

**Effect of Ethanol Extract of Radish (*Raphanus sativus* L.)
Seeds and *Lactobacillus acidophilus* on Enteropathogenic
Escherichia coli *in vitro* and *in vivo*.**

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Abstract

The antibacterial activity of each of ethanol extract of radish seeds and *L. acidophilus* against Enteropathogenic *E. coli* was studied using well diffusion method (*in vitro*) and mice (*in vivo*), The growth kinetics of mixed culture of two bacterial species were also studied.

The results showed a strong antibacterial activity against *E. coli* for each of ethanol extract of radish seeds (*R. sativus*) and *L. acidophilus* . It was found that the diameters of inhibition zones were 28 and 25mm. respectively .It was found also that the *L. acidophilus* inhibits the growth of *E.coli* in mixed culture, and the inhibition begun after 36 hr. but after 72 hr. there was no any growth of *E. coli*.

The histopathological study of intestine and liver in mice revealed that the ethanol extract and *L. acidophilus* exhibited inhibition in the growth of *E. coli* and prevent any inflammation or harmful histological changes, Such findings confirms the beneficial effects of *Lactobacillus* as probiotic and that ethanol extract of radish seeds as treatment compound for diarrheal diseases caused by bacterial pathogens. It was concluded that the extract and *Lactobacillus* holds great promise as antimicrobial and anti-inflammatory therapeutic

Introduction

For a long period of time, plants have been a valuable source of natural products for maintaining human health (26).

Raphanus sativus L. belong to the family Brassicaceae and It's common name is Radish, leafy Daikon and Fodder Radish. Radishes have long been grown as a food crop, but they also have various medicinal actions (5). The leaves, seeds and old roots are used in the treatment of asthma and other chest complaints. The seeds are taken internally in the treatment of indigestion, abdominal bloating, acid regurgitation, diarrhea and bronchitis (4). It is also active against many food born pathogenic and food spoilage bacteria (28).

Escherichia coli belong to the normal intestinal micro flora of humans. Pathogenic strains are characterized by the ability to produce toxins. Diarrhea is caused by enteropathogenic *E.coli* strains which are resistant to high range of antibiotics (14). Probiotics microorganisms are defined as viable nutritional agents confirming benefits to the health of the host (21). There are many evidences confirmed that administration of selected microorganisms are beneficial in the prevention and treatment of ceratin intestinal infections (6). *Lactobacillus* regarded as one of the most important probiotics to limit the course of acute diarrhea (9).

The purpose of the present study was to investigate the antibacterial activity of radish (*Raphanus sativus* L.) seeds and *L. acidophilus* against enteropathogenic *E.coli* by well diffusion assay and in mixed culture, as well as in mice.

Materials and Methods

Plant material:

Radish seeds used in this study were collected from the local Iraqi markets in Baghdad and Identified as *Raphanus sativus* by Dr. Ali Almosawy from Biology Department, College of Science.

Test microorganisms:

The bacterial isolates used in this study (Enteropathogenic *Escherichia coli* and *Lactobacillus acidophilus*) were obtained from the stock culters of Institute of Genetic Engineering and Biotechnology for Post Graduate Studies.

Preparation and Standardization of Inoculums:

Four to five colonies from pure culture of the test bacteria were transferred to 5 ml of Mueller Hinton Broth (MHB). The broth was incubated at 37°C for 24 hr. The turbidity of the culture was compared to 0.5 Mcfarland Nephelometer standard to get 150×10^6 CFU/ml (23).

Preparation of Plant extract:

The ethanol extract of *R. sativus* seeds was done according to Parekh *et al.* (17). Ten grams of dried seeds were crushed in electrical grinder. Crushed seeds were extracted with 100 ml of 80% ethanol kept on rotary shaker to 24 hr. Thereafter, it was filtered through eight layered Muslin cloth then through Whatman No.1 filter paper, and centrifuged at 5000 rpm for 15 min. The supernatant was collected and the solvent was evaporated at 40°C by rotary evaporator to dryness with the aid of vacuum, and weighed.

