

Antibacterial Activity of *Linum usitatissimum* L. Seeds and Active Compound Detection

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ABSTRACT

In the present study, antibacterial properties of four different extracts from *Linum usitatissimum* L. seeds were screened against four types of Gram-positive and negative bacteria: *Staphylococcus aureus*, *Bacillus cereus*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* using agar-well diffusion method and comparing their antibacterial activities with the antibiotics Ampicillin, Cefalexin, Chloramphenicol and Tetracycline. Petroleum ether extract demonstrated significant inhibitory effects against all tested bacteria using all extract concentrations compared with used antibiotics except chloramphenicol, the clearest activity was seen against *K. pneumoniae* using the extract concentration 50mg/cm³. Ethanol extract possessed considerable antibacterial activities against the pathogenic bacteria, the highest inhibitory effect was observed against *B. cereus* using the extract concentration 200mg/cm³, followed by aqueous extract which revealed good inhibitory action against *Ps. aeruginosa* using the same concentration. The weakest extract used was the chloroform extract which was only active against *S. aureus*. Thin-Layer Chromatography of petroleum ether extract indicated the presence of active fatty acids.

Linum usitatissimum L.

Linum usitatissimum : *Staphylococcus aureus* *Bacillus cereus* *Klebsiella pneumoniae* *Pseudomonas aeruginosa*
Ampicillin Cefalexin Chloramphenicol
Tetracycline
Chloramphenicol

*K. pneumoniae*50mg/cm³*B. cereus*200mg/cm³*Ps.**aeruginosa**S. aureus*

INTRODUCTION

Plants have long provided man kind with a source of medicinal agents, with natural products once serving as the source of all drugs (Balandrin et al., 1993). Dependence on plants as the source of medicine is prevalent in developing countries where traditional medicine plays a major role in health care (Farnsworth, 1994 ; Rabe and van Staden, 1997). The rural population of a country is more disposed to traditional ways of treatment because of its easy availability and cheaper cost (Brantner and Grein, 1994). Herbal therapy, although still an unwritten science, is well established in some cultures and traditions, and has become a way of life in almost 80% of the people in rural areas, especially those in Asia, Latin America and Africa (Jäger et al., 1996). Medicinal plants, which form the backbone of traditional medicine, have in the last few decades been the subject of very intense pharmacological studies, This has been brought about by the acknowledgement of the value of medicinal plants as potential sources of new compounds of therapeutic value and as sources of lead compounds in drug development. Besides, there also exists a very large market of minimally processed medicinal plant parts especially in Europe and America, which are usually dispensed as over-the-counter medication (Shale et al., 1999).

Linum usitatissimum L. (flax, linseed) is a species of the family *Linaceae*. It is an erect, herbaceous annual which branches corymbosely above the main stem. Two types of *L. usitatissimum* are cultivated: the linseed type, grown for oil extraction from the seed, is a relatively short plant which produces many secondary branches compared to the flax type, grown for the fibre extraction from the stem, which is taller and is less branched (Oomah et al., 2007). Seeds contain 35-45% oil which comprises mainly linoleic and linolenic acids and 20-25% protein, the seed also contains cyanogenic glycosides (prussic acid) in small quantities these glycosides stimulate respiration and improve digestion (Coşkuner and Karababa, 2007). Seeds are used in breads and cereals, it can also be sprouted and used in salads (Bown, 1995). Linseed has a long history of medicinal use, its main effects being as a laxative and expectorant that soothes irritated tissues, controls coughing and relieves pain in addition of being analgesic, demulcent, emollient, laxative, pectoral and resolvent (Duke and Ayensu, 1985). The methanol extract from these seeds have been reported to have excellent antiviral activities (Sökmen, 2001). Extracted oil from flax seeds contains 4% L-glutamic acid, which is used to treat mental deficiencies in adults. It also has soothing and lubricating properties, and is used in medicines to soothe tonsillitis, sore throats, coughs, colds, constipation, gravel and stones (Phillips and Foy, 1990).

MATERIALS AND METHODS

Plant material

Linum usitatissimum seeds were purchased from the local market in Mosul city Nineveh province, and identified at College of Agriculture and Forestry.

Extraction procedure

Dried and powdered seeds (100 g) were extracted using a soxhlet extractor with solvents of increasing polarity beginning with petroleum ether followed by ethanol then water and finally chloroform, each extraction was carried out for 8-10 hours continuously (Ashnagar et al., 2005). The solvents were removed using a rotary vacuum evaporator at 40°C to give concentrated extracts which were frozen and freeze-dried until use.

Preparation of extract concentrations

1gm of each extract (petroleum ether, ethanol and chloroform) was dissolved in 5ml DMSO (Dimethylsulfoxide) and in distilled water regarding aqueous extract to give an extract concentration of 200mg/cm³ which was used as a standard concentration in providing next dilutions (100, 50, 25 and 12.5mg/cm³) Erturk et al., 2006, then were sterilized via pasturalization at 62°C for 15 minutes, and using membrane filtration through a 0.25 µm regarding aqueous extract.

