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Synergistic antibacterial activity between *Thymus vulgaris* and *Pimpinella anisum* essential oils and methanol extracts

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Abstract

Essential oils (EOs) and methanol extracts obtained from aerial parts of *Thymus vulgaris* and *Pimpinella anisum* seeds were evaluated for their single and combined antibacterial activities against nine Gram-positive and Gram-negative pathogenic bacteria: *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Proteus vulgaris*, *Proteus mirabilis*, *Salmonella typhi*, *Salmonella typhimurium*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The essential oils and methanol extracts revealed promising antibacterial activities against most pathogens using broth microdilution method. Maximum activity of *Thymus vulgaris* and *Pimpinella anisum* essential oils and methanol extracts (MIC 15.6 and 62.5 μg/ml) were observed against *Staphylococcus aureus*, *Bacillus cereus* and *Proteus vulgaris*. Combinations of essential oils and methanol extracts showed an additive action against most tested pathogens especially *Pseudomonas aeruginosa*.

Keywords: Synergistic antibacterial activity; Essential oils; Methanol extract; *Thymus vulgaris*; *Pimpinella anisum*

1. Introduction

Essential oils (EOs) also called volatile or ethereal oils are aromatic oily liquids obtained from different plant parts and widely used as food flavours (Burt, 2004). Volatile oils have been shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties (Kordali et al., 2005). Some oils have been used in cancer treatment (Sylvestre et al., 2006), while others have been used in food preservation (Faid et al., 1995), aromatherapy (Buttner et al., 1996) and fragrance industries (Van de Braak and Leijten, 1999). There has been an increased interest in looking at antimicrobial properties of extracts from aromatic plants particularly essential oils (Milhau et al., 1997).

Phytomedicines derived from plants have shown great promise in the treatment of intractable infectious diseases including viral infections (Cowan, 1999). Single and poly herbal preparations have been used numerously throughout history for the treatment of various diseases. Many studies have been carried out to extract various natural products for screening antimicrobial activity but attention has not been focused intensively on studying the combinations of these products for their antimicrobial activity (Abu-Shanab et al., 2004).

Thymus vulgaris L. (Thyme), locally known "zaatar" member of the Lamiaceae family, is widely used in Iraqi folk medicine for its expectorant, antitussive, antibroncholitic, antispasmodic, anthelmintic, carminative and diuretic properties. Iranian and Turkish *Thymus* species have been, respectively, reported for their antibacterial (Rasooli and Mirmostafa, 2002) and antimicrobial activities (Tepe et al., 2004).

Pimpinella anisum L. (Anise), member of Umbelliferae family, is an annual herb with white flowers and small green to yellow seeds, which grows in Iraq, Turkey, Iran, India, Egypt and many other warm regions of the world (Pourgholami et al., 1999). Members of this genus are cultivated for their aromatic seeds used in medicine as a condiment, mild expectorant and also in treating dyspeptic complaints (Fujimatu et al., 2003). In addition, essential oils of some Iraqi *Pimpinella* species have been used to treat some diseases like seizures and epilepsy.

Due to the combination uses of these two plants in Iraqi folk medicine (Majeed and Mahmood, 1988), this study aimed to determine single and combined antibacterial effects of these plants against some widely spread pathogens.

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2. Materials and methods

2.1. Plant materials

Aerial parts of *Thymus vulgaris* and *Pimpinella anisum* seeds were purchased from the local market in Mosul city, Nineveh province, Republic of Iraq and identified at Department of Biology, College of Education, University of Mosul. Voucher specimen of the plants (No. 103, 104, respectively) were dried and deposited at the herbarium of Biology Department, University of Mosul.

2.2. Essential oil extraction

The aniseeds and dried flowering parts of thyme were submitted to steam distillation in a Clevenger-type apparatus for 3 h. The essential oils obtained were separated from water and dried over anhydrous Na_2SO_4 then stored at 4 °C until use (yields 2.07% from aniseeds and 1.67% from thyme aerial parts).

2.3. Methanol extracts

Powdered samples 100 g from each plant part were extracted with methanol (MeOH) using a soxhlet extractor for 10 h continuously or until the used solvent turned pure and colourless (Chhabra et al., 1982). The solvent was removed using a rotary vacuum evaporator at 40 °C to give concentrated extracts, which were frozen and freeze-dried until use.