Antimicrobial activity of extract:

Antimicrobial activity (*in vitro*) was performed by well diffusion assay. Mueller Hinton Agar (MHA) plates were seeded with 0.1 ml of standardized inoculums of the test microorganism. The inoculums was spreader evenly over plates with a glass spreader. The seeded plates were allowed to dry in the incubator at 37°C for 20 minutes. A cork poorer of 6 mm was used to cut uniform wells on the surface of MHA. Stock solutions were obtained by dissolving 1g of extract in 10 ml of 5% DMSO. 100 µl of extract was introduced in the wells (this test was performed at five concentration 2.5, 5, 10, 20 and 40 mg/ml). The inoculated plates were incubated at 37°C for 24 hr. The zone of inhibition was measured to the nearest millimeter (mm). Antibiotic Streptomycin (30 µg/ml) was used as positive control while the solvent (5% DMSO) was used as a negative control. Each experiment was performed in triplicate (20).

Antibacterial activity of *L. acidophilus*:

The antimicrobial activity of *L. acidophilus* (cell-free filtrate) against *E. coli* (*in vitro*) was performed by well diffusion assay. *L. acidophilus* was inoculated in MRS broth at 37°C for 24 hr. Activated culture was centrifuged at 4000 rpm for 15 min and the clear supernatant was sterilized by filtration (0.45 µ) to obtain cell-Free filtrate.

Pathogenic test bacteria were incubated in BHI broth at 37°C for 24 hr.

Petri dishes containing 20 ml of Mueller Hinton agar were prepared previously and inoculated with 0.1 ml of broth culture of pathogenic bacteria. After solidified, dishes were incubated for 24 hr. in a refrigerator. 4 wells were made in each dish and filled with 100 µl of cell- free filtrate of *L. acidophilus* and incubated at 37°C for 24 hr. The diameters of the inhibition zone were measured in mm. (11)

Growth kinetics in mixed culture:

Associative growth of *E. coli* and *L. acidophilus* was done in sterilized skim milk at 30°C. Each strain was inoculated in 10 ml of a sterile skim milk (10% w/v). 10 ml of mixed culture was distributed in sterile media. The growth kinetics are carried out simultaneously with the regular length of time intervals. *E. coli* isolate was used as test which is inoculated in skim milk at 2% to give approximately 5×10^3 CFU/ml, in pure and mixed culture with *L. acidophilus* (8×10^4 CFU/ml). Antimicrobial effect of *L. acidophilus* against *E. coli* was measured by preparing decimal dilutions in sterile physiological saline, then 0.1 ml of appropriate dilutions were spread in duplicate on selective agar plates (13).

Experimental Animal test:

Thirty adult male mice obtained from the animal house of Institute of Genetic Engineering and Biotechnology for Post Graduate Studies were used and housed in stainless-steel cages under strict hygienic conditions at 25-28 °C (8-20) hr. light and free access to feed and water. Animals were divided into 4 groups (6 in each) as following:

First group was used as a negative control group that injected intra peritoneally (i.p.) with normal saline (0.1 ml/ mice) at intervals parallel to the treated groups. The second group was injected i.p with Enteropathogenic *E. coli* (0.1 ml/ mice) (1.5×10^8 CFU/ml) as a positive control. Third group was injected with *E. coli* as the second group but synergistically received orally with ethanol extract of *R. sativus* L. seeds 0.1 ml (120 mg/kg body weight). The last group was injected as a second group but synergistically received orally 0.1 ml containing 10^7 (CFU/ml) *L. acidophilus*. After daily treatment with *R. sativus* and *L. acidophilus* (for 15 days), mice were killed by decapitation after ether anesthesia (25).

Histopathology:

Livers and intestines of all animals were harvested and fixed in 10% buffered formalin into labeled bottles. Tissues were processed routinely and embedded in Paraffin wax. Section of 5 micron thick were cut, stained with haematoxylin and eosin and examined under light microscope (11).

Results and Discussion:

Results of antibacterial activity of ethanol extract of *R. sativus* L. seeds and *L. acidophilus* (cell-free filtrate) on enteropathogenic *E.coli* are shown in table(1)

Table (1): Antibacterial activity of ethanol extract of *R.sativus* L. seeds and *L. acidophilus* on enteropathogenic *E.coli*.