Microbial cultures

Bacterial strains tested included the gram positive strains: *Staphylococcus aureus*, *Bacillus cereus*, and the gram negative strains: *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* which all had been identified and kindly provided by the postgraduate students of Department of Biology/College of Science and were further identified by using characteristics features including microscopical, cultural and biochemical tests to insure.

Preparation of inoculum

All bacterial strains were cultured overnight in Nutrient broth (Oxoid), incubated at 37°C and used as inoculum. The turbidity of the suspensions were adjusted to (1.5–3×10⁸ cells/ml) in comparison with McFarland turbidity standard.

Antibacterial activity

The antibacterial tests were performed using agar-well diffusion method described by (Bauer et al., 1966). Agar plates were prepared using sterile Nutrient agar (Oxoid). The bacterial inoculum was evenly spread onto the surface of the agar plates using sterile swab sticks. Wells (6 mm diameter) were punched in the plates using a sterile stainless steel borer. Ten microliters of each extract concentrations were added to each well. Commercial antibiotics Ampicillin 250mg, Cefalexin 250mg, Chloramphenicol 250mg and Tetracycline 250mg were purchased from a local pharmacy, each antibiotic capsule (250mg) was dissolved in 10ml distilled water or ethanol (70% or absolute depending on antibiotic) to produce antibiotic solutions with a concentration 25mg/cm³ as described by (Adomi, 2006) with the assistance of a pharmacist. Ten microliters of each antibiotic solutions were filled in

each well and used as positive control, and ten microliters of DMSO and water per well was used as negative control. Diffusion of the extracts and antibiotics was allowed at room temperature for 30 minutes. The agar plates were then incubated at 37°C for 24 h. The plates were observed for the presence of a clear zone around the wells. The size of the zones of inhibition was measured and the antibacterial activity expressed in terms of the average diameter of the zone inhibition in millimeters. The absence of a zone inhibition was interpreted as the absence of activity. Each experiment was tested in triplicate.

Active compound detection from petroleum ether extract

Thin layer chromatography (TLC) was used to identify the active ingredients of the petroleum ether extract. TLC plate (Merck) used was 0.25mm in thickness and 20×20cm in dimension, the plate was activated before use at 100°C in oven for one hour, then left to cool to room temperature. A capillary tube was used to apply the petroleum ether extract on an invisible line used as a start point, the plate was placed inside the TLC tank which contained the solvent system (methanol : benzene : water) at a ratio of (1:2:7 v/v/v), the placement of the plate was vertical so that the side of plate containing the spot was in touch with developing solvents, the tank was covered and left until the developing solvent was raised to 13cm in distance, then the plate was up-lifted and left horizontally to dry out at room temperature for 1-2 hours. The TLC plate was immersed in a 15ml solution of (NH₄OH : Ethanol) at a ratio of (5 : 95) for 5 minutes then washed with distilled water and analyzed by UV (254 and 366nm) Harborne, 1973.

Qualitative analysis of active compounds

The rate of flow (R_f) on TLC plate was measured according to the following equation.

$$R_f = \frac{\text{Distance of material}}{\text{Distance of solvent}}$$

(Mikes,1979)

RESULTS AND DISCUSSION

The results of the antibacterial screening from *L. usitatissimum* seed extracts are presented in (Table 2). All the concentrations of the petroleum ether extract showed antibacterial activity against all tested bacteria, with inhibition zones between 10.2-23.5mm in diameter, *K. pneumoniae* (Figure 1) showed highest susceptibility towards petroleum ether extract compared with the antibiotics ampicillin and cefalexin, with an inhibition zone of 23.5mm using the extract concentration 50mg/cm³, this extract concentration was the optimal concentration inhibiting *S. aureus* and *K. pneumoniae*, the scientific explanation for this situation is that the cytoplasmic membrane of bacteria contains pores that prevents high concentration of extracts from insertion, in fact petroleum ether extract was able to

breakthrough cell wall and cytoplasmic membrane and accomplish antibacterial activity, this was also described by (Kreander et al., 2006), who studied the effect of plant extracts on bacterial cytoplasmic membrane permeability, meanwhile *B. cereus*, *S. aureus* and *Ps. aeruginosa* showed moderate susceptibility towards petroleum ether extract, the highest calculated inhibition zone regarding these bacteria was 16.7mm.

Petroleum ether extract activity (Figure 4) can be due to its content of palmitic acid, linoleic acid and oleic acid as indicated by the thin-layer chromatography techniques and by calculating R_f as indicated in (Table 1), these acids are the main essential fatty acids in linseed oil, and are reported to contain antibacterial properties (Dilika et al., 2000 ; Mokbel and Hashinaga, 2005). In addition to Lignans which are active phenolic compounds that have anti-estrogenic, antioxidative, antiviral, antibacterial, antitumor and anti HIV properties (Mohagheghzadeh et al., 2006).

Table 1: The rate of flow (R_f) values of detected and standard fatty acids.

Detected compounds	Standard R_f *	Calculated R_f
Palmitic acid	0.76	0.75
Linoleic acid	0.72	0.70
Oleic acid	0.66	0.65

* Standard (R_f)'s were obtained from (Harborane,1973).