2.4. Test organisms

All microorganisms were obtained from Department of Biology, College of Science, University of Mosul, Iraq. Seven strains of Gram-negative bacteria: *Escherichia coli*, *Proteus vulgaris*, *Proteus mirabilis*, *Salmonella typhi*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and two strains of Gram-positive bacteria: *Staphylococcus aureus* and *Bacillus cereus* were used. The cultures of bacteria were maintained in their appropriate agar slants at 4 °C throughout the study and used as stock cultures.

2.5. Antibacterial activity assay

Antibacterial activity was determined based on a micro-well dilution method (Swanson et al., 1992). Bacterial strains were cultured overnight at 37 °C on Mueller Hinton broth and adjusted to a final density of 10^6 cfu/ml, and used as an inoculum. The essential oils and methanol extracts of each plant were dissolved in dimethyl sulfoxide (DMSO) and then in Mueller Hinton 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 sterile test tubes containing Mueller Hinton broth. The 96-well plates were prepared by dispensing into each well 95 μl of Mueller Hinton broth and 5 μl of the bacterial inoculum. A 100 μl (50 μl + 50 μl in case of combination) from each plant essential oil and methanol extract initially prepared was added into the first wells. Then, 100 μl from their serial dilutions was transferred into six consecutive wells. The last well containing 195 μl of Mueller Hinton broth without compound and 5 μl of the inoculum on each strip was used as negative control. The final volume in each well was 200 μl. Maxipime (Bioanalyse) at the concentration range of 500.0–7.8 μg/ml was prepared in Mueller Hinton broth and used as standard drug for positive control. Contents of each well were mixed on a plate shaker at 300 rpm for 20 s prior to incubation at 37 °C for 24 h. Each experiment was tested in triplicate. As an indicator of bacterial growth, 40 μl *p*-iodonitrotetrazolium violet (INT) dissolved in water were added to the wells and incubated at 37 °C for 30 min (Buwa and van Staden, 2006). The lowest concentration of each extract showing no growth was taken as its minimal inhibitory concentration (MIC) and confirmed by plating 5 μl samples from clear wells on Mueller Hinton agar medium. 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.

3. Results

The antibacterial activities of *Thymus vulgaris* and *Pimpinella anisum* essential oils and methanol extracts were assayed in vitro by a broth micro-dilution method against nine pathogenic bacteria. Table 1 summarizes the microbial growth inhibition by each plant essential oil and their combination effects.

According to the results, thyme essential oil was found to be active against all pathogenic bacteria except *Pseudomonas aeruginosa*. The strongest antibacterial activity was seen against *Bacillus cereus* with a MIC value of 15.6 μg/ml followed by *Staphylococcus aureus* and *Proteus vulgaris* MIC 31.2 μg/ml. While *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* resisted *Pimpinella anisum* essential oil and the best MIC value 62.5 μg/ml was against *Bacillus cereus* and *Proteus vulgaris*.

Combinations between the two essential oils possessed high inhibitory activities against most pathogenic bacteria compared with the standard antibiotic Maxipime which was resisted by *Bacillus cereus*, *Salmonella typhi*, *Salmonella typhimurium* and *Pseudomonas aeruginosa*. The strongest combination effect was seen against *Proteus vulgaris* with a MIC value of 15.6 μg/ml, in addition *Pseudomonas aeruginosa* was inhibited using the largest concentration of the combined essential oils.

Staphylococcus aureus showed best susceptibility towards the methanol extract of *Thymus vulgaris* with a MIC value of 15.6 μg/ml (Table 2) followed by *Bacillus cereus* and *Proteus vulgaris* MIC 31.2 μg/ml. On the other hand, the methanol extract of *Pimpinella anisum* demonstrated moderate activities against tested bacteria. The best activity was seen against *Staphylococcus aureus* and the lowest activity was against *Salmonella typhimurium* MIC 62.5 and 500.0 μg/ml, respectively, while *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* resisted all the methanol extract concentrations of the two plants. Combi-

Table 1
Single and combined antibacterial activity of *Thymus vulgaris* and *Pimpinella anisum* essential oils

Microorganisms	Gram +/−	MIC values (μg/ml)			
		Essential oils		Combined EOs	Maxipime
		Thymus vulgaris	*Pimpinella anisum*		
Staphylococcus aureus	G+	31.2	125.0	62.5	7.8
Bacillus cereus	G+	15.6	62.5	62.5	>500.0
Escherichia coli	G−	62.5	>500.0	250.0	15.6
Proteus vulgaris	G−	31.2	62.5	15.6	62.5
Proteus mirabilis	G−	62.5	125.0	31.2	62.5
Salmonella typhi	G−	250.0	500.0	125.0	>500.0
Salmonella typhimurium	G−	125.0	250.0	62.5	>500.0
Klebsiella pneumoniae	G−	500.0	>500.0	250.0	31.2
Pseudomonas aeruginosa	G−	>500.0	>500.0	500.0	>500.0

nations of the methanol extracts showed positive results against most tested bacteria, especially against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The largest effect was observed against *Proteus vulgaris* MIC 15.6 μg/ml.