Pathogenic Bacteria	Diameter of Inhibition Zone in(mm)*						
	Ethanol extract of <i>R. sativus</i> seeds					<i>L. acidophilus</i>	Streptomycin antibiotic (30 µg/ml)
	2.5mg/ml	5mg/ml	10mg/ml	20mg/ml	40mg/ml		
Enteropathogenic <i>E. coli</i>	10	10	17	23	28	25	27

*Diameter of Inhibition zone included the well (6 mm),

** The result present 3 duplicates.

Results indicates that the ethanol extract radish seeds have been strongly active against *E. coli* especially at concentration 40 mg/ml with highest inhibition zone (28mm), in comparison with the antibiotic Streptomycin. It was also found the similar result with cell-free filtrate of *L. acidophilus* , but with an inhibition zone of 25 mm. It was observed that the lower concentrations of ethanol extract (10mg/ml and 20mg/ml) was also exhibit moderately antibacterial activity (17 and 23mm), respectively.

Present findings are in a fair correlation with the study carried out by Abdou *et al.* (1) who recorded that crude juice of radish (*R.sativus*) was found to be strongly active against *E.coli*, *Salmonella typhi* and *Bacillus subtilis*. The methanolic extract and crude water extract of *R. sativus* seeds displayed highest antimicrobial activity against *Enterobacter*, *Lactobacillus* and *Bacillus* (20). In contrary,

some researchers found that different species of bacteria such as *Bacillus*, *S. typhi*, *Micrococcus*, *E. coli* and *Niesseria* were resistant to the juice of white radish (24). In another study radish was found to contain raphanin which is an antibacterial and antifungal (19).

Strain of *Lactobacillus* isolated from cow milk samples in Nigeria was tested to inhibit growth of some pathogenic bacteria, Results indicated the inhibitory effect on *E. coli* and *Pseudomonas aeruginosa* (15). The antagonistic activity of Lactobacilli may be due to production of organic acid resulting in pH decrease. Lactic acid bacteria have been shown to inhibit (*in vitro*) growth of many enteric pathogens and used in both humans and animals to treat a broad range of gastrointestinal disorders (16; 22).

Results of growth kinetics of mixed culture of *E. coli* and *L. acidophilus* were summarized in (Figure, 1).

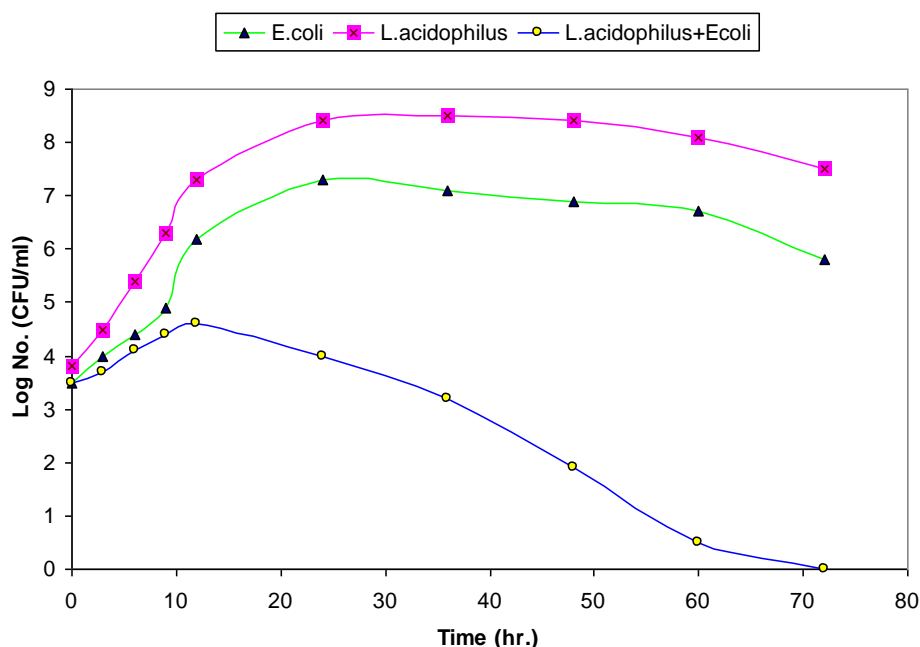


Figure (1): Growth of Enteropathogenic *E. coli* in absence and presence of *L. acidophilus* in Skim milk.

