



RESEARCH ARTICLE

In vitro antimicrobial activity of Anise seed (*Pimpinella anisum* L.)Huda S. A. A. Mohamed¹, Warda S. Abdelgadir², Aisha Z. I. Almagboul³¹ Faculty of Pharmacy, University of Science and Technology, Omdurman, Sudan² Food Research Centre, P. O. Box 213, Ministry of Science & Communications, Khartoum, Sudan.³ Medicinal and Aromatic Plant Research Institute (MAPRI), National Centre for Research, Khartoum, Sudan**Manuscript Info****Manuscript History:**

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gentamicin, nicin***Corresponding Author****Huda S. A. A. Mohamed****Abstract**

Medicinal plants synthesize a vast array of secondary metabolites that are important for human life. For Medicinal purpose, antimicrobial activity of substances derived from plant extracts has been recognized for many years. The antimicrobial activity of the petroleum ether, chloroform, ethyl acetate, methanol and aqueous extracts of the seeds of *Pimpinella anisum* L. (Apiaceae) was tested for their potential antimicrobial activities against two Gram positive (*Bacillus subtilis*, *Staphylococcus aureus*), three Gram negative (*E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) bacteria and against two standard fungi namely *Aspergillus niger* and *Candida albicans* using the cup-plate-agar diffusion method. The Petroleum ether, chloroform, ethyl acetate and methanol extracts of *P. anisum* (1:10 and 2:10) were highly active (30-40 mm) against *B. subtilis*. The ethyl acetate extract exhibited moderate activity (15 mm) against *E. coli* and low activity (13 mm) against *Ps. aeruginosa*. The methanol extract of *P. anisum* showed high activity (16 mm) against *E. coli*, low activity (13mm) against *Ps. aeruginosa*. The methanol extract have variable activity against all test organisms. All the tested organisms were resistant to anise seed aqueous extract. The results were comparable to those of the standard drug gentamicin and nicin.

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INTRODUCTION

Plant-derived drugs remain an important resource, especially in developing countries, to combat serious diseases. Some plants contain bioactive compounds such as glycosides, alkaloids and terpenes which may be used as drugs and antimicrobial agents (Kurita et al., 1982). Many extracts and essential oils have been derived from plants and found to have antibacterial, fungicidal and insecticidal properties (Hänsel et al., 1999).

Pimpinella anisum L. is an annual herb and a grassy plant with white flowers and a small green to yellow seed. The plant is self fertile; prefer light sandy and medium loamy and well drained soil. When threshed out, the fruit or the so- called seed (part used) may be easily dried in trays, in a current of air in half-shade, out-of-doors, or by moderated heat. The taste is sweet and spicy, and the odor aromatic and agreeable (Pourgholami et al., 1999).

The plant is indigenous to Near East and widely cultivated in Mediterranean rim (Turkey, Egypt, Syria, Spain, etc. and in Mexico and Chile. It has been used as an aromatic herb and spice since Egyptian times and antiquity and has been cultivated throughout Europe (Hänsel et al., 1999). Chemical studies have demonstrated that *Pimpinella anisum* seed contain trans-anethole as the main compound (80-95%) or more (Fujita and Nagasawa, 1960), estragole (Zargari, 1989), eugenole (Monod and Dortan 1950), pseudoisoeugenol (Reichling et al., 1995), methylchavicol and anisaldehyde (Wagner et al., 1984), terpene hydrocarbons (Kartnig et al., 1975), polyenes and polyacetylenes (Schutle et al., 1970) as the major components. An unusual compound is the phenol ester 4-methoxy-2-(1-propene-yl)-phenol-2-methyl-butyrate, which is characteristic for anise (5%).

In folk medicine, anise is used as an appetizer, tranquillizer and diuretic drug and was reported that it has several therapeutic effects on several conditions such as digestive, gynecologic, neurologic and respiratory disorders (Lawless, 1999).

The essential oil is used as an expectorant, carminative and in cough mixtures especially in pediatrics, and the important phenyl- propane, such as trans-anethole and estragole, have a stabilizing influence on the autonomic nervous system (Hänsel et al., 1999).

