by *Bifidobacterium* at pH5m pH7 and pH9 and at 37˚C represented by OD 620nm.

Table 1: Effect of time and temperature on OD (620 nm)/(Polystyrene)

Time (hr.)	Temperature (C)			LSD value
	5	37	40	
24	0.0568	0.0666	0.0952	0.0272 *
48	0.0582	0.0721	0.1201	0.0328 *
72	0.0599	0.0852	0.1523	0.0394 *
LSD value	0.0255 NS	0.0295 NS	0.0351 *	---
* (P<0.05), NS: Non-significant.				

Table 2:Effect of time and temperature on OD (620 nm)/ (stainless steel)

Time (hr.)	Temperature (C)			LSD value
	5	37	40	
24	0.0982	0.122	0.2018	0.046 *
48	0.1218	0. 199	0.262	0.059 *
72	0.1928	0.233	0.355	0.044 *
LSD value	0.041 *	0.055 *	0.058 *	---
* (P<0.05).				

Table 3: Effect of time and temperature on OD (620 nm)/(galvanized iron)

Time (hr.)	Temperature (C)			LSD value
	5	37	40	
24	0.121	0.5015	0.601	0.227 *
48	0.3223	0.598	0.820	0.219 *
72	0.395	1.744	1.925	0.563 *
LSD value	0.278 NS	0.429 *	0.453 *	---
* (P<0.05) , NS: Non-significant.				

Table 4: Effect of time and PH on OD (620 nm)/(Polystyrene)

Time (hr.)	PH			LSD value
	4	7	9	
24	0.0411	0.0412	0.0368	0.0219 NS
48	0.0801	0.0832	0.0790	0.0266 NS
72	0.1491	0.1582	0.2058	0.174 NS
LSD value	0.0623 *	0.0591 *	0.084 *	---
* (P<0.05) , NS: Non-significant.				

Table 5: Effect of time and PH on OD (620 nm)/(stainless steel)

Time (hr.)	PH			LSD value
	4	7	9	
24	0.0354	0.092	0.0573	0.0337 *
48	0.084	0.155	0.121	0.0428 NS
72	0.222	0.242	0.260	0.0441 NS
LSD value	0.0732 *	0.0927 *	0.0941 *	---
* (P<0.05) , NS: Non-significant.				

Table 6: Effect of time and PH on OD (620 nm)/(galvanized iron)

Time (hr.)	PH			LSD value
	4	7	9	
24	0.312	0.401	0.599	0.315 NS
48	0.402	0.521	1.202	0.529 *
72	0.987	2.489	2.763	0.882 *
LSD value	0.367 *	0.694 *	0.884 *	---
* (P<0.05).				

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Starvation of Green algae *Scenedesmus quadricauda* to stimulate the production of total lipid and total protein

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University of Baghdad-College of Science

Abstract: In this study Identification and Isolate the Species of green algae of *Scenedesmus quadricauda* belonging to the Division Chlorophyta, Ch-10 culture media was used for their cultivation. Determine the effects of the phosphorus and nitrogen deficiencies (50% phosphorus ,50% nitrogen , 100% nitrogen , 50% nitrogen plus phosphorus) on the lipid and protein contents was studies, in suitable laboratory conditions (25 ±2C°, 200µE/m^2/sec) for 16:8 hrs. Light: dark and harvested culture end of the exponential phase. Highest lipid accumulation of 33 % was recorded in the culture without nitrogen (100%N),also the lowest dry Weight 0.15mg/l in the same treatment .protein and lipid values found as 42.8% and 15.23%, for the control group, respectively. Also recorded at treatment (50% nitrogen plus phosphorus 50%) 24.1%, 15.23% lipid and protein content, respectively.

Key words: *Scenedesmus quadricauda*, nitrogen starvation, lipid content.

تجويع الطحالب الخضراء*Scenedesmus quadricauda* لتحفيز انتاج الدهون والبروتين الكلي

غيداء الربيعي و عبداللطيف محمد جواد

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الخلاصة: في هذه الدراسة تم عزل وتنقية نوع من الطحالب الخضراء وهي *Scenedesmus quadricauda* التي تنتمي إلى شعبة Chlorophyta، وقداستعمل الوسط الزرعي Ch-10 لتنميتها. وتهدف الدراسة تأثيرالنقص الفوسفوروالنيتروجين بنسب (50٪ الفوسفوروالنيتروجين 50٪، 100٪ نيتروجين و 50٪ نيتروجين بالإضافةإلى الفوسفور) على محتوى الدهون والبروتين للكتلة الحية. في ظروف ثابتة (25م˚ شدة أستضاءة و200 مايكروأنشتاين /م^2/ثا) ولمدة 16:8 ساعة، وحصدت المزرعة نهاية الطورالاسي . وسجلت أعلى محتوى للدهون 33٪ في معاملة خالي من النيتروجين (N٪100) ،وأقل وزن الجاف 0.15 / mg لترفي نفس المعاملة.كما وجدمحتوى البروتين والدهون عندمعاملة السيطرة 42.8٪ و 15.23٪ على التوالي. وسجل محتوى الدهون والبروتين في معاملة (50٪ النيتروجين بالإضافة إلى الفوسفور 50٪) بلغت 24.1٪و 15.23٪ على التوالي .