The figure shows that no growth was occurred for *E. coli* after 72 hr. of incubation in the presence of *L. acidophilus*. The initial numbers of *L. acidophilus* and *E. coli* were 8×10^3 CFU/ml and 5×10^3 CFU/ml respectively, After 24 hr. of incubation , number of *L. acidophilus* was 1×10^8 CFU/ml and the number of *E. coli* was 3×10^7 CFU/ml in the pure culture while in mixed cultures the number of *E. coli* was reduced to 1×10^4 CFU/ml. after 24hr. incubation.

The study of Godiso *et al.* (7) revealed significant reduction of enterotoxigenic *E. coli* from 32×10^3 CFU/ml to 0.8×10^3 CFU/ml after 60 min. in mixed culture of Yakult drink in the presence of *L. casei*. Three isolates of *Lactobacillus* demonstrated an inhibitory effect on *E. coli*, *Listeria monocytogenes* and *S. enterica* and the antibacterial agents are expected to be peptidic antibacterial agent such as bacteriocin (12).

Fig.2 (A and B) shows the histopathological section of mice intestines treated with enteropathogenic *E. coli*(Positive control) . It illustrates that necrosis was detected in the intestinal villi involving intestinal glands with bleeding, while there were no observable microscopic lesions in the intestine of the (negative) control group(Fig.3.A). The intestines of all the animals infected with *E. coli* and treated with *R. sativus* extract (Fig. 2.B) and *L. acidophilus* (fig. 3.C) were also appeared to be normal without observable microscopic lesions.

