ISSN: 0067-2904
GIF: 0.851

Antibacterial and Antibiofilm Activity of Flaxseed Oil

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Abstract:
The present study aimed to explore the antibacterial and antibiofilm activity of flaxseed oil on some locally isolated bacterial pathogens. No inhibitory effect was noticed against *Escherichia coli* or *Enterococcus faecalis*. However, variable effects were developed against Methicillin resistant *Staphylococcus aureus* (MRSA), methicillin sensitive *Staphylococcus aureus* (MSSA), *Klebsiella pneuminae* and *Staphylococcus epidermidis*. The flaxseed exhibited antibiofilm activity against all tested bacterial isolates (MSSA, MRSA, *S. epidermidis* and *K. pneumoniae*) and showed various degrees of inhibition against them. Experimental wounds were healed by application of flaxseed oil. In conclusion**,** flaxseed oil is a good alternative medication can be used to treat wound infection caused by bacteria.

Keywords: Flaxseed,MSSA,MRSA,Antibacterial ,Antibiofilm

الفعالية الضد بكتيرية و ضد الغشاء الحياتي لزيت بذور الكتان

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الخلاصة
هدفت هذه الدراسة لايضاح الفعالية الضد بكتيرية وضد الغشاء الحياتي لزيت بذور الكتان على بعض الممرضات البكتيرية المعزولة محليا.لم تلحظ اية فعالية تثبيطية ضد بكتريا الايشيريكية القولونية او المكورات المعوية البرازية. في حين كانت هناك تاثيرات متغايرة ضد المكورات العنقودية الذهبية المقاومة للمثيسيلين و المكورات العنقودية الحساسة للمثيسلين و الكليبسيلا الرئوية و المكورات العنقودية البشروية. اظهرت بذور الكتان فعالية ضد الغشاء الحياتي للبكتريا قيد الدراسة (المكورات الذهبية المقاومة للمثيسيلين و المكورات العنقودية الحساسة للمثيسلين و الكليبسيلا الرئوية و المكورات العنقودية البشروية) و بدرجات تثبيط متفاوتة. تم شفاء الجروح التجريبية بفعل زيت بذور الكتان.تم الاستنتاج من الدراسة ان زيت بذور الكتان من البدائل العلاجية الناجحة و من الممكن ان يستعمل لعلاج خمج الجروح المصابة بالبكتريا قيد الدراسة.

Introduction

Infectious disease caused by bacteria, viruses and fungi are still a serious problem in public health. In Iraq it is estimated that over 90% of bacterial pathogens are resistant to antibiotics [1-4].The increase in multidrug resistance of pathogenic bacteria has led to an urgent need for identifying alternative strategies to counter bacterial infection [5]. A biofilm is a structured consortium of bacteria embedded in a self-produced polymer matrix consisting of polysaccharide, protein and DNA. Bacterial biofilms cause chronic infections because they show increased tolerance to antibiotics and disinfectant chemicals as well as resisting phagocytosis and other components of the body's defence system [6]. The latest researches have been focused on identifying the potential antimicrobial agents from the natural resources. The antimicrobial activity of plant extracts has been known for many years, as plants are known to produce useful antimicrobial phytochemicals [7].

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Flaxseed is the seed from the flax plant (*Linumusitatissimum* L.), which is a member of the Linaceae family. The plant is not a new crop and native to West Asia and the Mediterranean [8].
Flax plant has a long history of traditional use both as a source of oil and fibre and is grown for commercial use in over 30 countries of the world. There has been a growing interest in the probiotic properties of flax and in its beneficial effects on coronary heart disease, some kinds of cancer and neurological and hormonal disorders [9-11].

Flaxseed is rich in fat, protein and dietary fibre. Chemical analysis of flaxseed averaged 30 to 40% oil, 20 to 25% protein, 20 to 28% total dietary fibre, 4 to 8% moisture and 3 to 4% ash and the oil contains vitamins A, B, D and E, minerals and amino acids. By virtue of the presence of physiologically active food components that may provide health benefits beyond basic nutrition, flaxseed is often grouped into one of several categories: ‘‘functional food’’, ‘‘bioactive food’’ and an ‘‘endocrine active food’’ [12].

Flaxseed is rich in secoisolariciresinoldiglucoside (SDG), the precursor of lignans, which has many favorable actions on human health. SDG is known to exhibit anticancer properties by inhibiting cell proliferation and growth, especially in breast and prostate cancer [13].SDG has also anti-viral, antibacterial and anti-fungal properties, is an antioxidant and it has been shown to enhance immune system functioning. Moreover flaxseed contains phenolic acids, flavonoids, and other phenylopropanoids [14, 15].