الكلمات المفتاحية : *Scenedesmus quadricauda*، نقص النيتروجين والفسفور ، محتوى الدهون.

Introduction

Microalgae are photosynthetic microorganisms which transformt sunlight, water and CO2 to sugars, from which macromolecules such as lipids and Triacylglycerol's (TAGs) [1].In recent years, a study on microalgae lipid for biodiesel sources has been done in many Local and countries Studies [2,3,4] Microalgae, as the simple cellular structure ,short production cycle, high intracellular lipid content, and rapid growth rat [5].Also are far more efficient and can be grown in fresh water brackish and marine water, utilize waste water and do not compete with food crops for land and water Its potential wide scale production and could be used for nutritional purposes, such as a source of protein,lipid, carbohydrates, vitamins [6].common species of microalgae have also been used for the production of biodiesel ,but most of the work has been concentrated on microalgae because of their high lipid content such as *chlorella* sp. *Senedesmus* sp. [7].

Algae represent valuable sources of a wide spectrum of complex lipids with different potential applications in food, cosmetic and pharmaceutical industries [8]. Lipids of microalgae also possess antibacterial, antiviral, antitumor , antiinflammatory, antiprolierative and antioxidant activity [9].

Several studies have shown that the quality and quantity of lipids within the cell can differ as result of changes in growth conditions, such as phosphate limitation, nitrogen deficiency, salt stress, alteration of temperature, light intensity and iron content of the medium also affect algal growth along with lipid content [10, 11]. The lipid extraction from algae has already been studied and tested using different solvents like isopropanol/hexane, ethanol/hexane, and methanol/chloroform [6].

The present study was conducted to investigate the: growth response and lipid content of a freshwater green alga, *Scenedesmus quadricauda*, by different the concentrations of phosphor and nitrogen (50% phosphorus ,50% nitrogen, 100% nitrogen, 50% nitrogen plus phosphorus) in the growth medium.

Materials and methods

It was isolated *Senedesmus quadricauda*from pond in Baghdad uni. The followers of method Streak plating [12] it has known algae using an optical microscope Olympus compound and the adoption the source , *S.quadricauda*is a genus of algae,specifically of the chlorophyceae,colony composed of 4-8 ovate cells with short spines,usually stronglyrecuved; cells 4-7.4 in

diameter,9-16 long widely distributed in many lakes and ponds[13].

growth condition

The cultures were grown in 1000 ml Erlenmeyer flasks with 500 ml ch-10 medium, The culture were grown in Ch-10 medium and the content of the medium consist of the following composition (g/l): 5.8Sodium Meta Silicate, 57.56 $Ca(NO_3)_2.4H_2O$, 10 K_2HPO_4, 25 $MgSO_4.7H_2O$, 4.36 EDTA .Na_2, 3.15 $FeCl_3.6H_2O$, 20 Na_2CO_3, 1 EDTA.Na_2, 2.68 H_3BO_3, 1.81 $MnCl_2.4H_2O$, 0.222 ZnSO4.$7H_2O$, 0.390 Na Mo $O_4.5H_2O$, 0.079 $CuSO_4.5H_2O$, 0.0494 $Co(NO_3)_2.6H_2O$. [12] .

Culture was kept at a constant room temperature(batch culture) incubated in 25±2C˚ with 200µE / m^2 /Sec for 8:16 h dark: light. Were hand shaken two to three times daily to avoid sticking. All the glassware and media were always sterilized prior to inoculation. This was referred to as control culture. All the experiments were carried out in triplicates [6].The experiment was performed in (50% and 100% nitrogen, 50% nitrogen plus 50% phosphorus and 50% phosphorus) deficiency according to the amount in ch-10 medium.

Determine the growth curve

Growth curve was determined for the purpose of identifying growth phases. Then the deposition cultures at the end of doubling phase (exponential), Exponential phase on the ninth day of the algae, then harvested and dried [14].

