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Isolation and identification of anethole from *Pimpinella anisum* L. fruit oil. An antimicrobial study

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ABSTRACT

The present study was conducted to isolate the most important bioactive compound from *Pimpinella anisum* L. fruit oil. The plant essential oil was extracted via steam distillation. Anethole was separated using a separatory funnel and detected on thin layer chromatography TLC plates in comparison with standard anethole that, served as positive control. Moreover, infrared FTIR spectrometer and High Performance Liquid Chromatography HPLC analysis were used to confirm the purity and identification of anethole. The isolated material was investigated for its antimicrobial activity against seven selected pathogenic microorganisms. The Gram-positive bacteria were *Staphylococcus aureus*, *Bacillus cereus*; and Gram-negative bacteria included;*Escherichia coli*, *Proteus mirabils*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*; and the yeast *Candida albicans*. Anethole at different concentrations (1:1, 1:5, 1:10, 1:20) was active against all tested bacteria except for *P. aeruginosa*, and the highest inhibitory effect was observed against *B. cereus* (zone of inhibition: 22.3 mm) using the disc diffusion method. The minimal inhibitory concentration MIC of anethole was determined using a broth microdilution method in 96 multi-well microtiter plates. MIC values ranged from 31.2–500.0 µg/ml, and the most promising results were observed against *S. aureus*, *B. cereus* and *E. coli* (MIC 31.2 µg/ml) while, *P. mirabilis* and *K. pneumonia* ranked next (MIC 62.5 µg/ml). Furthermore, anethole achieved considerable antifungal activity against the yeast *C. albicans* (zone of inhibition range: 10.6–17.2 mm; MIC: 500.0).

Keywords: Isolation and identification, Anethole, *Pimpinella anisum*, HPLC, FTIR, Antimicrobial activity.

INTRODUCTION

Plants are a large source of new bioactive molecules with therapeutic potentials. Only a small percentage of living plants on Earth have been phytochemically investigated. Plants are thus an enormous reservoir of pharmaceutically valuable molecules to be discovered (Balandrin et al., 1985). Although there are drugs effective against bacteria and yeasts, plant extracts as well as essential oils and other compounds are of considerable interest because of their antimicrobial activities (Lima, et al., 1993).

Anise (*Pimpinella anisum* L.) is an annual aromatic herb, belonging to the family Apiaceae. The genus Pimpinella contains 23 species, 3 of which are endemic in Iraq (Al-Rawi and Chakravarty, 1988). Anise is native to the Middle East and it has been known since the time of ancient Egypt (Hemphill and Hemphill, 1988).

Anise oil is the essential oil obtained by steam distillation from dry ripe fruits of anise *P. anisum*. Essential oil of anise fruits contains from 80 to 95%, or more, *trans*-anethole as the main compound, followed by chavicol methyl ether (estragole), anisaldehyde and cis-anethole (Hänsel et al., 1999).

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Anise oil has been used in Iraqi folk medicine for the treatment of some diseases, including seizures and epilepsy (Al-Rawi and Chakravarty, 1988). Anise has mild estrogenic effects, which explains the use of this plant in folk medicine for increasing milk secretion and for amenorrhea. Products that contain anise fruits extracts or anise essential oil may cause contact dermatitis, probably due to their anethole content (Kosalec et al., 2005).

In spite of all the information available on *P. anisum* fruit oil, we were not able to find an extensive isolation study of anethole. Thus, we report here the isolation and identification of anethole from *P. anisum* fruit oil using different spectral techniques, and its antimicrobial activity against some selected pathogenic microorganisms.

MATERIALS AND METHODS

Chemicals and Reagents

Diethyl ether $(CH_3CH_2)_2O$, sodium bisulphide $NaHSO_4$,Hexane C6H14, Dichloro methane CH_2CL_2 dichloro , vanillin–H_2SO_4, methanol and dimethylsulfoxide "DMSO" were supplied from BDH Analar (England). *trans*-anethole (standard) =99% purity (molecular weight 148.20, mp 20-21°C, bp 234-237°C, molecular formula $CH_3CH{=}CHC_6H_4OCH_3$) and *p*-iodonitrotetrazolium violet (INT) were obtained from Sigma-Aldrich chemical com.

Table 1. Antibacterial activity of anethole isolated from *P. anisum* fruits oil.