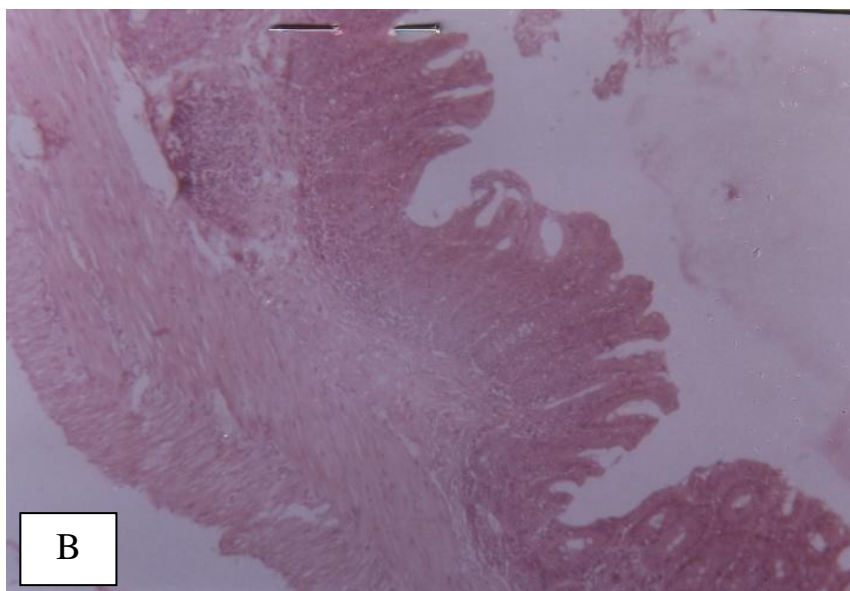
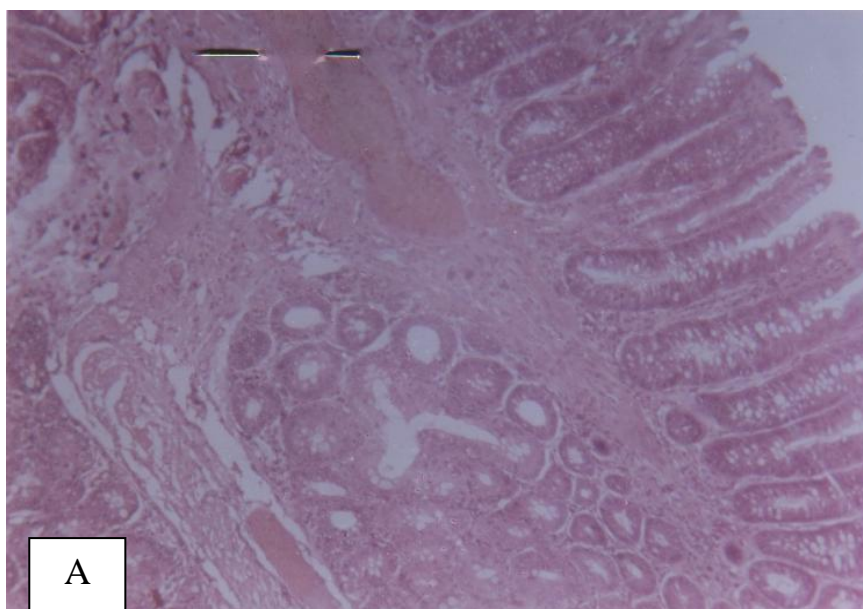
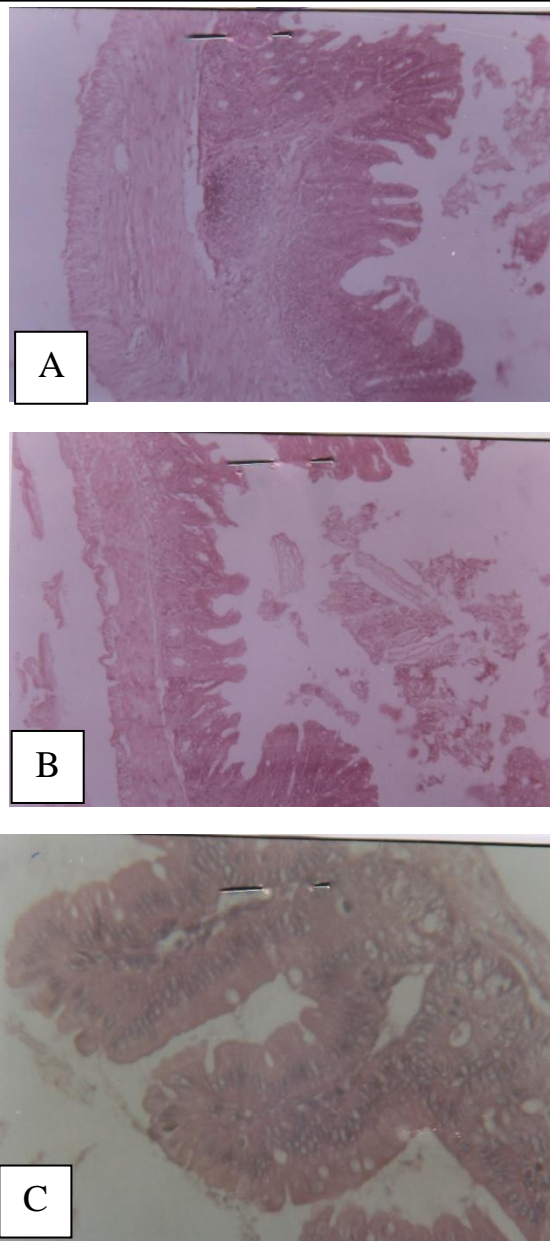
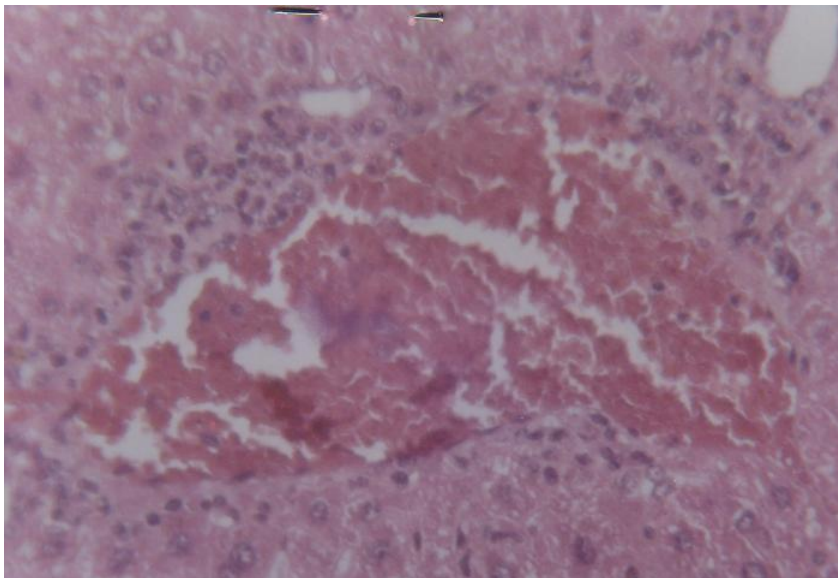