The aim of the this study was to investigate the In vitro antimicrobial activity of the petroleum ether, chloroform, ethyl acetate, methanol and aqueous extracts of the seeds of *Pimpinella anisum* L. (Apiaceae) against two Gram positive (*Bacillus subtilis*, *Staphylococcus aureus*), three Gram negative (*E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) bacteria and two fungi (*Aspergillus niger*, *Candida albicans*).

MATERIALS AND METHODS

Five hundred gm fresh dried, well packed *Pimpinella anisum* seeds were purchased from Omdurman local market (Fig. 1) and authenticated by a scientist at the Medicinal and Aromatic Plants Research Institute, National Centre for Research (MAPRI).



Fig. 1. *Pimpinella anisum* seeds

Plant extraction

Hundred grams of the dried *P. anisum* seeds were milled to a coarse powder using an electrical grinder and were extracted with 500 ml petroleum ether (40-60°C) for 4-6 hours using Soxhlet apparatus. The extract was filtered and evaporated under reduced pressure using rotary evaporator. The extracted plant material was then air-dried, repacked in the Soxhlet apparatus and successively extracted with chloroform (63°C), ethyl acetate (64-70°C) and methanol (62°C) using the same procedure.

Each residue was weighed and the yield percentage was determined. The petroleum ether was dissolved in petroleum ether and the chloroform extracts was dissolved in a mixture of methanol and petroleum ether (2:1, v/v), whereas both of the ethyl acetate and methanol extracts were dissolved in methanol, all extracts to a final volume of 20 ml (conc. 100mg/ml).

The aqueous extract was prepared by boiling 10 g of the dried ground aniseed in 20 ml distilled water for 2 hours in a water bath at 70°C.

Test organisms

All reference micro-organisms were kindly provided by the Department of Microbiology, Medicinal and Aromatic Plant Research Institute. Two strains of gram positive and three strains of gram negative bacteria (*Bacillus subtilis*, NCTC 8236, *Staphylococcus aureus* NCTC 5953, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 53657, *Pseudomonas aeruginosa* ATCC 27853) and two fungi (*Aspergillus niger* ATCC 9763, *Candida*

albicans ATCC 7596) were used. The cultures of bacteria were maintained in their appropriate agar slants at 4°C throughout the study and used as stock cultures.

Preparation of bacterial suspensions

One ml aliquots of a 24 hours broth culture of the test organisms were aseptically distributed onto nutrient agar slopes and incubated at 37°C for 24 hours. The bacterial growth was harvested and washed off with sterile normal saline, and finally suspended in 100 ml of normal saline to produce a suspension containing about (10^8 - 10^9) colony forming units per ml. The suspension was stored in the refrigerator at 4°C until used.

The average number of viable organisms per ml of the stock suspension was determined by means of the surface viable counting technique (Miles and Misra, 1938). Serial dilutions of the stock suspension were made in sterile normal saline in tubes and 0.02 ml volume (one drop) of the appropriate dilutions were transferred using an automatic microtitre pipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for 2 hours at room temperature for the drop to dry, and then incubated at 37°C for 24 hours. After incubation, the number of developed colonies in each drop was counted. The average number of colonies per drop (0.02 ml) was multiplied by 50 and the dilution factor to give the viable count of the stock suspension expressed as the number of colony forming units per ml (CFU/ml) of suspension.

Preparation of fungal suspension

The fungal cultures were maintained on Sabouraud dextrose agar, incubated at 25°C for 4 days. The fungal growth was harvested and washed with sterile normal saline and finally suspended in 100 ml of sterile normal saline and refrigerated until used.

In vitro testing of extracts for antimicrobial activity

The cup-plate-agar diffusion method (Kavanagh, 1972) was used to assess the antibacterial activity of the prepared extracts. Three ml of each of the five standardized bacterial stock suspensions (10^8 - 10^9 C.F.U/ml) were thoroughly mixed with 300 ml of sterile melted nutrient agar which was maintained at 45°C.

