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Effect of Thymol and Carvacrol, the Major Components of *Thymus capitatus* on the Growth of *Pseudomonas aeruginosa*

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This study was designed to evaluate the antibacterial activity of *Thymus capitatus* and two of its major components namely thymol and carvacrol. The dried leaves were extracted using soxhlet apparatus. Thymol and carvacrol were isolated using normal phase column chromatography. The antibacterial activity of the leaves extract, thymol and carvacrol were estimated using agar disc diffusion and broth dilution methods against four bacterial strains. The leaves extract was found to show a broad spectrum of antibacterial activity against all the tested bacterial strains. *Pseudomonas aeruginosa* was the most sensitive bacteria. The purified compounds exhibited relatively stronger antibacterial activity than the extract. Thymol fraction exhibited stronger antibacterial activity than carvacrol. Thymol fraction exhibited MIC values ranging from 0.005 – 0.008 mg/mL while carvacrol fraction exhibited MIC values ranging from 0.007 – 0.008 mg/mL.

Keywords: *Thymus capitatus*, thymol, carvacrol, *Pseudomonas aeruginosa.*

In south of Jordan and other parts of the Middle East, the species *Thymus capitatus* (*T. capitatus*) of Lamiaceae family, is cultivated as a spicy herb commonly named Za'ater[1, 2]. Its edible parts, the young stems and leaves are consumed raw as salad ingredients, garnishes or seasonings additives for flavoring foods. Traditionally it is used as medicinal plant in folk medicine, to provide remedy mainly for respiratory infections.

Chemically, plant essential oils are complex mixture derived mainly from terpenes and their hydroxylated compounds. They have many biological applications as antibacterial, antifungal and antioxidant agents in addition to their functions as resources for pharmaceutical industries[3-7]. The biological activities of essential oils depend on their chemical composition which varies according to several conditions such as geographical origin, environmental, agronomic, stage of plant development and the extraction method[8, 9].

Thymus plant essential oils possess several pharmacological properties, such as spasmolytic, antiseptic, expectorant, antispasmodic, and anti-inflammatory effects[10-12]. The antimicrobial activities of thyme essential oil are mostly attributed to the active monoterpene phenolic components[13]. These terpene phenols interact with the amine and hydroxylamine groups of the proteins on bacterial membrane altering their permeability and resulting in bacterial death[14].

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Parallel to the decline in antibiotic discovery, the problem of antibiotic resistant microbes was emphasised. Usually, most common resistant bacteria in hospitals are *Staphylococcus aureus*, *Enterococci* and gram-negative rods, including the *Enterobacteriaceae* and *Pseudomonas aeruginosa*[15]. The *Pseudomonas aeruginosa* (*P. aeruginosa*) is an opportunistic pathogen causing life threatening infections and septic mortality in burn patients particularly when nosocomially acquired[16]. Furthermore, in burn population there is great risk for the transmission of resistance from one species of *Pseudomonas* to another as well as to other gram-negative organisms including *Enterbacter* sp., *Acinetobacter* sp., and *Escherichia coli*[17]. Because of the multi-resistant potential, *P. aeruginosa* strain has adopted high virulence ability and limited susceptibility to antimicrobial agents which render the treatment of this opportunistic infection very complicated[18]. Numerous medicinal plants are utilized as antimicrobial crude drugs or sources for novel compounds with anti-microbial activity that can kill antibiotic-resistant bacterial strains by alternative modes of action[19].

In a preliminary work, we have shown previously that the ethanol but not aqueous extract of leaves from Jordanian *T. capitatus* exhibited broad spectrum antibacterial activities[20]. The present work was undertaken to assess the antibacterial potential of purified essential oil components including thymol and carvacrol from this herb species against the multi-drug resistant strain *P. aeruginosa* isolated from burn patients. Such antibacterial activity towards the opportunistic bacteria was compared with certain Gram positive and Gram negative strains.

MATERIALS AND METHODS

Plant materials

Collective samples of the aerial parts from *Thymus capitatus* growing wild in south district of Jordan were cultivated during summer season. The taxonomic identification of *T. capitatus* was characterized at the department of biology, Mutah University - Jordan. The plant leaves were separated from the stem, and subjected to soxhlet extraction.

Extraction

The finely ground sample (15 g) of *Thymus capitatus* leaves was extracted with ethanol using Soxhlet apparatus for 24 h. The mixture was filtered and dried using a rotary evaporator. The final dried materials were stored in labelled sterile bottles and kept as aliquots until it was used.