Figure (2): Histopathological section of small intestine from mice treated with enteropathogenic *E. coli* , stained with hematoxylin and eosin under light microscope(x10).
(A) Shows bleeding or congestion of blood vessels (B) shows necrosis in villi involving intestinal glands.

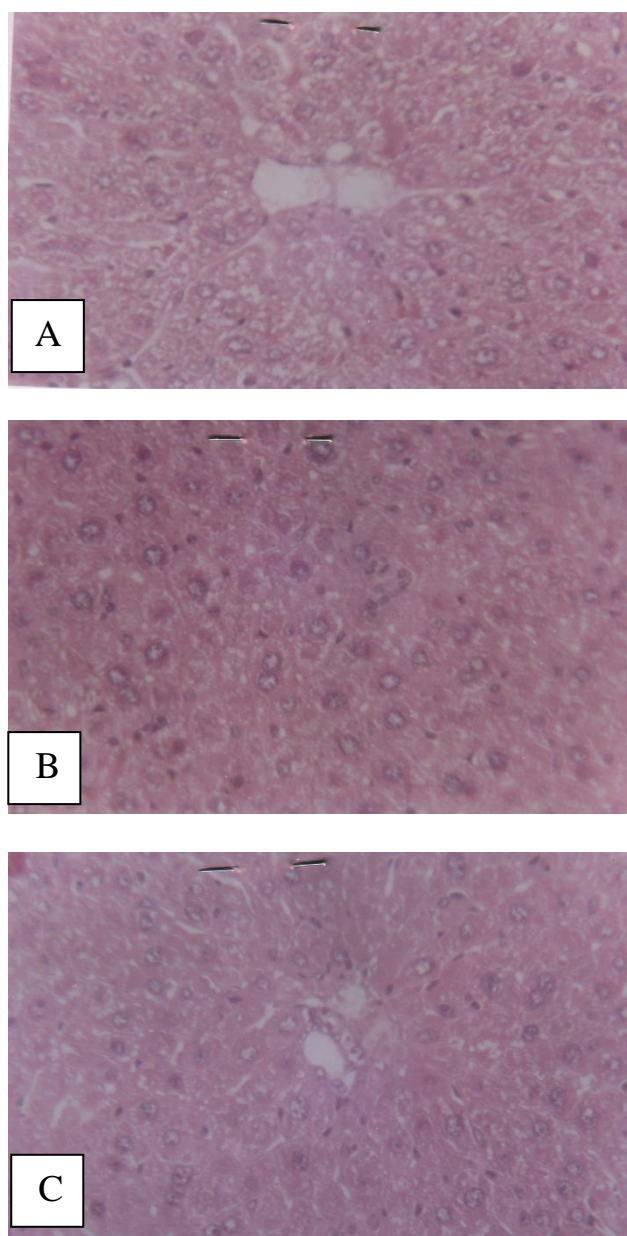


Figure, (3): Histopathological section of small intestine from mice, stained with hematoxylin and eosin under light microscope .**(A)** Treated with normal saline (Control group); Normal villi , intestinal glands and duodenal gland (x10). **(B)** Treated with *E. coli* and *R. sativus* extract. Normal villi, intestinal glands and duodenal gland (x10). **(C)** Treated with *E. coli* and *L. acidophilus* .Normal villi, intestinal glands and duodenal gland (x10).