The present work aimed to investigate the in vitro and in vivo antimicrobial effects and antibiofilm properties of flaxseed oil on some pathogenic bacterial isolates.

Materials and Methods:

Flaxseed preparation: Flaxseed (300 g) was milled in a coffee grinder and used for analysis by Soxhlet extraction in *n*-hexane for 8 h and oven dried until constant mass.

Microorganisms: Methicillin susceptible *S. aureus* (MSSA), Methicillin resistant *S.aureus* (MRSA), *S. epidermidis, E. faecalis, E. coli* and *K. pneumoniae* were obtained from Department of Biology, College of Science, University of Baghdad.

Bacterial suspension preparation: Few discrete pure colonies, grown on nutrient agar, were taken to a sterile normal saline tube with turbidity adjusted to approximately 1.5×10^8 CFU/ml in comparison to McFarland turbidity standard (tube no. 0.5).

Determination of Minimum inhibition concentration (MIC):

The antibacterial activity of flax seed oil was studied by employing agar method as described by Valgas *et al*. [16] In brief, double serial dilutions were performed using DSMO as a diluent. The bacterial inoculum (prepared as described previously) was uniformly spread using sterile cotton swab on a sterile Petri dish Mueller Hinton agar. 50 µl of each dilution was added to each of the 3 wells (6 mm diameter holes cut in the agar gel, 20 mm apart from one another). DMSO alone was considered as control. The systems were incubated for 24 hrs at 37ºC, under aerobic conditions. After incubation, confluent bacterial growth was observed. Inhibition of the bacterial growth was measured in mm. Tests were performed in triplicate.

Biofilm quantification protocol:

Biofilm formation and flaxseed oil tolerance was quantified using the static microtiter plate biofilm assay following the procedure of Folkesson *et al*. [17]In short; bacterial cultures of the investigated strains were grown in 5ml Brain Heart infusion Broth at 37°C overnight. The cultures were diluted to trubidity equivalence to MacFarland standard No. 0.05 and inoculated in triplicate in a 96-well microtiter plate (flat-bottom). Test plates were transferred to plastic bags to avoid evaporation and statically incubated for 24 hrs at 37°C. Subsequently, the growth medium was carefully removed from the biofilm microtiter wells, washed twice with 0.9% NaCl solution, and covered with 0.1% crystal violet for 15 min. The wells were again washed twice with 0.9% NaCl where after surface bound crystal violet was extracted by addition of 96% ethanol. Absorbance of stained wells was determined at 490 nm with a microplate reader (Beckman coulter, Austria)

Antibiofilm activity of flaxseed oil:

Before biofilm formation: Bacterial suspension contained flaxseed oil at a given concentration of indicated MIC (depends on bacterial species) was added to the wells. Afterward, biofilm protocol mentioned earlier was followed.

After biofilm formation: In regard to already formed biofilms, biofilms were grown in microtitre plates without the oil as for biofilm quantification protocol. There after flaxseed oil was carefully

added to the wells at the indicated MIC (depends on bacterial species). After 24 hrs static additional incubation, the procedure was preceded as for biofilm quantification. In parallel with each quantification, viable count was determined as well. Briefly, cells were vigorously re-suspended and the total colony forming units/ml (CFU/ml) were determined by serial dilution and plating on nutrient plates without prior washing. Consistent removal of the bacterial cells was confirmed by crystal-violet staining of the wells. Controls were accomplished by adding phosphate buffered saline instead of flaxseed oil. For microtitre biofilm assays and tolerance assays, experiments were performed in triplicate. It is worthy to noticed that the biofilm-associated cells were truly dispersed to single cells by vigorous aspiration was ascertained by observing the dislodged biofilm under a microscope prior to plating.

HPLC analysis:

The sample hydrolysate were separated on FLC (Fast Liquid Chromatographic) column , 3 urn particle size (50 × 4.6 mm I.D) C-18DB column, Mobile phase were 0.1%TFA:acetonitrile (55:45 V/V),detection UV set at 280 nm and flow rate was 1.5 ml/min.

In vivo study:

Animals: Eight weeks old female white mice BALB/C each weight 21 - 24 g were used. They were housed in plastic cages under standard conditions of temperature, light, feed and water. All animals were randomly assigned to groups; A through I (three animals per group).