Isolation of thymol and carvacrol

The ethanol extract of *T. capitatus* leaves was suspended in a diluted solution of 100 ml pure toluene according to Pothier *et al.*[22]. The standard solutions for thymol and carvacrol were prepared using 96% ethanol (1 mg per 1ml). The suspensions were mixed by vortex and filtrated through sterile syringe filter with 0.2-0.45µm.

In order to determine the best mobile phase, several mixture of hexane–ethyl acetate or toluene–ethyl acetate were prepared and evaluated using TLC. Spots and bands on TLC plats were visualized by UV irradiation (254 and 366 nm). Rf was determined by dividing the Distance travelled by spots (mm) on the distance travelled by solvent front (mm).

The isolated thymol and carvacrol were identified by comparing their Rf with the Rf of the standard thymol and carvacrol (purchased from Sigma). They were identified also by comparing their lambda max (λ) with the lambda max (λ) of the standard thymol and carvacrol. Lambda max (λ) was determined using Spectrophotometer from 200-400 nm. The λ_{thymol} and $\lambda_{carvacrol}$ were 292 and 288, respectively.

Chromatography

The isolation of thymol and carvacrol were done using normal phase column chromatography. The stationary phase (Silica gel 70-230 mesh) was packed in a glass column (5 cm x 1.5 m) and eluted with toluene–ethyl acetate (99:1 to 95:5, 90:10 to 70:30). The eluent was collected in fractions of 1 mL. The chemical profile of each fraction was evaluated using thin-layer chromatography (TLC) and visualized with UV (254 nm and 365 nm). The Rf for each fraction was determined and the similar fractions were collected to give 25 fractions (F1-F25). Comparing with the R*f* of the standard thymol (R*f* 48) and carvacrol (R*f* 45), and by using lambda max, fractions that contains thymol and carvacrol were companied together. Sub-fraction contains thymol and carvacrol was further purified using normal phase column chromatography eluted with toluene–ethyl acetate (90:10 then 93:7). The fractions were

collected in 0.5 mL and the similar fractions were companied together to yield thymol fraction and carvacrol fraction.

Antimicrobial activity

Microorganisms

The test bacterial strains *Escherichia coli*, *Enterobacter aerogenes* and *Staphylococcus aureus* were obtained from the Dr. Khaled Khleifat (Department of Biology, Mu'tah University (Jordan)). Their morphological characteristics were re-verified and their biochemical identity was verified using the REMEL kit (RapIDTM ONE and RapID TM NF plus systems) procedure by Dr. Khaled Khleifat. While *Pseudomonas aeruginosa* was obtained from Mr. Amjed Al-Tarawneh. Later on *P. aeruginosa* was isolated clinically from hospitalized infected burn patients. The isolated *P. aeruginosa* was characterized by using Api 20 NE testing system by Mr. Amjed AlTarawneh. The test organisms were sub-cultured at 37°C and maintained on nutrient agar media.

Disc diffusion method

The essential oil extract or the standard or the isolated thymol and carvacrol were dissolved in 10 % aqueous dimethyl sulfoxide (DMSO). The samples were filtrated through sterile syringe filter with 0.2 - 0.45 mili-pore. An inoculum containing 10^6 bacterial cells/ml was spread on nutrients agar plats. Then sterile filter papers (6 mm diameter) containing the tested samples were laid down on the surface of inoculated agar plate together with a negative control. The plates were incubated for 24 h at 37°C and the zone of bacterial growth inhibition was measured as millimeter diameter[23].

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

Minimum Inhibitory Concentration (MIC) was performed as previously described[24] with some modification. MIC was measured by determining the smallest amount of extract, the isolated thymol or carvacrol, the standard thymol or carvacrol or standard antibiotic needed to inhibit the growth of a test microorganism. Using 50 mL flasks and nutrient broth containing different concentrations of the samples or solvent control and the test microorganism (10^7 CFU/mL) were incubated at 37°C. After 24 h incubation periods, the turbidity was measured at 600 nm.

The MBC was determined by transferring and spreading the treated culture broth of the sample containing the concentrations equal to and higher than the MIC on agar plates. MBC was interpreted as the plate that showed no growth at all after incubation at 37°C for 24h.