The bacteria infected group showed necrosis of the hepatic cells of the liver. The polymorphonuclear lenkocytes appeared in the lesions(Fig.4), while in Fig. 5(A, B and C) which represent the control group and treated groups , liver parenchyma was in good morphology, hepatocytes were arranged around the central vein, no inflammation or necrosis was noticed.



Figure, (4): Histopatholoical section of the liver from mice treated with *E. coli*, stained with hematoxylin and eosin under light microscope (x40). Necrosis of the hepatic cells was noticed.



Figure, (5): Histopathological section of the liver from mice, stained with hematoxylin and eosin under light microscope. (A) Control group, Note the central vein (x10). (B) Treated with *E. coli* and *L. acidophilus* (x40). (C) Treated with *E. coli* and *R. sativus* extract (x40).

Previous studies indicate that fresh juice of Radish possesses gastro protective potential related to the mucous secretion stimulation (potentiation of defensive factors), and the increase in NP-SH (Non-protein Sulfhydryl) concentration is probably due to the prostroglanidin- inducing abilities , mediated through it's antioxidant activity (2).

In the present study treatment with *R. sativus* extract and *L. acidophilus* was significantly and substantially prevented the histopathological changes of the intestine and liver. It was concluded that the extract and *Lactobacillus* possess great promise as antimicrobial and antiinflammatory therapeutics. The histopathological photos indicate that lactic acid bacteria have significant role in stimulation of defense mechanism which is capable to overcome the pathogenic effect of *E. coli* as Perdigon *et al.* (18) who returned that different strains of *Lactobacillus* and *Streptococcus thermophilus* were capable of stimulating non- specific immunity (macrophages) and specific immunity (lymphocyte B and T) in mice.

Hedault *et al.* (8) confirmed that supernatant of *L. casei* is able to prevent the invasion of Caco-2- cells by *S. typhimurium*. Lactic acid bacteria secretes anti-inflammatory metabolites, such as lipoteichoic acids from *L. johnsonii* and *L. acidophilus* antagonize the responsiveness of human intestinal epithelial cell to lipopolysaccharide(27). A number of researchers using animal models and many clinical studies in humans have confirmed the benifical effects of lactic acid bacteria on diarrheal disease by multiple mechanisms, the transient passage of lactic acid bacteria in the digestive tract may be represent a microbial barrier against the development of pathogenic bacteria, probably due to the release of compounds contributing to maintenance of colonization resistance of pathogens (2; 3; 10).

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تأثير المستخلص الأيثانولي لبذور نبات الفجل *Raphanus sativus* L. وبكتريا *Lactobacillus acidophilus* في بكتريا *Escherichia coli* الممرضة خارج وداخل الجسم الحي.

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الخلاصة

درست الفعالية المضادة لكل من المستخلص الايثانولي لبذور نبات الفجل *R. sativus* وبكتريا *L. acidophilus* تجاه بكتريا *E. coli* الممرضة باستخدام طريقة الانتشار في الحفر وفي الفئران كما درست حركات النمو للمزروع المختلط لنوعي البكتريا.

أظهرت النتائج وجود فعالية مضادة قوية لكل من المستخلص الايثانولي لبذور نبات الفجل وبكتريا *L. acidophilus* تجاه بكتريا *E. coli* الممرضة إذ وصل قطر منطقة التثبيط (28) و(25) ملمتر. كما أن المزرعة المختلطة لكل من نوعي البكتريا أظهرت تأثيراً مثبطاً من قبل بكتريا *L. acidophilus* تجاه بكتريا *E. coli* حيث بدأ التثبيط واضحاً بعد 36 ساعة من النمو ولم يكن هنالك أي نمو لبكتريا *E. coli* بعد 72 ساعة.

بينت الدراسة النسيجية لكل من الأمعاء والكبد في الفئران أن كل من المستخلص الايثانولي وبكتريا *L. acidophilus* أحدثتا تثبيطاً لنمو بكتريا *E. coli* ومن ثم منعنا حدوث الالتهاب أو أية تغييرات نسيجية ضارة. مما يعكس التأثير المفيد لبكتريا *Lactobacillus* كعلاج حيوي والمستخلص الأيثانولي لبذور نبات الفجل كمادة علاجية للحد من حالات الاسهال الناتج عن البكتريا الممرضة.