Twenty ml aliquots of the inoculated nutrient agar were distributed into sterile Petri-dishes. The agar was left to solidify and each plate was divided into two halves. Using a sterile cork borer (No. 4), two wells were punched in each half (10 mm in diameter) and the agar disks were removed. Alternate cups were filled with 0.1 ml of each of the extracts using Transfer pipette adjustable volume automatic microtitre pipette, and allowed to diffuse at room temperature for 2 hours. The plates were then incubated in the upright position at 37°C for 18 hours. Two replicates were carried out for each extract against each of the test organisms. Simultaneously; positive controls involving the addition of the respective solvents instead of the extracts were carried out separately. After incubation, the diameters of the resultant growth inhibition zones were measured, and mean values were tabulated.

Testing for antifungal activity

The same procedure as for the bacteria was adopted. Instead of nutrient agar, Sabouraud dextrose agar was used. The inoculated media was incubated at 25°C for two days for the *Candida albicans* and three days for *Aspergillus niger*.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC concentrations were evaluated on the plant extract that showed antimicrobial activity. For bacteria, the test was performed in five concentrations of the extract (100, 50, 25, 12.5, 6.25 mg/ml). The Petri dishes were divided into three segments for the bacteria and two segments in case for the fungi. The organisms to be tested were grown in broth over night and diluted in broth to contain 10^8 /ml. A standard loop- full of dilute culture (0.01ml) was spotted onto the surface of each segment and then incubated at 37°C for 18 hours for the bacteria and at 25°C for 2 days for the fungi.

The end point (MIC) is the least concentration of antimicrobial agent that completely inhibits growth. Results are reported as MIC in mg/ml of crude extract.

Antibacterial and antifungal activity of reference drugs against the standard organisms

One antibacterial reference drug (Gentamicin) and one antifungal reference drug (Nystatin) were prepared in suspensions of three concentration (40, 20 and 10 µg/ml) and 50, 25, and 12.5 µg/ml respectively, using sterile distilled water to evaluate their antibacterial and antifungal activity against tested organisms using cup-plate diffusion method. The cups were filled with 0.1 ml of each of the three concentrations of reference drugs using automatic microtitre pipette, and allowed to diffuse at room temperature for two hours. The plates were then

incubated at 37°C for 18 hours. Two replicates were carried out for each concentration against each of the test organisms simultaneously. After incubation, the resultant growth inhibition zones were measured and the mean values were tabulated.

Identification of the active ingredients

Head Space injection method (Gas Chromatography-Mass Method) was used to detect the presence of volatile materials in the petroleum ether extract. A certain vial was half-filled with the oil and heated at 120°C for 15 minutes. Vapours of the volatile materials were separated in the upper half of the vial. The head space injector drew the volume of the upper layer before injection into the column.

Saponification and methylation were carried out to convert fixed oil to volatile oil

Saponification

Two gram of the oil and 50 ml of 0.5N alcoholic KOH was refluxed for one hour. After Saponification, diethyl ether was added followed by sufficient amount of water which resulted in formation of two phases: upper phase (ether phase) and lower phase (alcohol phase). The alcohol layer was repeatedly extracted with diethyl ether three times (3x50 ml). Then the ether insoluble fraction (lower phase-alcohol layer) was acidified with 10% HCl using methyl orange as an indicator. It was then extracted with diethyl ether three times (3x50 ml). The diethyl ether extract was washed with water till a neutral reaction, dried with anhydrous Na₂SO₄ and the solvent was then removed.

Methylation

Hundred ml of absolute sodium hydroxide were added to obtain fatty acid and 15 drops of cons.H₂SO₄ were added to the mixture, and the mixture was refluxed. After cooling, it was transferred to separatory funnel and distilled water was added and the methyl esters were extracted with diethyl ether (3x50 ml). The diethyl ether extract was washed with 10% Na₂CO₃, then with water till a neutral reaction (Phenolphthalein), dried with anhydrous Na₂SO₄.