Injection protocol: Mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight) [18].Thereafter, transcutaneous 10 mm in length wounds were performed on the backs of the mice, 2-3 drops of inoculum material mentioned in Table-1, was applied to the wound. Concerning flaxseed oil the application was achieved after the onset of inflammation signs twice a day at the ¼ MIC (except for *K. pneumoniae* the application was at the neat concentration) for 4-5 successive days.

Table 1-Animal groups included in the present study

Animal group	Inoculum
A	Flaxseed oil
B	*live cells of MSSA
C	*live cells of MRSA
D	*live cells of *S. epidermidis*
E	*live cells of *K. pneumonia*
F	*live cells of *S. aureus* + flaxseed oil (MIC)
G	*live cells of MRSA+ flaxseed oil (MIC)
H	*live cells of *S. epidermidis* + flaxseed oil (MIC)
I	*live cells of *K. pneumoniae* + flaxseed oil (neat)

*** 1.5×10^8 CFU/ ml, MIC= minimum inhibition concentration.**

Histological technique:

Mice were sacrificed after three days. Injured skin specimens were removed, fixed with 10% formalin for 24 hrs at room temperature (20-25 ºC), then embedded in paraffin according to standard histological methods, after fixation tissue was held in 70% alcohol until proceeded and embedded in paraffin using standard techniques[19]. The sections were examined by light microscope under magnification power 100X.

Statistical analysis:

All experiments carried out in this study were replicated three times. Mean values with standard deviations were reported when and where necessary. Analysis of variance (ANOVA) was performed and differences in mean values determined using Tukey's studentized test at $P< 0.05$ and employing ANOVA and TUKEY procedures of statistical analytical system, respectively.

Results and Discussion:

Figure-1 depicts the *in vitro* effect of flaxseed oil on bacterial isolates. No inhibitory effect was noticed against *E. coli* or *E. faecalis*, perhaps it can be attributed to possession of resistance genes need to be revealed. However, variable effects were developed against MRSA, *K. pneuminae* and *S. epidermidis*.Upon such results *E. coli* and *E. faecalis* were eliminated from further studies.