Dry weight measurement

Dry cell weight was determined according to Rai et al. [15]. A known volume of algal culture was centrifuged at 5000 rpm for 10 min and the harvested biomass was dried at 40 C˚ for a period of 48 hours until to reach a constant weight.

Analytical methods

Sample of *S.quadricauda* were collected in the end of exponential phase ,for lipid extract cell were separated from the medium by centrifugation at 5000 rpm for 10 min. then , biomass was dried at 40C˚ for 2h , pulverized in a mortar and stored at -20C˚ for later analysis.Then, extracted using a mixture of chloroform: methanol (2:1,v/v).about 120 ml of solvents were used for every gram of dried sample in each extraction step. The solid phase was separated carefully using filter whatman paper Millipore filter 0.45µm . The solvent phase was evaporated in a

rotary evaporator under vacuum at 60C° and the procedure was repeated three times until entire lipid was extracted [16].Also, Kjeldahl method also adopted to determine the quantity of protein [17].

Statistical Analysis

The Statistical Analysis System- SAS [18] was used to effect of treatments in study parameters. Least significant difference –LSD test was used to significant compare between means in this study.

Results and Discussion

As show in Fig. 1, a lag phase could not be observed in the growth curve During the first three days, indicating that *S. quadricauda* can adapt well in the media culture ch-10, while Exponential phase began on the fourth day until the ninth day and end then went to the death phase Because of the exhaustion of nutrients in the media.

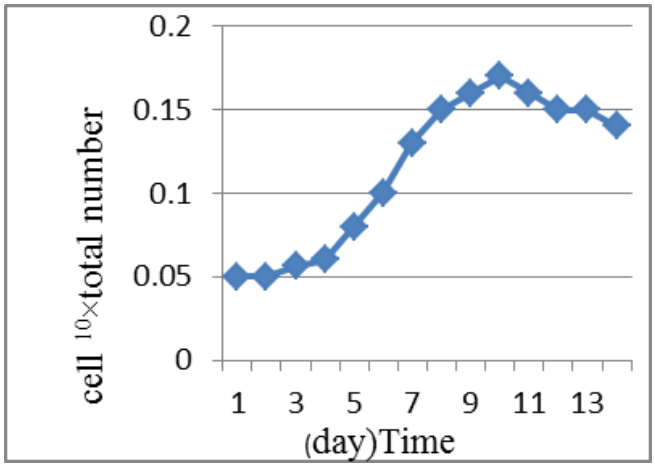

Figure 1: Growth curve of algae *S.quadricauda*

The results showed in this study, Growth and lipid accumulation under phosphorus and nitrogen deficiency is shown in Table 1. The S. *quadricauda* strains showed increase in lipid accumulation under the stress factors, nutrient deficiencies (50% N, 100% N, 50% N +50% P, 50% P). With nitrogen having more effect on biomass reduction than phosphorus.

It is known that the different nitrogen sources and levels were effective on the growth of microalgae and biochemical composition [19,

,20].Nutrient limitation is an efficient trigger to increase lipid content per unit algal biomass, as reported by many other researchers [21,22,23]. as cells grow and divide they require a supply of nitrogen. If the supply of nitrogen is limited in proportion to other elements, photosynthesis may continue but the resultant compounds will include a smaller proportion of nitrogen and more energy such as lipids [24].

Table 1. Effect of difference treatments in DW, Lipid and Protein content

Mean ±SE			Treatment
Protein(%)	**Lipid(%)**	**DW (mg/L)**	
42.8 ± 1.06	15.23 ± 0.62	0.356 ± 0.052	Control
20.3 ± 0.72	20.50 ± 0.94	0.246 ± 0.033	50 %N
16.8 ± 0.31	33.0 ± 1.07	0.157 ± 0.004	100 %N
23.3 ± 0.66	24.1 ± 0.59	0.229 ± 0.017	50 % N +P
30.8 ± 0.47	19.8 ± 0.62	0.180 ± 0.007	50 %P
* 5.084	* 3.792	0.1094	LSD value
(*P<0.05.)			

In this study, different nutritional combinations were tried on *S.quadricauda and* it was reported that the highest lipid accumulation was short of the 100% N. that the highest percentage of lipid content in the cell when free of nitrogen 100% in the medium, where record 33% of the lipid with a reduction in dry weight of 0.15 g/l in rural compared to control (suitable conditions) record dry weight 0.35 g/l and the lipid content of 15 %. Results also show when deficiency of nitrogen 50% plus phosphorus 50%,record dry weight of 0.22 grams/ L and lipid content 24.1 % (Fig. 2 and 3).