4. Discussion

Plant essential oils and extracts have been used for many thousands of years (Jones, 1996), especially in food preservation, pharmaceuticals, alternative medicine and natural therapies (Lis-Balchin and Deans, 1997). It has long been acknowledged that some plant essential oils exhibit antimicrobial properties (Finnemore, 1926) and it is necessary to investigate those plants scientifically, which have been used, in traditional medicine to improve the quality of healthcare. Essential oils are potential sources of novel antimicrobial compounds especially against bacterial pathogens (Prabuseenivasan et al., 2006).

In this paper, the essential oils and methanol extracts of *Thymus vulgaris* and *Pimpinella anisum* inhibited bacterial growth but their effectiveness varied. MIC values showed by the essential oils and the methanol extracts (single and combined) were in the range of 15.6–500.0 μg/ml and 7.8–62.5 μg/ml regarding the standard drug Maxipime (Tables 1 and 2).

The Gram-positive bacterium *Staphylococcus aureus* and *Bacillus cereus* were more sensitive to the essential oils than the Gram-negative bacterium except *Proteus vulgaris*. It has frequently been reported that Gram-positive bacteria are more susceptible to essential oils than Gram-negative bacteria (Mann et al., 2000). The tolerance of Gram-negative bacteria to essential oils has been ascribed to the presence of a hydrophilic outer membrane that blocks the penetration of hydrophobic essential oils into target cell membrane.

The antibacterial activities of *Thymus vulgaris* essential oil and methanol extract could be associated to the presence of phenolic compounds like carvacrol, thymol, γ-terpinene and p-cymene, which are all reported to have antibacterial properties (McGimpsey et al., 1994; Cosentino et al., 1999; Juliano et al., 2000). While chemical studies have demonstrated that *Pimpinella anisum* seeds contain anethole, estragole, eugenol, coumarins and terpene hydrocarbons (Monod and Dortan, 1950; Chandler and Hawkes, 1984; Burkhardt et al., 1986) which are good antibacterial compounds (Cowan, 1999).

The MIC values indicated that the essential oil of *Thymus vulgaris* was more efficient than that of *Pimpinella anisum*. Recent studies have shown that essential oils of oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), bay (*Pimenta racemosa*) and clove (*Eugenia caryophyllata* synonym: *Syzygium aromaticum*) were found to be strongly antimicrobial among the many tested (Smith-Palmer et al., 1998; Hammer et al., 1999; Dorman and Deans, 2000).

Some significant infections should not be treated with single antibiotic, due to that bacteria can rapidly develop resistance

Table 2
Single and combined antibacterial activity of methanol extracts from *Thymus vulgaris* and *Pimpinella anisum*

Microorganisms	Gram +/−	MIC values (μg/ml)			
		Methanol extract		Combined methanol extracts	Maxipime
		Thymus vulgaris	*Pimpinella anisum*		
Staphylococcus aureus	G+	15.6	62.5	31.2	7.8
Bacillus cereus	G+	31.2	125.0	62.5	>500.0
Escherichia coli	G−	250.0	>500.0	125.0	15.6
Proteus vulgaris	G−	31.2	125.0	15.6	62.5
Proteus mirabilis	G−	62.5	125.0	62.5	62.5
Salmonella typhi	G−	62.5	250.0	125.0	>500.0
Salmonella typhimurium	G−	250.0	500.0	125.0	>500.0
Klebsiella pneumoniae	G−	>500.0	>500.0	250.0	31.2
Pseudomonas aeruginosa	G−	>500.0	>500.0	500.0	>500.0

when such a single antibiotic is used. According to different reports, multiple drug resistances to *Pseudomonas aeruginosa* are spreading in the world and making the therapeutic management of these patients more problematic (Obritsch et al., 2005).

Many phytomedicines exert their beneficial effects through the additive or synergistic action of several chemical compounds acting at single or multiple target sites (Adwan et al., 2006). In the present study, the data showed that the combination effects of these plants had antibacterial enhancement (additive effects) against most pathogenic bacteria especially *Pseudomonas aeruginosa* which resisted all single actions of essential oils, methanol extracts and the standard drug.

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