Microorganisms	Zone of inhibition (mm)					
	Anethole concentrations				Control	
	1:1	1:5	1:10	1:20	G	A
S. aureus	20.4	18.4	15.5	12.7	19.1	N.T
B. cereus	22.3	20.6	18.5	16.4	19.6	N.T
E. coli	21.2	19.8	17.6	14.3	21.3	N.T
P. mirabilis	18.4	16.6	15.8	13.9	19.4	N.T
K. pneumonia	19.2	16.5	14.6	11.4	18.3	N.T
P. aeruginosa	00.0	00.0	00.0	00.0	16.0	N.T
C. albicans	17.2	15.7	12.3	10.6	N.T	10.2

G: Gentamycin, A: Amphotericin B, 10 µg/disc each, N.T: Not tested

Table 2. Minimum inhibitory concentration (MIC) of anethole isolated from *P. anisum* fruits oil.

Microorganisms	MIC values (µg/ml)		
	Anethole	Control	
		G	A
S. aureus	31.2	15.6	N.T
B. cereus	31.2	15.6	N.T
E. coli	31.2	7.8	N.T
P. mirabilis	62.5	15.6	N.T
K. pneumonia	62.5	31.2	N.T
P. aeruginosa	>500.0	31.2	N.T
C. albicans	500.0	N.T	7.8

G: Gentamycin, A: Amphotericin B, N.T: Not tested

Plant materials

P. anisum fruits were obtained commercially from a local market in Mosul city, Nineveh province, Iraq and identified by a botanical taxonomist at college of Agriculture and Forestry, University of Mosul.

Essential oil extraction and isolation of *trans*-anethole

The air-dried, powdered anise fruits 100g was submitted for 3 h to steam distillation in a Clevenger-type apparatus. The oil was dried over anhydrous sodium sulphate Na_2SO_4 and stored at 4 °C in dark glass containers until use. 1.0 ml (density = 1.04 g/ml) of the plants fruit essential oil was dissolved with 50 ml of diethyl ether and transferred to a 125 ml separatory funnel. 25 ml of sodium bisulphide was added. The funnel was shaken vigorously and two layers were resulted, aqueous layer which was drained and anethole in the organic layer (Cheronis and Entrikin, 1963).

Characterization of *trans*-Anethole

Thin-layer chromatography (TLC)

The isolated compound was dissolved in appropriate solvents. 5 µl of reference solution of anethole and 5 µl of investigated anise oil were applied to silica gel plates, Merck (Germany) 20×20 cm, 0.25 mm in thickness. Plates were developed using the solvent system hexane: CH_2CL_2 (5:1 v/v). The separated zones were visualized with freshly prepared vanillin reagent (1% of vanillin in 2% sulphuric acid in ethanol v/v) and heated at 100°C for 10 minutes. Chromatograms were then examined under daylight within 10 minutes.

FTIR studies

Infrared (IR) spectra of menthol was recorded in College of Education, Department of Chemistry, University of Mosul, using a computerized Tensor 27 FTIR spectrometer, Bruker Co. (Germany), in the range 400–4000 cm^{-1} by KBr pellet technique.

High-performance liquid chromatography (HPLC)

HPLC analysis were performed in College of Science, University of Mosul, using a Shimadzo LC 2010 HPLC system (Kyoto, Japan), equipped with a Shimadzo LC 2010 UV-VIS detector with a thermostatted flow cell and a selectable two wavelengths of 190–370 nm or 371–600 nm. The detector signal was recorded on a Shimadzo LC 2010 integrator. The column used was a C18 block heating-type Shim-pack VP–ODS (4.6 mm interior diameter ×150 mm long) with a particle size of 5 µm. Anethole was separated using a mobile phase of methanol: water (75:25 v/v) at a flow rate of 1.0 ml/min, column temperature 30 °C. Injection volume was 40 µl and detection was carried out at 346 nm

Antibacterial activity

Microbial cultures

Six strains of bacteria and one yeast were used as test microorganisms. The bacterial strains included Gram-positive: *Staphylococcus aureus* and *Bacillus cereus*; Gram-negative: *Escherishia coli, Proteus mirabilis, Klebsiella pneumonia, Pseudomonas aeruginosa*; and the yeast *Candida albicans.* All microorganisms were clinical isolates, obtained from the Microbiology Laboratory at Department of Basic Science, College of Nursing, University of Mosul, Iraq, and very carefully identified using standard microbiological methods.