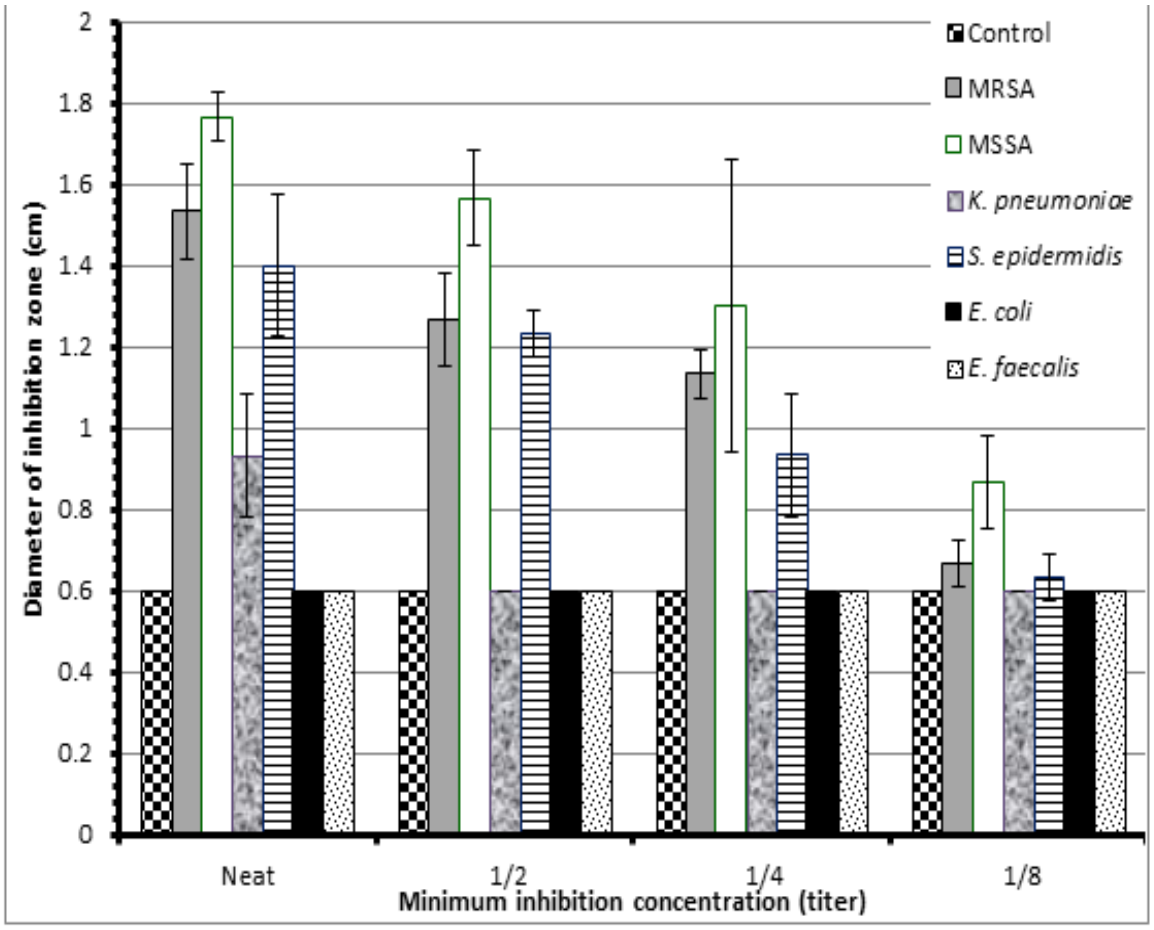

Figure 1-Inhibitory effect of flaxseed oil on bacterial isolates. The well diameter is 6 mm, i.e. there is no inhibition zone.

Rajesha *et al*.[20] found that the SDG in hull fraction showed maximum activity 31.5 mm at MIC 100 ppm against *E. coli*, while minimum inhibitory activity was 3.1 mm with MIC at 300 ppm against *Bacillus subtilis*.

Antibiofilm activity of flaxseed oil:

The extract of flaxseed oil was evaluated for its antibiofilm activity. It was specifically selected for its antibacterial properties Figure-1. The flaxseed exhibited antibiofilm activity against all tested bacterial isolates (MSSA, MRSA, *S. epidermidis* and *K. pneumoniae*) and showed various degrees of inhibition against them.

Concerning antibiofilm activity before biofilm formation, it is markedly seen that biofilm was significantly reduced Figure-2 evaluated by microtiter plate assay method. Additionally, the bacterial count in the biofilm was reduced as well Table-2. It is obviously noticed that biofilm (measured by OD_{490}) of MRSA and *S. epidermidis* was higher than biofilms of other tested pathogens; nevertheless, the viable count in these two species were undetectable. i.e. flaxseed oil succeeded in preventing them from forming biofilm and kills all live cells inside the biofilm.