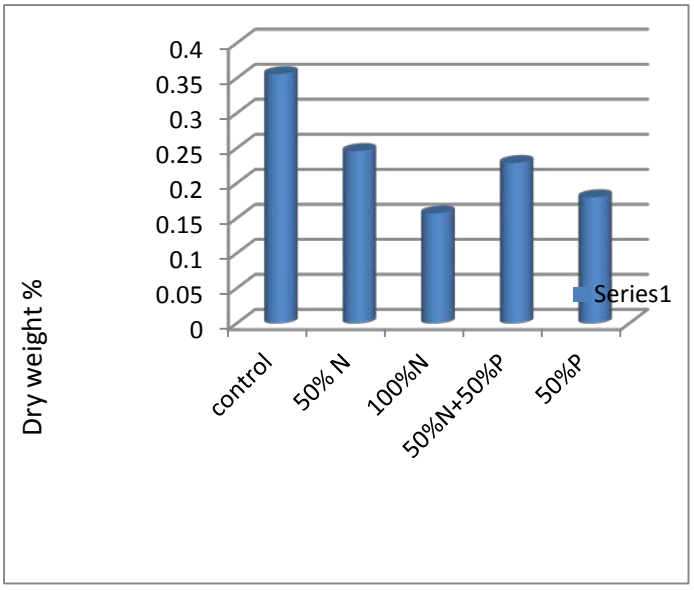

Figure 2: The effect of nutrients ratio on the dry weight(DW mg/L) on *Senedesmusquadricauda*

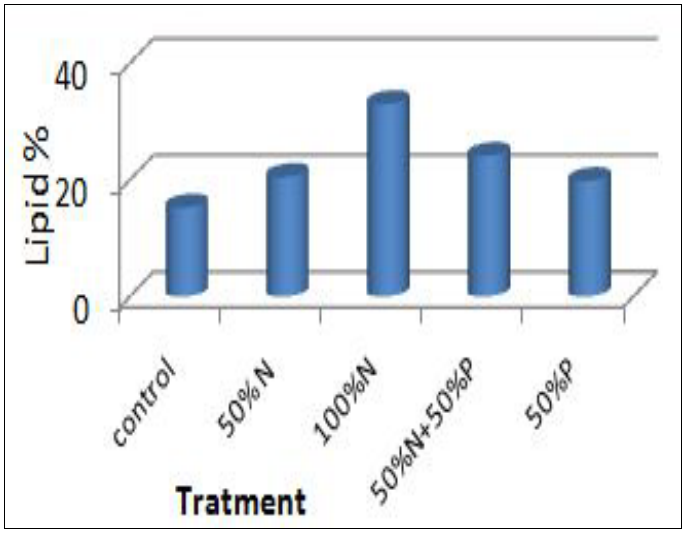

Figure 3: The effect of different nutrients ratio of the lipid content on *Senedesmusquadricauda*

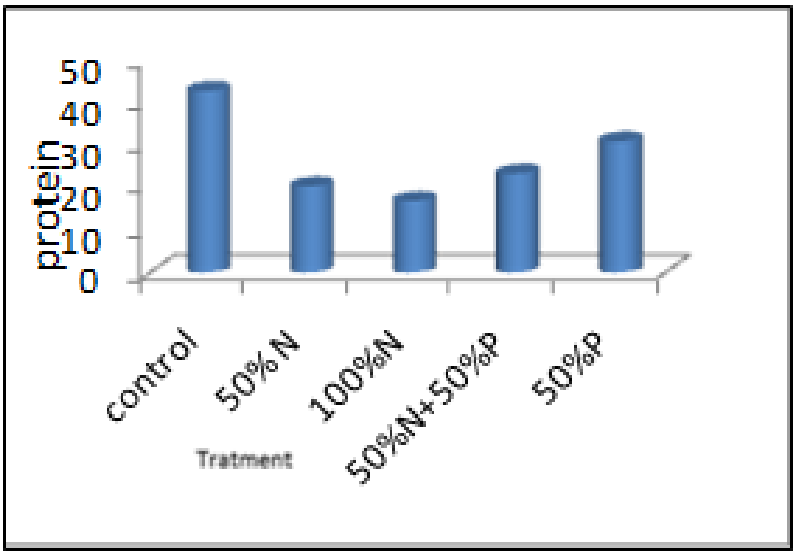

Figure 4:The effect of different nutrients ratio of the protein content on *Senedesmus quadricauda*

This could be ascribed to the fact that under nitrogen starvation, NADPH consumption was decreased due to unavailability of nitrogen pool, which blocks the amino acid synthesis pathways, thus resulting into accumulation of excess NADPH in the cells, The another possible reason could be that under nitrogen starvation, the carbon dioxide fixed is converted into carbohydrate or lipid rather than protein due to unavailability of nitrogen source [25, 26]. Under P-starvation, (at p50% p) dry Weight record for *S. quadricauda* 0.24 mg/l and content of lipid was 20.5%, This is consistent with Yasemin *et al.* [27] and Subramaniyan *et al.* [28] where lipid accumulation was enhanced when growth was restricted due to unavailability of phosphate.