UV Spectrophotometric study of the extract

An aliquot of the extract was dissolved in methanol, and the methanolic solution was scanned between 200-700 nm using methanol as blank.

High Performance liquid Chromatography (HPLC)

Column, C18/ Mobile phase, acetonitrile (CH₃CN) 50% +50%, 1% acetic acid

Flow rate, 1 ml/min and the Detector set at 290 nm

Methanolic solution of the sample and the standard anethole and anisaldehyde were injected into the chromatographic instrument according to the conditions shown above.

RESULTS

Extracts yield

The petroleum ether, chloroform, ethyl acetate and methanol extracts of *pimpinella anisum* seeds yielded 3.68, 2.612, 2.481 and 13.55 gm respectively after evaporation and dryness and the water extract yielded 0.066 gm.

Antimicrobial activity

The antimicrobial activity of different *P. anisum* seed extracts expressed as inhibition zone (mm) is summarized in Table 1. At the concentration 1:10, the petroleum ether extract of *P. anisum* seeds showed very high activity (29 mm) against *B. subtilis*, no activity against *S. aureus*, *E.coli*, and *Ps. aeruginosa* while full inhibitory activity was observed against the gram negative bacteria *K. pneumoniae* and the two fungi *A.niger* and *C. albicans*. The chloroform extract exhibited high activity (20 mm) against *B. subtilis* and inactive against the rest of the organisms tested. The ethyl acetate extract showed weak activity (11-13 mm) against *S. aureus* and *Ps. aeruginosa* and the fungi *A. niger* and *C. albicans*, while inactive against *B. subtilis*, *E. coli* and *K. pneumoniae*. The methanol extract exerted high activity (19 mm) against *B. subtilis* and low activity (11-13 mm) against the rest of the organisms tested. The aqueous extract was devoid of any activity against all the test organisms

Doubling the concentration (2:10), the petroleum ether extract of *P. anisum* showed very pronounced activity (40 mm) against *B. subtilis* and no activity against *S. aureus*, *E. coli* and *Ps. aeruginosa*.

Table 1: Antimicrobial activity of different concentrations of *Pimpinella anisum* L. seed extracts

Conc. Of extract	Solvents	Bacteria					Fungi	
		B.s	S.a	E.c	K.p	Ps.a	A.n	C.a
Inhibition zones (mm)								
1:10	Pet. eth	29	(-)	(-)	(F)	(-)	(F)	(F)
2:10		40	(-)	(-)	ND	(-)	ND	ND
1:10	CHCl ₃	20	(-)	(-)	(-)	(-)	(-)	(-)
2:10		30	(-)	13	13	(-)	(-)	(-)
1:10	EtoAc	(-)	(-)	(-)	(-)	12	13	11
2:10		14	12	15	(-)	13	13	12
1:10	MeoH	19	11	11	12	12	13	12
2:10		30	12	16	12	13	13	12
1:10	H ₂ O	(-)	(-)	(-)	(-)	(-)	(-)	(-)

Conc.: concentration; (-) : no activity; (F): Full inhibition; ND: Not done

Pet.eth: Petroleum ether; CHCl₃ : Chloroform; EtoAc: Ethyl acetate; MeoH: Methanol

B.s: Bacillus subtilis ; S.a: Staphylococcus aureus; E.c: Escherichia coli; K.p: Klebsiella pneumoniae; P.sa: Pseudomonas aeruginosa; A.n: Aspergillus niger; C.a: Candida albicans

Interpretation of results: >15= High activity, 15= Moderate activity, <15= Low activity

The chloroform extract also showed pronounced activity (20 mm) against *B. subtilis* (Plate 3), low activity (13 mm) against *E. coli*, *K. pneumoniae* and no activity against *Ps. aeruginosa* and the two tested fungi. The ethyl acetate extract showed moderate activity (15 mm) against *E. coli*, low activity (12-14 mm) against *Ps. aeruginosa* and the two fungi. The methanolic extract exerted pronounced activity (30 mm) against *B. subtilis*, high activity (16 mm) against *E. coli* (Plate 4) and low activity (11-13 mm) against *K. pneumoniae*, *Ps. aeruginosa* and the two tested fungi (Table 1).