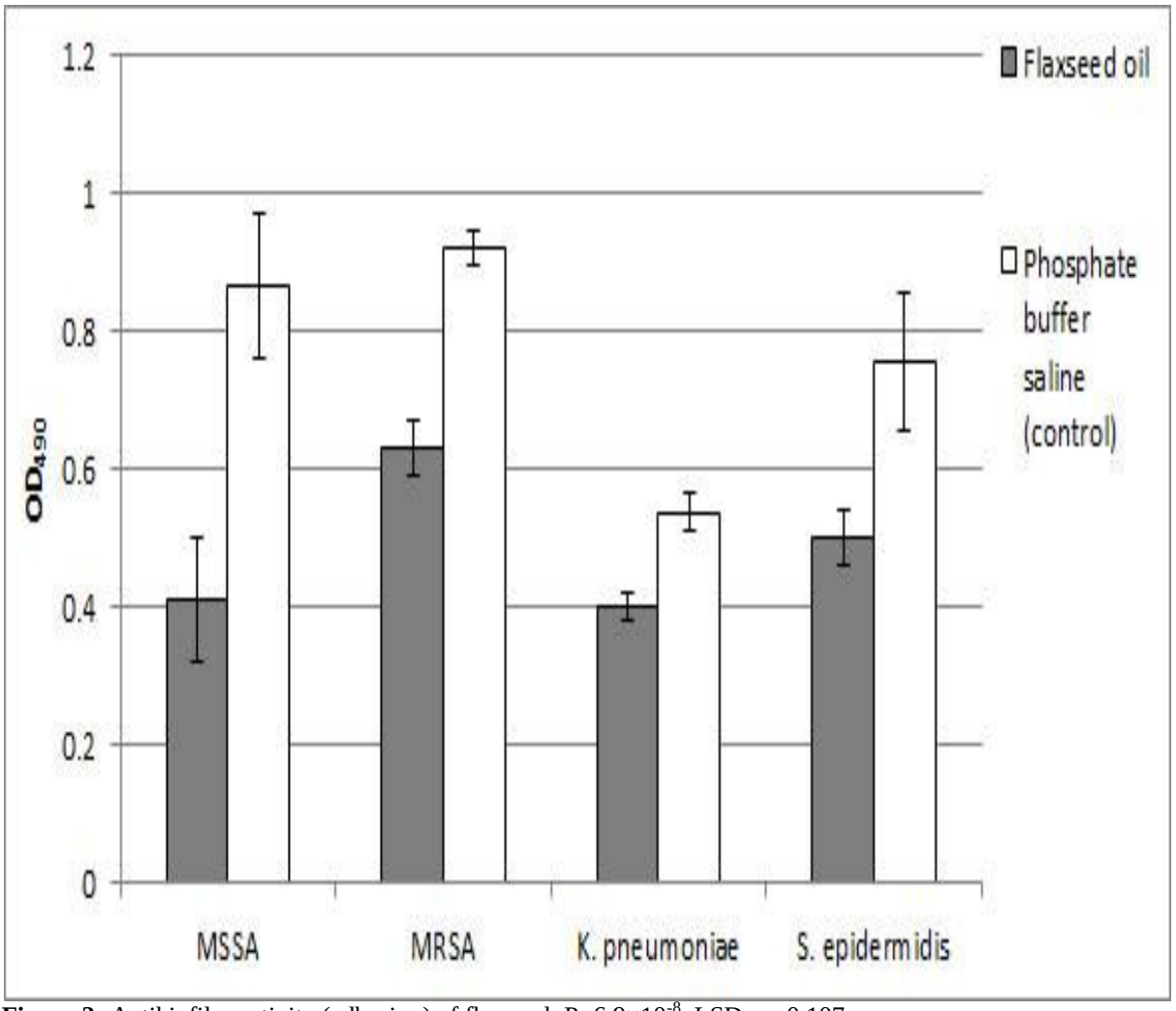

Figure 2- Antibiofilm activity (adhesion) of flaxseed. $P=6.9\times10^{-8}$, $LSD_{0.05}=0.107$

Table 2- Inhibitory effect of flaxseed oil on bacterial count in biofilm (before biofilm formation)

Bacteria	Flaxseed oil		Phosphate buffer saline (control)	
	Viable Number (CFU/ml)×10^6	SD	Viable Number (CFU/ml)×10^6	SD
MSSA	48.33333	2.516611	203000	2645.751311
MRSA	ND	-	41333.33	2516.611478
K. pneumonia	303.3333	25.16611	27000	2645.751311
S. epidermidis	ND	-	73333.33	3214.550254

SD=standard deviation, ND=not detectable, $P=3.4\times10^{-19}$, $LSD_{0.05}= 1243.49$

Similar results were obtained in case of activity of flaxseed oil on previously formed biofilm. Figure-3 and Table-3 illustrate that flaxseed oil was able to lower, significantly, the biofilm thickness (as it measured by OD_{490}) and reduced the bacterial count in these formed biofilm.