In this study it found that the decline in the proportion of protein. In contrast increase lipids in all groups (50% and 100% nitrogen, 50% nitrogen plus 50% phosphorus and 50% phosphorus). Also It observed a higher level of protein is 42.8% and the corresponding the15.23% lipid content in the control treatment, while the lowest level of 16.8% protein and the corresponding 33% for lipid content when nitrogen deficiency 100%.

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Comparison of three methods (Microscopy, Immunochromatography and Real-time PCR technique) for the detection of *Giardia lamblia* and *Cryptosporidium parvum*

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Abstract: Giardiasis and Cryptosporidiosis are a significant health problem in countries with poor sanitation and unsafe water, human are infected by feco-oral route either directly or indirectly and in all age groups. Microscopical, immunochromatography and molecular assay are the most important methods for detection. To compare of direct microscopic examination with immunochromatograic test and real-time PCR assay for the detection of *Giardia lamblia* and *Cryptosporidium parvum*. We studied 180 children (93) females and (87) males, aged from 5 -15 years, who attended the out patients clinical of the Central Teaching Hospital for Pediatric in Baghdad city, during the period from June 2014 to October 2014, with gastrointestinal complaints. All specimens examined by light microscopy. Parasitic infection also examined by one step colored chromatographic immunoassay and real-time PCR technique. Out of one hundred eighty stool specimens, 95 had positive results of parasitic-infection while 85 with negative result. Most parasitic infection recorded in age group 5-10 years and in males, based on lab diagnosis 40% (38/95) of stool specimens were positive for *Giardia lamblia* and 4.21% (4/95) were positive for *Cryptosporidium parvum* microscopically, 40% (38/95) and 7.36% (7/95) of the stool specimens were found to be positive for *Giardia lamblia* and *Cryptosporidium parvum* respectively by immunochromatography assay. According to RT-PCR 47.36% (45/95) was positive for *Giardia lamblia* while 7.36% (7/95) was positive for *Cryptosporidium parvum*. Sensitivity and specificity of PCR was 100% compared to sensitivity of microscopy and immunochromatography assay. Microscopy exhibited many false positive and negative cases with two parasites. More sensitive and specific method for the detection of intestinal protozoa is molecular technique. It also offers the opportunity of familiarizing DNA detection in many laboratories and should be considered the gold standard methods for the diagnosis of parasitic disease.

Key word: Giardiasis, cryptosporidiosis, diarrhea, immunochromatograic test, real-time PCR.

مقارنة استخدام ثلاث طرق (المجهر الالكتروني، الاستشراب المناعي و تقنية الوقت الحقيقي لتفاعل سلسلة البلمرة) للكشف عن طفيلي الجيارديا لامبليا وطفيلي الابواغ الخبيئة

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الخلاصة: يعد داء الجيارديات وداء الابواغ الخبيئة من المشاكل الصحية العامة خصوصا في الدول التي تعيش تحت خط الفقر وتستخدم مياة شرب غير صالحة. الانسان يتعرض للاصابة بهذا المرض عن طريق التلوث بالبراز سوء كان بطريقة مباشرة او غير مباشرة، كل الفئات العمرية معرضة للاصابة ، الفحص المباشر ، الطرق المناعيه و الجزيئية من اهم الطرق المستخدمة للكشف عن كلا الطفيليين. في هذه الدراسة، تم مقارنة طرق مختلفة للكشف عن كلا الطفيليين، اذ تم دراسة 180 طفل، 93 (اناث) و 87 (ذكور) تراوحت اعمارهم بين 5-15 سنة، حضروا الى العيادة الخارجية في مستشفى الطفل المركزي في بغداد للفترة من حزيران 2014 ولغاية تشرين الاول 2014 كانو يعانو من الالم معدية معوية، تم تشخيص الاصابة باستخدام الفحص المباشر للبراز ومقارنتة بطريقة الاستشراب المناعي وتقنية الوقت الحقيقي لتفاعل سلسلة البلمرة، ضمن ال 180 عينة التي فحصت 95 عينة كانت موجبة لفحص الطفيليات بيمنا كانت 85 سالبة، معظم الاصابات سجلت في الفئة العمرية من 5-10 سنة وضمن مجموعة الذكور، اعتمادا على الفحص باستخدام المجهر اوضحت الدراسة ان نسبة الاصابة بالجيارديا لامبليا كانت 40% بينما سجلت 4.21% لطفيلي الابواغ الخبيئة، وكانت النسب 40%، 7,36% لكلا الطفيليين باستخدام طريقة الاستشراب المناعي. اعتمادا على تقنية الوقت الحقيقي لتفاعل سلسلة البلمرة فقد كانت نسبة الاصابة بطفيلي الجيارديا 47,36 بينما بلغت7,36% لطفيلي الابواغ الخبيئة. الحساسية والخصوصية كانت 100% بالنسبة لتقنية الوقت الحقيقي لتفاعل سلسلة البلمرة، مقارنة مع المجهر والاستشراب المناعي, اظهر فحص المجهر عدد من النتائج الخاطئة، وتعد الطرق الجزيئية من اهم الطرق للكشف عن كلا الطفيليين ويجب ان تضاف الى المختبرات لتشخيص الاصابات الطفيلية.