Inoculum preparation

Nutrient broth and Sabouraud dextrose agar (SDA) were used for growing and diluting the microorganism suspensions. Bacterial strains were grown to exponential phase in nutrient broth at 37 °C for 18 h and adjusted to a final density of 10^8 cfu/ml by diluting fresh cultures and comparison to McFarland density. *C. albicans* was aseptically inoculated on petri dishes containing autoclaved, cooled, and settled SDA medium. The petri dishes were incubated at 31 °C for 48 h to give white round colonies against a yellowish background. These were aseptically subcultured on SDA slants. The yeast colonies from SDA slants were suspended in sterilized 0.9% sodium chloride solu-

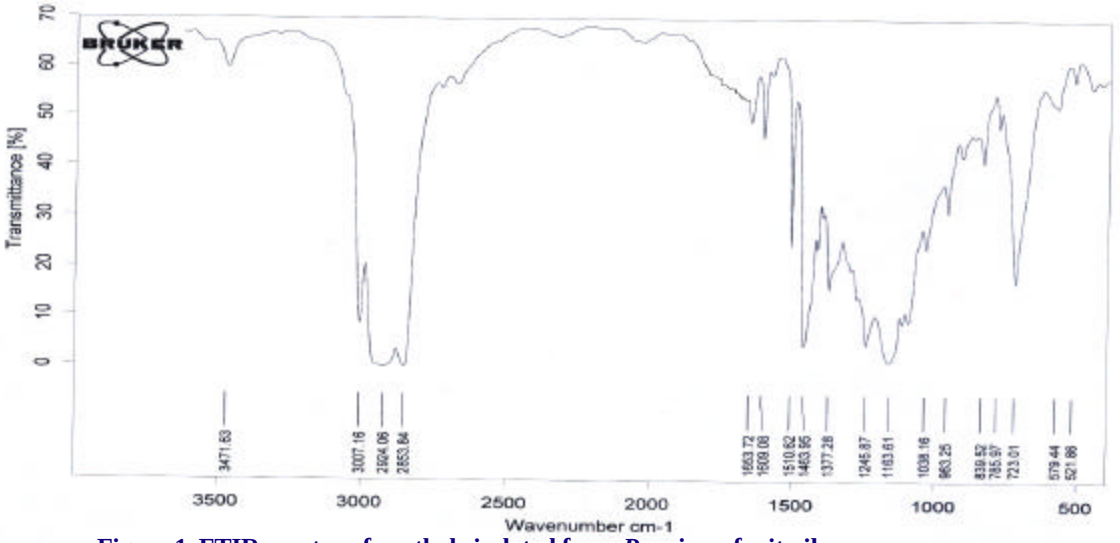
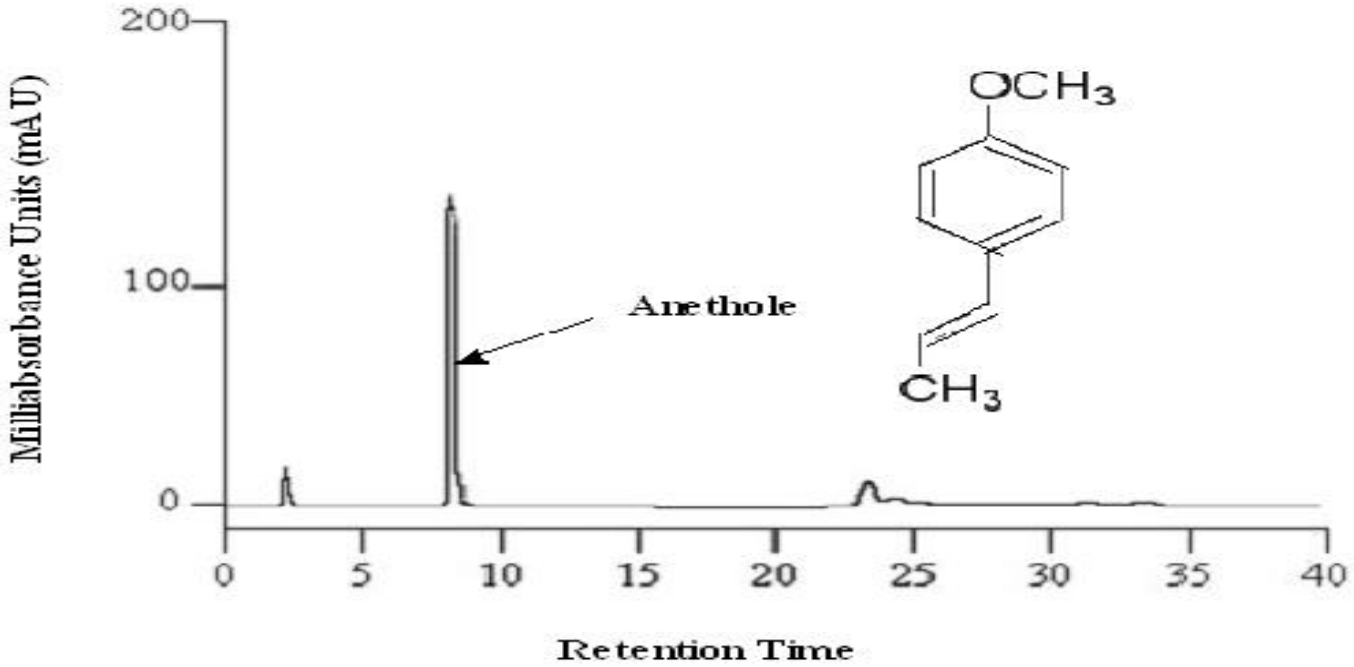