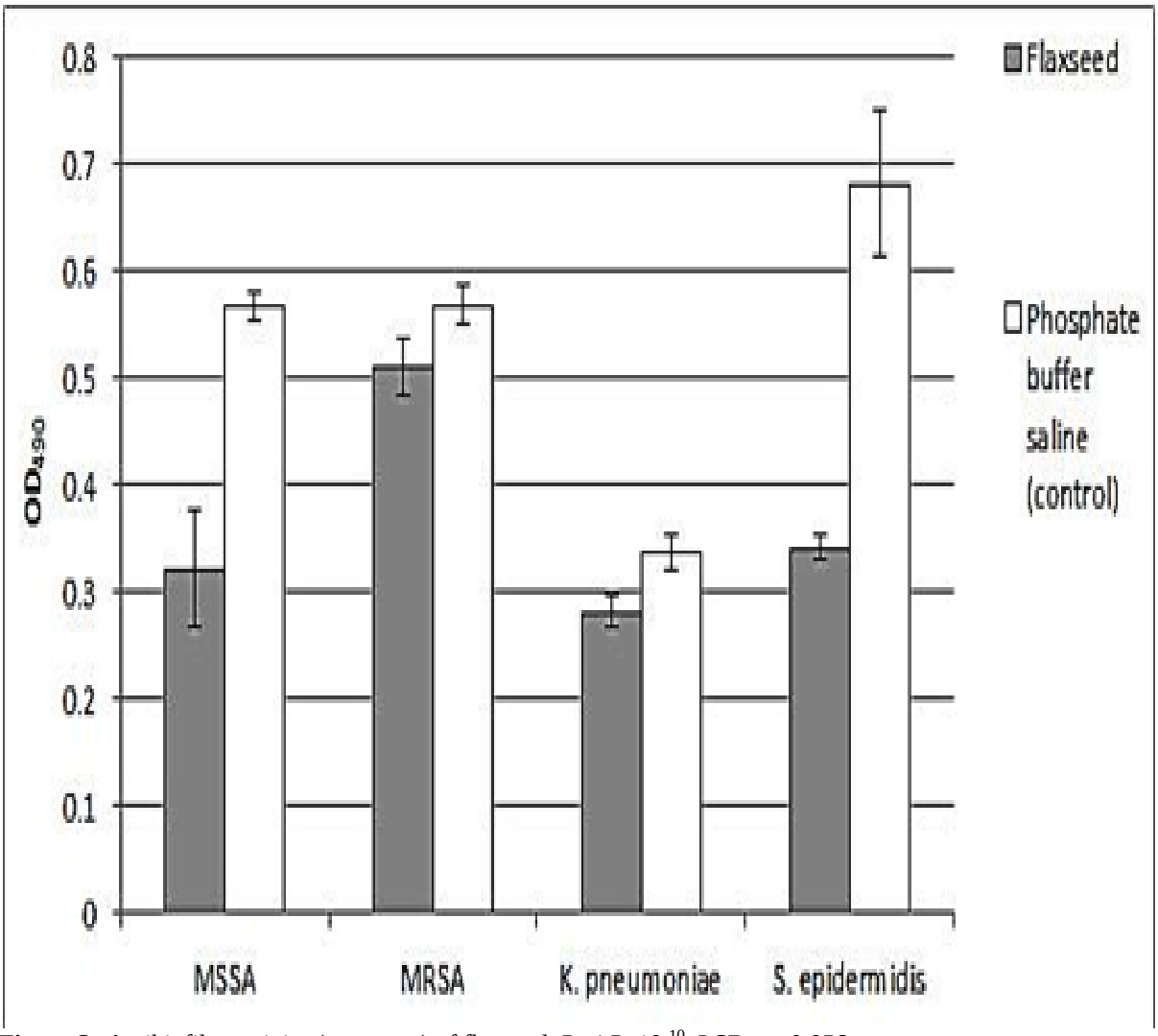

Figure 3- Antibiofilm activity (treatment) of flaxseed. $P=4.5\times10^{-10}$, $LSD_{0.05}=0.056$

Table 3- Inhibitory effect of flaxseed oil on bacterial count in biofilm (after biofilm formation)

Bacteria	Flaxseed oil		Phosphate buffer saline (control)	
	Viable Number (CFU/ml)×10^6	SD	Viable Number (CFU/ml)×10^6	SD
MSSA	235.6667	6.027714	363333.3	20816.65999
MRSA	10200	200	138000	3000
K. pneumonia	1143.333	15.27525	64666.67	3055.050463
S. epidermidis	976.6667	30.5505	86666.67	1527.525232

SD=standard deviation, ND=not detectable, $P=1.46\times10^{-24}$, $LSD_{0.05}$= 3230.82

High performance liquid chromatography:

High performance liquid chromatographic analyses were carried out and the SDG peaks were identified and quantified by comparison with those of the SDG standards, and its amount were also calculated as well. Findings obtained from HPLC analysis revealed that SDG was the predominant lignan in the extract, which has shown maximum absorbance at 280 nm and the retention time was found to be 3.833 min in a concentration of 33.28 µg/ml as it shown in Figure-4; accordingly, all the antibacterial activity in the flaxseed extract could be attributed to this constituent.

In the analyzed samples of defatted and dried flaxseed, no significant difference in lignan content was observed when compared to non-defatted flaxseed samples [21].