Introduction

Intestinal opportunistic parasitic infections are important causes of diarrhea which is a serious health problem in tropical regions. *Giardia spp*. and *Cryptosporidium spp*. are common parasitic causes of human diarrhea with the prevalence rate of 1% - 3% in the industrialized world and 4% - 17% in developing countries [1].

Giardia lamblia occurs by ingestion of cysts in contaminated food, drinking water or by faecal-oral route from person to person. The incubation time is between 1 to 3 weeks. The symptoms of giardiasis are acute or chronic diarrhea, but asymptomatic cyst elimination is also occurring. Acute symptoms include watery diarrhea, loss of appetite, nausea, abdominal cramps and weight loss [2].

Cryptosporidium parvum is one of several species of the genus Cryptosporidium that commonly causes cryptosporidiosis in human. Cryptosporidium was detected in up to 0.2 % of healthy individuals and

about 2 % of patients with diarrhea in developed countries. The infection occurs after the ingestion of oocysts in contaminated water and food, as well as by faecal-oral route from person to person. In immunocompetent persons the disease manifests itself after 2 to 10 days as a self-limiting watery diarrhea and may be accompanied by nausea, abdominal pain and weight loss. Immunocompromised individuals often develop serious, chronic, and sometimes fatal illness [3].

Diagnosis of these parasites is usually performed by microscopy. However, microscopy lacks sensitivity and specify. Replacing microscopy with more sensitive and specific nucleic acid baserd methods is hampered by higher costs, in particular in developing countries [4].

Some researchers which revealed that enzyme-linked immunosorbent assay (ELISA), direct fluorescent-antibody assay (DFA) and polymerase chain reaction (PCR) are more specific and sensitive alternative methods so basically have been introduced for all three of these parasitic infections but more expensive compared with direct microscopic examination [5].

So basically, the present study was aims to evaluate a different methods use for detection of *Giardia lamblia* and *Cryptosporidium parvum* in stool specimens and related with age and gender.

Patients and Methods:

Study population

We collect one hundred eighty stool samples in a clean, dry, tight fit cover from children, (93) was females and (87) was males, aged from 5 -15 years, who attended the out patients clinical of the central teaching hospital for pediatric in Baghdad city during the period from June 2014 to October 2014**.**

Microscopic Examination

Each specimen examined within half hour in microbiology laboratory at hospital by direct wet mount methods with normal saline and lugols iodine for the detection trophozoite and cyst stage of *G. lamblia* [6]. And modified acid fast staining was performed for oocyte of *Cryptosporidium parvum* [8].

Immunochromatograic test

The Cer Test Crypto-Giardia card is a one-step colored chromatographic immunoassay (E-50018 Zaragoza- Spain) used for detection of *Giardia lamblia* and

Cryptosporidium parvum in stool samples which consist of two strips, **strip A** consist of a nitrocellulose membrane pre-coated with mouse monoclonal antibodies on the test line (T), in the results window, against *Cryptosporidium* and rabbit polyclonal antibodies, on the control line (C), against a specific protein. While **strip B** consist of a nitrocellulose membrane pre-coated with mouse monoclonal antibodies on the test line (T), in the results window, against *Giardia* and rabbit polyclonal antibodies, on the control line (C), against a specific protein. This technique was performed according to manufacturer's instructions. In brief stool samples were collected in clean containers, take out the cap of the stool collection tube to collect faecal sample and add it to the stool collection tube then small amount of liquid was add to tube for liquefaction tube was closed. After shaking 4 drops putted in the circular window marker with letter A and 4 drops was putted in the circular window with letter B marked. Finally the result read according to manufacturer's instructions