The petroleum ether extract showed full inhibition at the concentration of 50 and 25 mg/ml and showed inhibition zones of 15 and 20 mm for the concentration of 12.5 mg/ml for *A. niger* and *C. albicans* respectively. The concentration of 6.25 mg/ml showed no activity against both fungi.

From the result it is clear that the effects of *P. anisum* extracts on *Bacillus subtilis* was comparable to those of the standard drug, gentamicin (Table 3), the case which is not applicable to other bacteria. The same effects were seen for fungi (Table 4).

Minimum Inhibitory Concentration (MIC)

The *P. anisum* petroleum ether extract showed Minimum Inhibitory Concentration (MIC) at 6.25 and 12.5 mg/ml concentration for all tested organisms (Table 2).



Plate 3: Inhibition zone of *P. anisum* seeds chloroform and aqueous extract (10%), *B. subtilis*

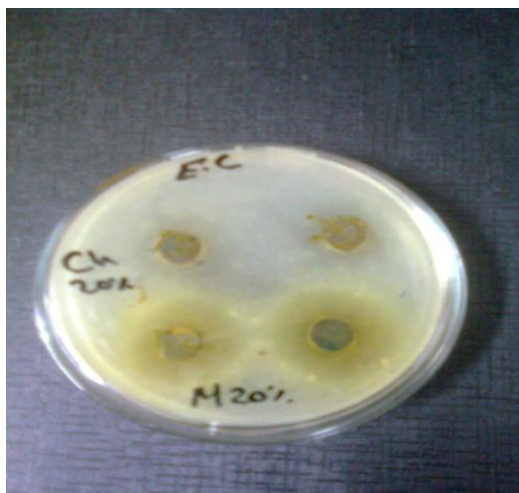


Plate 4: Inhibition zone of *P. anisum* seeds chloroform and methanol extract (20%), *E. coli*

Table 2: Minimum inhibitory concentration of petroleum ether extract of *P. anisum* seeds

Concentration mg/ml	Petroleum ether extract				
	<i>Bacillus subtilis</i>	<i>Escherichia coli</i>	<i>Klebsiella pneumoniae</i>	<i>Aspergillus niger</i>	<i>Candida albicans</i>
100	-	-	-	-	-
50	-	-	-	-	-
25	-	-	-	-	-
12.5	+	+	+	+	+
6.25	+	+	+	+	+

- = No growth; + = Growth

Table 3: Antibacterial activity of standard antibacterial drugs.

Drug	Concentration on $\mu\text{g/ml}$	MDIZ (mm)				
		<i>Bacillus subtilis</i>	<i>Staphylococcus aureus</i>	<i>E. coli</i>	<i>Pseudomonas aeruginosa</i>	<i>Klebsiella pneumoniae</i>
Gentamicin	40	30	18	24	20	16
	20	20	17	23	15	13
	10	20	14	20	14	(-)

MDIZ = Mean diameter of growth inhibition zone, (-): No activity

Table 4: Antifungal activity of standard antifungal drug.

Drug	Concentration $\mu\text{g/ml}$	MDIZ (mm)	
		<i>Aspergillus niger</i>	<i>Candida albicans</i>
Nystatin	50	28	17
	25	26	14
	12.5	23	(-)

MDIZ = Mean diameter of growth inhibition zone ; (-): No activity

Identification of the active ingredient of *P. anisum*

GC/MS Method

Despite the characteristic smell of the extract, however no volatile material was obtained by the use of head-space injection system. The sample was then checked for fixed oils constituent after Saponification and methylation, again no positive result was obtained. This negative result does not necessarily exclude absence of volatile or non volatile materials. It could be due to improper conditions used i.e. improper temperature gradient or improper column.

UV spectrum Method

Using spectrophotometry, the sample was scanned between 200-700 nm. This revealed the presence of a peak at about 256nm indicating the presence of a UV absorbing component.