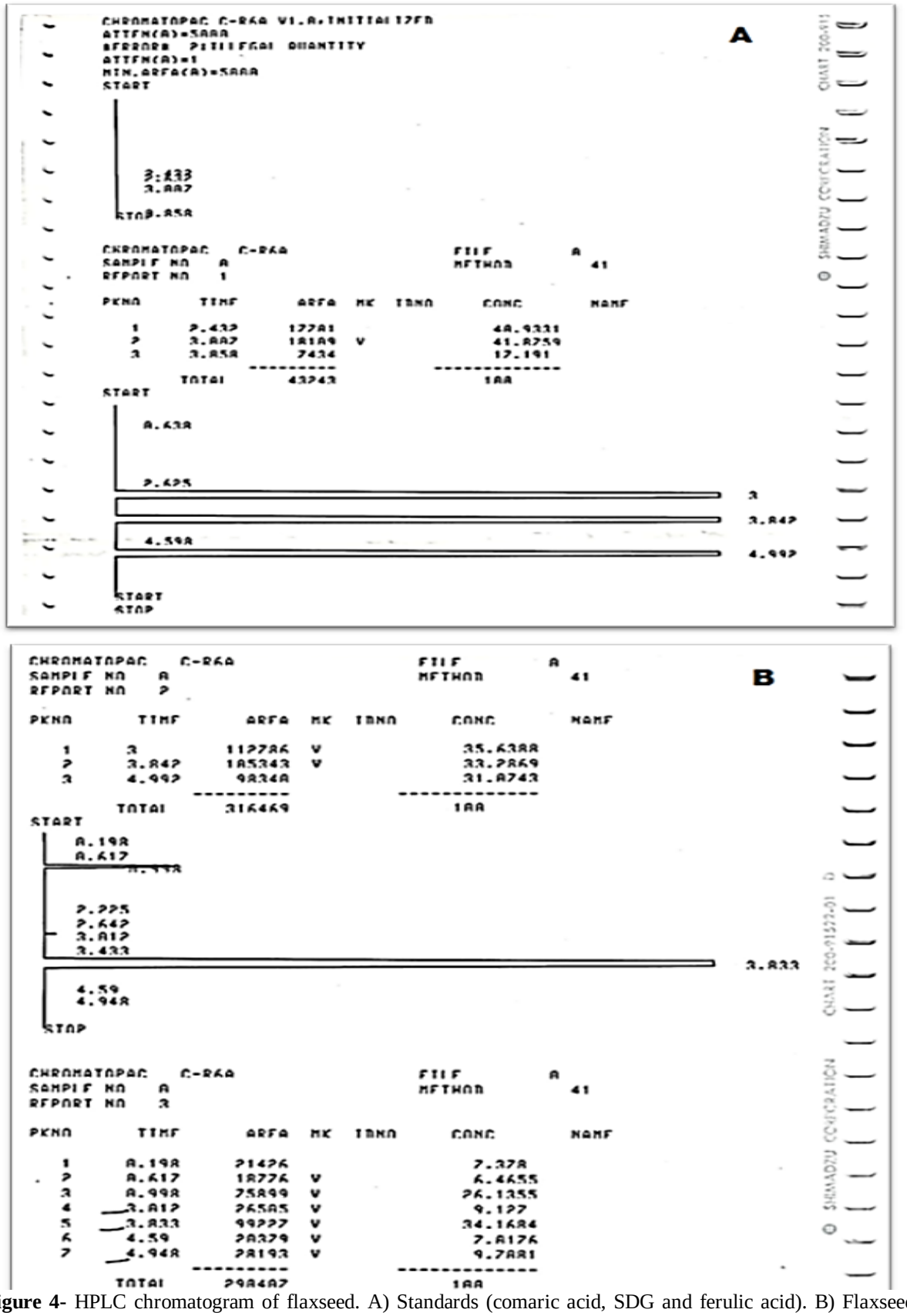

Figure 4- HPLC chromatogram of flaxseed. A) Standards (comaric acid, SDG and ferulic acid). B) Flaxseed extract.

Histopathology study

Flax seed gum is an inexpensive, abundant, natural material with no side effects [22] .Our findings depicted in Figure-5 evidently demonstrated the harmless effect of flaxseed oil on skin tissue. On the other hand, all tested bacterial pathogen successfully caused experimental wound infection in murine model as it is shown in Figure-5.