Figure 1. FTIR spectra of anethole isolated from *P. anisum* fruit oil.



tion (normal saline), which was compared with McFarland solution. According to the manufacturer's directions, 1 ml of yeast suspension in normal saline was added to 74 ml of sterile medium and kept at 45 °C to give a concentration of 2×10^7 cells/ml.

Disc diffusion assay

A modified agar diffusion method (Mothana and Lindequist, 2005) was used to determine antimicrobial activity. Nutrient agar was inoculated with microbial cell suspension (200 µl in 20 ml medium) and poured into sterile petri dishes. Sterile filter paper discs 6 mm in diameter were impregnated with 20 µl of anethole in different concentrations (1:1, 1:5, 1:10, 1:20 initially prepared by diluting in DMSO and sterilized by filtration through 0.45 µm Millipore filters), and placed on the inoculated agar surface. Standard 6 mm discs containing gentamycin 10 µg/disc and amphotericin B 10 µg/disc (Bioanalyse) were used as positive controls. The plates were incubated overnight at 37 °C and the diameter of any resulting zones of growth inhibition was measured (mm). Each experiment was tested in triplicate.

Microdilution assay

Minimal inhibitory concentrations of anethole isolated from anise fruits were determined based on a microdilution method in 96 multi-well microtiter plates as previously described (Al-Bayati, 2008). Briefly, bacterial strains were cultured overnight at 37 °C on nutrient broth and adjusted to a final density of 10^8 cfu/ml, and used as an inoculum. Anethole was dissolved in DMSO and then in n utrient broth

to reach a final concentration of 500.0 µg/ml. Serial twofold dilutions were made in a concentration range from 7.8 to 500.0 µg/ml. In each microtiter plate, a column with broad-spectrum antibiotic was used as positive control (gentamycin in serial dilutions 500.0–7.8 µg/ml). As an indicator of bacterial growth, 40 µl *p*-iodonitrotetrazolium violet (INT) dissolved in water was added to the wells and incubated at 37 °C for 30 min. The lowest concentration of compound showing no growth was taken as its minimal inhibitory concentration MIC. The colourless tetrazolium salt acts as an electron acceptor and is reduced to a red-coloured formazan product by biologically active organisms (Eloff, 1998). Where bacterial growth was inhibited, the solution in the well remained clear after incubation with INT.

As for *C. albicans*, a simple turbidity test (Al-Bayati and Sulaiman, 2008) was used to determine the MIC values of anethole. A volume of 0.1 ml from each serial dilution of anethole concentrations (500.0-7.8 µg/ml) was added into tubes containing 9.8 ml of sterile nutrient broth, and then the tubes were inoculated with 0.1 ml of yeast suspension and incubated at 31 °C for 48 h. Amphotericin B (500.0-7.8 µg/ml) was used as a positive control. The optical density was determined using a Spectro SC spectrophotometer (LaboMed, Inc.) at 630 nm. The MIC value was the lowest concentration of compound that showed no growth after 48 h of incubation in comparison with the control tube, which included 9.8 ml of nutrient broth and 0.1 ml of yeast suspension in addition to 0.1 ml of each compound concentration (unincubated).