The study results revealed that ethanol extract possessed good inhibitory activity against *B. cereus* (Figure 2) compared with the antibiotics ampicillin, cefalexin and tetracycline, the calculated inhibition zone reached 22.6mm in diameter using the extract concentration 200mg/cm³, *S. aureus* showed moderate sensitivity towards the ethanol extract using the highest extract concentration compared with ampicillin and tetracycline, *Ps. aeruginosa* and *K. pneumoniae* revealed weak sensitivity towards the extract compared with the tested antibiotics. The study results proved the suitability of ethanol in dissolving active components from plants in addition to natural products (Herrero et al., 2006). Ethanol extract activity against tested bacteria can be attributed to the linseed content of mucilage, gum, wax, cyanogenic and glycosides. Wax and cyanogenic glycosides have been reported to contain compounds that have antifungal and antibacterial effect (Aboaba et al., 2006 ; Reid et al., 2005), Mucilage which are saponins a thick, sticky substance that blocks cholesterol absorption and helps to balance blood-glucose levels, and does not dissolve in water, most of these compounds can be dissolved using ethanol (Maisch, 1889).

Aqueous extract demonstrated moderate activity against most tested bacteria compared with some of the tested antibiotics, *B. cereus* resisted all aqueous extract concentrations, while *Ps. aeruginosa* was more susceptible towards aqueous extract (Figure 3) compared with ampicillin and tetracycline with an inhibition zone of 17.7mm using the extract concentration 200mg/cm³, followed by *S. aureus* then *K. pneumoniae*. Previous

studies reported that aqueous extract from linseeds showed good activity against *Salmonella typhi* using the extract concentration 10mg/cm³ (Perez and Anesini, 1994). Aqueous extract activity of linseeds can be due to the presence of tannins in flax seeds (Wooten, 2006) which are mostly dissolved using water or a combination of acetone and water (Strumeyer and Malin, 1975).

This investigation showed that chloroform extract from linseeds had no activity against all tested bacteria, except a weak inhibition zone compared with the tested antibiotics was seen against *S. aureus* using the extract concentration 200mg/cm³, *Ps. aeruginosa* also showed a very weak susceptibility towards the same extracts, the weak actions mentioned above can be considered as resistant when compared with the inhibition zones of tested antibiotics, while *B. cereus* and *K. pneumoniae*, which were more susceptible towards petroleum ether and ethanol extracts showed complete resistance towards the chloroform extract using all extract concentrations, this can be due to the scanty of active compounds in chloroform extracts. The active compounds in plants play a cooperating role in showing inhibitory properties, if one or more of these compounds are separated it may decrease extract activity (Cowan, 1999).

To summarize the results, the petroleum ether, ethanol, aqueous and chloroform extracts from *L. usitatissimum* seeds showed antibacterial activity against most tested bacteria. However, the activity of the petroleum ether and ethanol extract revealed promising results against Gram-negative and Gram-positive pathogens, and this indicates the presence of potent antibacterial agents in the seeds of this plant. Therefore, further purification and isolation work is necessary to determine all types of agents responsible for the antibacterial effects of this medicinal plant.

Table 2: Antibacterial activity of different extracts from *Linum usitatissimum* L. seeds.

Microorganisms	Extracts	Zone of inhibition (mm)						
		Extract concentration (mg/cm ³)						Co
		200	100	50	25	12.5	AM	
<i>Staphylococcus aureus</i>	P.E	14.8	14.4	15.6	13.3	13.1	19.3	
	E	17.6	14.5	13.5	11.3	10.2		
	Aq	15.7	14.8	14.3	13.4	12.3		
	CH	13.5	12.3	10.2	9.6	9.2		
<i>Bacillus cereus</i>	P.E	16.7	15.5	14.3	13.4	12.1	16.2	
	E	22.6	19.8	17.5	15.6	13.4		
	Aq	-	-	-	-	-		
	CH	-	-	-	-	-		
<i>Klebsiella pneumoniae</i>	P.E	22.4	21.2	23.5	19.6	18.5	16.3	
	E	15.6	13.5	11.6	-	-		
	Aq	12.3	11.2	13.4	12.2	11.1		
	CH	-	-	-	-	-		
<i>Pseudomonas aeruginosa</i>	P.E	13.6	12.4	12.1	11.3	10.2	15.5	
	E	14.4	13.2	11.6	9.5	9.2		
	Aq	17.7	16.5	15.6	14.3	13.2		
	CH	10.5	10.1	-	-	-		

-: No activity, Extracts (P.E: Petroleum ether, E: Ethanol, Aq: Aqueous, CH: Chloroform),
AM: Ampicillin, CL: Cefalexin,

C: Chloramphenicol, TE: Tetracycline.

Chloramphenicol, TE: Tetracycline.

Fig. 1: Effect of petroleum ether extract concentrations against *K. pneumoniae*

Fig.2: Effect of ethanol extract concentrations against *B. cereus*

Fig. 3: Effect of aqueous extract concentrations against *Ps. aeruginosa*

Fig. 4: Thin-layer chromatography of petroleum ether extract

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