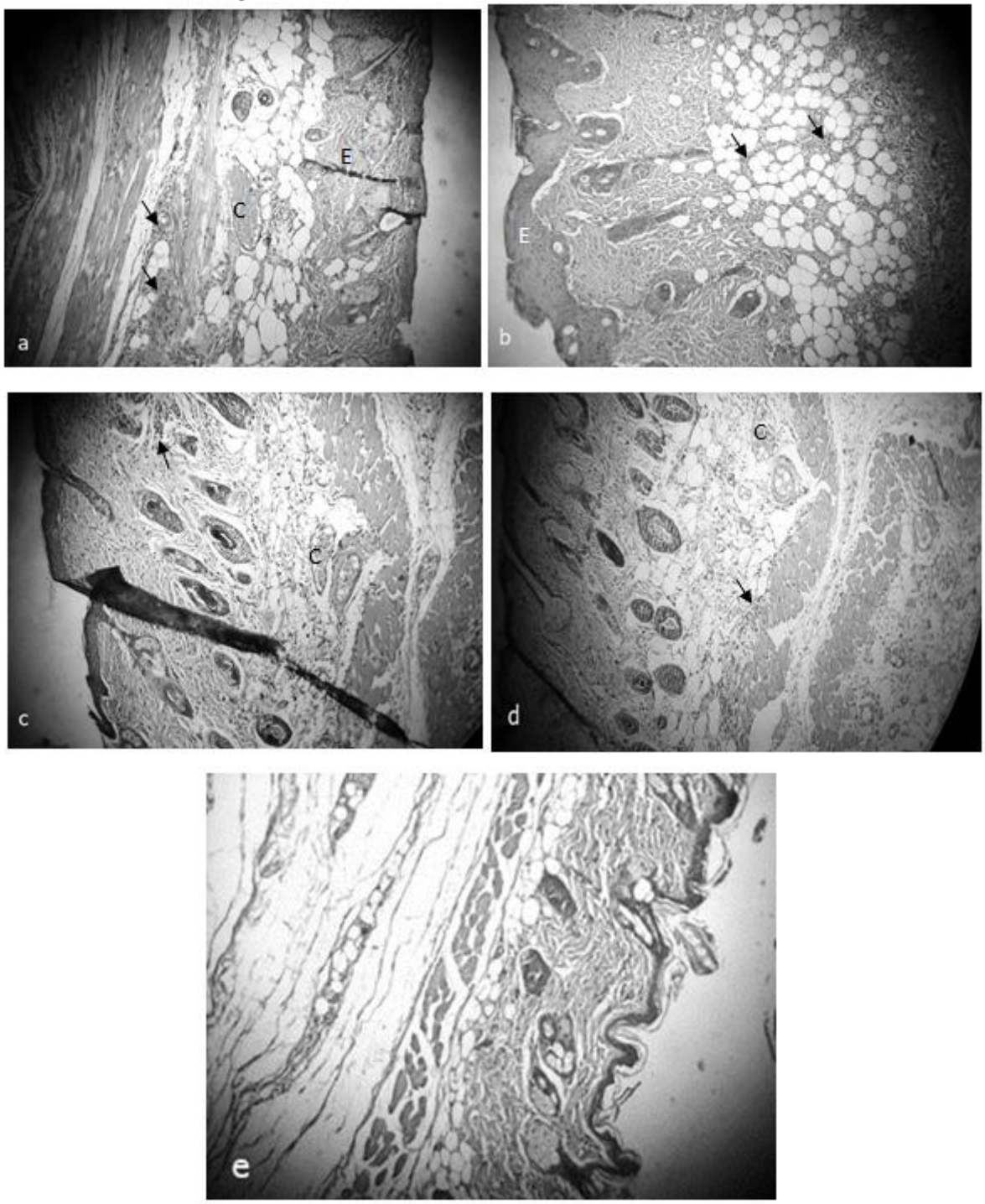

Figure 5- Histopathological sections of experimental wounds on murine model caused by a) MSSA, b) MRSA, c) *K. pneuminae*, d) *S. epidermidis* and e) control group. Infiltration of inflammatory cells (black arrow), expand of epidermis and dermis layers (E), congestion of blood inside the dilated blood vessels (C). 100 X. H & E.

That was very clear from the histopathological changes represented by infiltration of inflammatory cells (black arrow), expand of epidermis and dermis layers (E) in addition to congestion of blood inside the dilated blood vessels (C). However, the application of flaxseed oil on inflamed wounds healed them efficiently. Such curing action could be assigned to the active constituents represented mostly by SDG lignan.

Conclusions:

The present work emphasizes the role of a natural plant product, flaxseed oil, in inhibiting of bacterial infections as well as helps in eradication biofilm formed by these pathogens. What's more, flaxseed oil cured wound infections caused by these pathogens.

Acknowledgments:

The authors would like to thank Prof. Dr. Salim R. Hamoudi from the college of medicine, University of Baghdad for his kind help in examining the histopathologhical sections. Also we would like to offer our gratitude for Dr. Sadik Moula from the college of Science, University of Baghdad for his generous assistance in reviewing the statistical analysis.

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