RESULTS AND DISCUSSION

The present study was conducted to isolate the main bioactive compound from *P. anisum* fruit oil. Anethole was isolated from the extracted essential oil, and then detected on TLC plates in comparison with standard Anethole. A yellow-peach zone with a retention factor (R_f) value of 0.35 was identified as anethole in comparison with standard anethole that had the same R_f value. The FTIR spectrum confirmed the material isolated from *P. anisum* fruit oil as *trans*-anethole (Fig. 1). Significant peaks were found at: (1463.95–1609.08) cm^{-1} corresponding to phenyl ring; (3007) cm^{-1} ascribed to aromatic (C–H) bond; (2853–2924) cm^{-1} attributed to ($–CH_3$) group; (1653) cm^{-1} corresponding to (C=C) bond and (1245) cm^{-1} corresponding to aromatic ether (C–O–C). Moreover, anethole was analyzed using the HPLC system (Fig. 2) and identified by comparing its retention time (t_R) and UV spectrum with that of the standard compound. The retention time 7–9 min and UV spectra of the isolated compound on HPLC were completely identical to that of standard anethole. HPLC is the most widely used qualitative and quantitative determination and separation method. The method is popular because it is non-destructive and may be applied to thermally labile compounds (unlike GC); it is also a very sensitive technique since it incorporates a wide choice of detection methods.

After identification, anethole was investigated for its antimicrobial activity against six bacterial species and one yeast. The initial screening of antibacterial activity of anethole was assayed *in vitro* by the agar diffusion method using four concentrations (1:1, 1:5, 1:10, 1:20). All anethole concentrations were active against all tested bacteria except for *P. aeruginosa* (Table 1). The highest inhibitory effect was observed against *B. cereus* (zone of inhibition: 22.3 mm) using the concentration (1:1), while the weakest activity was demonstrated against the *K. pneumonia* (zone of inhibition: 11.4 mm) using the concentration (1:20).

In view of the results obtained by the disc diffusion method, the minimal inhibitory concentration MIC of anethole isolated from *P. anisum* fruit oil was determined by broth microdilution assay (Table 2). The highest MIC value (31.2 µg/ml) was observed against *S. aureus*, *B. cereus* and *E. coli* while, *P. mirabilis* and *K. pneumonia* ranked next (MIC 62.5 µg/ml). *C. albicans* remains the most common infection-causing fungus; about 45% of clinical fungal infections are caused by *C. albicans* (Gupta et al., 2004). The present study showed that anethole had potent anti-*C. albicans* activity (zone of inhibition range: 10.6–17.2 mm; MIC: 500.0).

The Gram-negative bacterium *P. aeruginosa* resisted all anethole concentrations and was only inhibited using the standard drug. Several studies have reported that the Gram-negative bacteria, Pseudomonas, and in particular *P. aeruginosa*, appear to be least sensitive to the action of essential oils (Dorman and Deans, 2000; Pintore et al., 2002; Burt, 2004). Furthermore, Several mechanisms of antimicrobial resistance are readily spread to a variety of bacterial genera. First, the organism may acquire genes encoding enzymes, such as ß-lactamases, that destroy the antibacterial agent before it can have an effect. In addition, bacteria may acquire efflux pumps that extrude the antibacterial agent from the cell before it can reach its target site and exert its effect. Finally, bacteria may acquire several genes for a metabolic pathway which ultimately produces altered bacterial cell walls that no longer contain the binding site of the antimicrobial agent, or bacteria may acquire mutations that limit access of antimicrobial agents to the intracellular target site via down-regulation of porin genes (Tenover, 2006).

The standard drug gentamycin was active against all reference bacteria (zone of inhibition range: 16.0–21.3 mm; MIC range: 31.2–7.8 µg/ml). In addition, amphotericin B demonstrated good antifungal activity against *C. albicans* (zone of inhibition: 10.2 mm; MIC: 7.8 µg/ml).

It can be concluded that anethole isolated from *P. anisum* fruit essential oils had good antimicrobial activities against seven different microorganisms and that anethole is very much responsible for the antimicrobial activity of the essential oil of this very important medicinal plant. Additional *in vivo* studies and clinical trials would be needed to justify and further evaluate the potential of this compound as an antimicrobial agent in topical or oral applications

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