Extraction of DNA from Stool

Giardia lamblia DNA and *Cryptosporidium parvum* DNA were isolated from purified *Giardia lamblia* cyst and *Cryptosporidium parvum* oocysts, according to the manufacturer's instruction (ZR fecal DNA MiniPrep^{TM} - Cat. No. D6010, USA). In brief 150 mg of fecal suspension was added to 750 µl of lysis buffer solution, followed by bead beating, then mixture was mixed and centrifuge at ≥10000xg for 1 minute for separation, after centrifugation 400 µl of supernatant was collected and centrifuge again at 7000 rpm for 1 minute, then solution was filter by used fecal DNA binding buffer., DNA was isolated and purified using fast-spin column technology for downstream molecular-based application (real time PCR).

Real Time PCR

All reagents and standard template Target specific for positive control and negative control was prepare according to the manufacturer's instructions (Taget Species dtec-RT-Qpcc test, Spain). Briefly, the reaction pre-mix reverse was prepare in a sterile 1.5ml tube on ice and consist of the follwing 4µl of the lyomix RT-qPCR mixed with 1µl of Target species dtec-RT-qPCR and DNase/RNase free water a final volume of 15µl. Then 5 µl of diluted standard template was added to each PCR tube to reach a final PCR

volume of 20µl. Amplification was done by 10 minutes at 50°C for retro transcription and 1 minutes at 95°C , for activation followed by 45 cycles of denaturation at 50°C for 10 minutes, hybridization, extention, data collection at 60°C for 60 Second**.**

Statistical analysis

Date was analyzed using statistical program for social sciences (SPSS) for windows 11.0. Differences in proportions were assessed by Chi-square test. P values < 0.05 were considered statistically significant.

Result

Out of total 180 enrolled cases, parasitic infection were found in 52.77% (95/180) of cases and non-parasitic infection were found in 47.22% (85/180) of cases as shown in table (1).

Table (1): Distribution of study group infected with diarrhea according to causative agent

Organisms	Frequency	Percentage
positive for two Parasite (diarrhea only)	95	52.77%
Negative for two Parasite (diarrhea only)	85	47.22%
Total	180	99.99%

Chi-square value 1.037- Non- significant

Table (2) summarizes the patients result of 52 cases included for detection of *Giardia lamblia* and *Cryptosporidium parvum* by use three methods, direct microscopic examination, immunochromatograic test and real time PCR, while other parasite not included. There were no samples containing co-parasitic infection.

Table (2): Distribution of study group suffering from diarrhea according to the type of diagnostic methods

Parasite	Microscopic Examination		Immunochromatograic test		One step real time PCR
Giardia lamblia	38(40%)		38(40%)		45(47.36%)
Cryptosporidium parvum	4 (4.21%)		7(7.36%)		7(7.36%)
Total	42		45		52

Chi-square value 12.084 – Non significant

The age range of the patients was 5-15 years, the group examined for *Giardia lamblia* and *Cryptosporidium parvum* by three methods which revealed that most infection occur in age group of 5-10 years with two parasites, but statistically non-significant with *Giardia lamblia,* while significant with *Cryptosporidium parvum* as shown in table (3) and (4).

Table (3): Prevalence of giardiasis according to age group

Age groups	Microscopic Examination		Immunochromatograic test		Real time PCR	
5-10 years	20(52.63%)		20(52.63%)		24(53.33%)	
11-15 years	18(47.36%)		18(47.36%)		21(46.66%)	
Total	38(99.99%)		38(99.99%)		45(99.99%)	

Non- significant P>0.05

Table (4): Prevalence of cryptosporidiosis according to age group

Age groups	Microscopic Examination		Immunochromatograic test		Real time PCR	
5-10 years	3(75%)		6(85.71%)		6(85.71%)	
11-15 years	1 (25%)		1 (14.29%)		1(14.29%)	
Total	4(100%)		7(99.99%)		7(99.99%)	

Significant **P<0.05**

Highest prevalence of parasitic infection was found in males than females, but without significant differences between them in giardiasis, while in cryptosporidiosis the difference was significant table 5 and 6.