Scanning of standard anethole and anisaldehyde showed that the anethole has λ_{\max} at about 256 nm while the anisaldehyde showed λ_{\max} 276nm. This suggests the presence of anethole in the sample.

HPLC Method

Using HPLC, three peaks were separated with retention time 2.5, 5.2 and about 8.9 minutes. Injection of standard anethole and anisaldehyde showed peaks at about 8.2 and 3.8 minutes respectively. This indicated that the sample most probably contain anethole confirming the UV finding. Beside the anethole peak, the chromatogram for the sample showed another two other components which were not identified.

DISCUSSION

In the present study, Anethole was found to be the main active ingredient in *Pimpinella anisum* seeds. Anethole is a monoterpene position isomer and it is the main constituent of essential oils from aromatic plants including anise, star-anise, and fennel (Ponte et al., 2012; Huxley, 1992). It is used in food and pharmaceutical industries and has also experimentally shown that it has no toxicity at low doses (Smith et al., 2002) and it is considered non-genotoxic and non-carcinogenic and, therefore, quite safe (Friere et al., 2005; Yea et al., 2006). On the other hand, 4-allylanisole was found to be the major compound of *Pimpinella anisum* L. grown in Morocco and Yemen with percentages of 76.70 and 85.28% of Moroccan and Yemen, respectively, in addition to other minor compounds such as limonene, and fenchone (AL Maofari et al., 2013). Their essential oils showed high activity against *E. coli*, moderate activity against *Staph. aureus* and weak action against *Klebsiella pneumoniae*.

The growth of *Bacillus subtilis* was inhibited by all the organic solvents extracts of *Pimpinella anisum*. *Bacillus subtilis* was found sensitive to the petroleum ether and methanolic extracts of the seeds of *Cannabis sativa* (Ali et al., 2012), the ethyl acetate leaf extract of *Mentha piperita* (Bupesh et al., 2007) and *Diospyros ebenum* (Barvalia et al., 2009). On the other hand, least activity was exerted against *Bacillus subtilis* by the methanol and aqueous extract of *Bidens pilosa* L., and was found highly resistant to petroleum ether, chloroform and acetone extracts showing no inhibition (Dagawal and Ghorpade, 2011).

Moderate to weak inhibition was exerted by the different extracts of the anise seed against *Staph. aureus*, *E. coli* and *Pseud. aeruginosa*. Variable antimicrobial activity of the ethanolic and water extracts of anise seed against *Staph. aureus*, *E. coli*, *Pseudomonas aeruginosa* and *C. albicans* was observed by Gülçin (2004). On the other hand, maximum activity of *Pimpinella anisum* essential oils and methanol extracts were observed against *Staphylococcus aureus*, *Bacillus cereus* and *Proteus vulgaris* and the combinations of essential oils and methanol extracts showed an additive action against most tested pathogens especially *Pseudomonas aeruginosa* (Al Bayati, 2008).

The petroleum ether and chloroform extracts were found active against *Pseud. aeruginosa* and the two fungi, *C. albicans* and *Aspergillus niger*. This result is similar to that reported by Yazdani et al. (2009) who found that the extracts of anise seeds inhibited only dermatophyte species, while extracts of star anise fruits inhibited growth of all dermatophytes and saprophytes tested. The high antifungal activity of *P. anisum* is probably due to the high concentration of anethole in the extract or as a result of the synergism of its components. Vat et al. (2011) reported that the chloroform extract of roots of *Murraya koenigii* (Linn.) Spreng. (Rutaceae) showed good inhibitory properties against *A. niger*, *P.aeruginosa*, and *C. albicans* and even at low concentrations and the Petroleum ether is inhibitory against *A. niger* and *P.aeruginosa*.

The aqueous decoction of *P. anisum* seed showed no activity against both gram positive and gram negative bacteria. This result is different from that reported by Chaudhry and Tariq, (2006) who found that the aqueous extract possesses broad antibacterial spectrum against gram positive and gram negative bacteria.

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