Table (5): Prevalence of giardiasis according to gender by different diagnostic method

Gender	Microscopic Examination	Immunochromatographic test	Real time PCR
Males	21(55.26%)	21(55.26%)	25(55.55%)
Females	17(44.73%)	17(44.73%)	20(44.44%)
Total	38(99.99%)	38(99.99%)	45(99.99%)

Non- significant P>0.05

Table (6): Prevalence of cryptosporidiosis according to gender by different diagnostic method

Gender	Microscopic Examination	Immunochromatographic test	Real time PCR
Males	3(75%)	5(71.43%)	5(71.43%)
Females	1 (25%)	2(28.57%)	2(28.57%)
Total	4(100%)	7(100%)	7(100%)

Significant P<0.05

In comparison with direct microscopic examination, the chromatographic immunoassay had a sensitivity of 84.44% (38/45) vs. 100% (7/7), whereas the one step real time PCR assay had sensitivity of 100% (45/45) vs. 100% (7/7) for the detection of *Giardia lamblia* and *Cryptosporidium parvum* as show in in table (7).

Table (7): Sensitivity and Specifity of diagnostic methods

Method	Microscopic Examination		Immunochromatograic test		Real time PCR	
	Sensitivity %	specificity %	Sensitivity %	specificity %	Sensitivity %	specificity %
Giardia lamblia	84	100	84	100	100	100
Cryptosporidium parvum	70	100	100	100	100	100

Discussion

Enteric protozoa remain to be the most commonly encountered parasitic diseases and to cause significant morbidity and mortality throughout both developed and developing regions of the world, affecting millions of people each year [8].

In 85(47.22%), a non-parasite faecal pathogen was demonstrated, while 95 of cases with parasitic infection, routine testing revealed *Giardia lamblia* rate was (40%), while *Cryptosporidium parvum* was (4.2%) and other different type of parasite. This result is agreed with that of [9] who found the intestinal parasite with the highest prevalence is *Giardia lamblia*, followed by *Cryptosporidium parvum/ Cryptosporidium hominis*. But this result is differing from those of [10] who reported that *Cryptosporidium* was detected in 15.7% of the diarrhoeic patients, while *Giardia* was detected in 4.6%. This may be related with difference in study area.

Immunochromatographic assay and real time PCR has been described previously for the simultaneous detection of *Giardia lamblia* and *Cryptosporidium parvum* in faecal samples [11, 12]. In the present study, the results obtained using these methods were compared retrospectively with the results obtained by routine microscopy in clinical laboratory practice for patients with diarrhea.

Real-time PCR had a similar sensitivity and specificity compared with the chromatographic immunoassay assay for detection of *Cryptosporidium parvum*, but different result occurs with *Giardia lamblia*. This may be related with non-specificity of antibody based methods owing to cross-reactivity with other microorganisms and low

sensitivity is reported to be problematic [13].

The PCR assay developed for detection of different intestinal protozoan parasites can be easily used for two parasites or more without any loss of sensitivity and specificity. Detection of parasite-specific DNA by PCR is more sensitive than microscopy [14, 15, 16].

Molecular methods such as PCR have proven to be highly sensitive and specific for the detection of *E. histolytica / E. dispar, G. lamblia* and *C. parvum / C. hominis* infections [17, 18]. Their use in routine diagnostic laboratories is still very limited. The introduction of molecular methods has been hindered by time-consuming methods for the isolation of DNA from faecal specimens and the presence of inhibitory substances in such samples. Furthermore, amplification of DNA was previously laborious and expensive, and cross contamination among samples was a notorious problem. However, newly developed methods have greatly reduced these obstacles [19]. Real time PCR reduces labour time, reagent costs and the risk of cross-contamination, and offers the possibility of detecting multiple targets in a single multiplex reaction [20].

According to age group, we showed that infection with of *Giardia lamblia* was more common in age group 5-10 years. This result agreement with [21] who found that the maximum infection rate was in age group less than 10 years (51.61%). This result may be due to the poor hygienic inhabits of children beside the other socioeconomic conditions and immune status. The same age group was also sensitive to *Cryptosporidium parvum* infection; this is agreed with that of [22] who revealed that, the prevalence rate was 13.51% with a peak among the age group (5-10)

Result showed that infection rate among males was higher than females. This result is agreed with that of [23] and [24] who found that there was no significant differences between infection in males and females. While result is differ from those of [25] who found that infection rates of giardiasis were 55% and 44.9% in males and females respectively, also [26]. As well as, result is differing from those of [27] who found that the rate of infection for females was higher (19%) than in males (16%). Regarding *Cryptosporidium parvum* infection study done by [22], who demonstrated significant relation between males and females.

Conclusion

In comparison with immunochromatography assay and real-time PCR assay. Microscopy exhibited many false positive and negative cases with two parasites. More sensitive and specific method for the detection of intestinal protozoa is molecular technique. It also offers the opportunity of familiarizing DNA detection in many laboratories and should be considered the gold standard methods for the diagnosis of parasitic disease

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