RESULTS AND DISCUSSION

Tawaha and Hudaib[21] showed that the main components of the essential oils of the *Thymus capitatus* leaves collected from Jordan were p-cymene (11.8 %), borneol (2.7 %), carvacrol (37.3 %) and caryophyllene oxide (1.6 %), and the lowest contents of γ-terpinene (3.7 %), thymol (26.0 %), and β-caryophyllene (2.5 %). Regarding the previously reported chemical composition of *T. capitatus* essential oil elsewhere, Bounatirou *et al.*,[2] showed that the main components of the oils were oxygen-containing phenolic monoterpenes, carvacrol and thymol in Tunisian *T. capitatus*. Recently, carvacrol (68.19%) and thymol (12.29%) were found to be the main compounds of the Libyan *T. capitatus* essential oil[7]. Cosentino *et al.*,[25] reported that *T. capitatus* from Italy contains thymol and p-cymene as major components of its essential oil. Mkaddem and co-authors[26] reported thymol (89.06%) as a major component of *T. capitatus* Hoff. et Link. essential oil followed by p-cimene (5.04%) and γ-terpinene (3.19%). The composition of essential oils from a particular species of plant can differ between harvesting seasons and between geographical sources[27].

In this study, the isolation of thymol and carvacrol from *T. capitatus* was performed using normal phase chromatography. Based on the literature, several trails were performed to isolate thymol and carvacrol using variable mobile phase. Tabanca and co-authors[28] reported the purification of thymol and carvacrol using column chromatography eluted with n-hexane-ethyl acetate (100:0 to 70:30). In another study, thymol and carvacrol were purified successfully using normal phase column chromatography eluted with different proportional of hexane-toluene, toluene-ethyl acetate and ethylacetate-ethanol[29]. In this study, toluene–ethyl acetate (90:10 then 93:7) was found to be the most suitable mobile phase to separate thymol and carvacrol[22].

The antimicrobial activities of extract, thymol and carvacrol fractions and the standard thymol and carvacrol were qualitatively and

quantitively assessed using disc diffusion method and MIC and MBC.

In this study, *T. capitatus* leaves extract exhibited broad spectrum activity as it was successfully inhibited the growth of all tested microorganism with varying degree (table 1). Results of the disc diffusion method showed that *P. aeruginosa* was the most sensitive strain (22 mm) followed by *S. aureus*, *E. aerogenes* and *E. coli*.

Thymol and carvacrol fractions and the standard thymol and carvacrol exhibited similar manner of inhibition as *P. aeruginosa* was the most sensitive strain followed by *S. aureus*, *E. aerogenes* and *E. coli*. The purified compounds exhibited relatively stronger antibacterial activity than the extracts. In general, the results also showed that there were no significant differences between thymol and carvacrol fractions and the standard thymol and carvacrol. The inhibition zones for P. aeruginosa were 22 and 23 for thymol fraction and standard thymol, respectively, and they were 19 and 22 for carvacrol fraction and standard carvacrol, respectively.

Quantitative analysis for the tested samples was evaluated using MIC and MBC (Table 2). The results of inhibition zone were reflected in lower MIC values. In most cases, the MIC was close to the MBC, indicating a strong antibacterial action of the tested samples. Again, *P. aeruginosa* was the most sensitive strain. Thymol exhibited stronger antibacterial activity than carvacrol. Thymol fraction exhibited MIC values ranging from 0.005 – 0.008 mg/mL while carvacrol fraction exhibited MIC values ranging from 0.007 – 0.008 mg/mL.

In this study, the ethanol extract of *T. capitatus* leaves produced positive antimicrobial activities against the multiresistant strain of *P. aeruginosa*. This plant extract also showed broad spectrum antibacterial activity towards the Gram positive bacterial strain *S. aureus* and the Gram

Table 1. Antibacterial activity using Disc Diffusion Method (20 µg/disc)

Bacteria	Inhibition zone (mm)					
	Extract	Fraction		Standard		Ciprofloxacin
		thymol	carvacrol	thymol	carvacrol	
E. coli	14	19	18	19	18	25
E. aerogenes	15	22	20	23	21	25
P. aeruginosa	22	28	19	28	22	26
S. aureus	18	26	25	26	24	25

Table 2. MIC and MBC (mg/mL)

Organisms	Fraction				Standard			
	Thymol		Carvacrol		Thymol		Carvacrol	
E. coli	0.008	>0.008	0.008	>0.008	0.0075	>0.008	0.008	>0.008
E. aerogenes	0.01	>0.013	0.008	>0.008	0.0075	0.013	0.008	>0.008
P. aeruginosa	0.005	0.0055	0.007	0.0073	0.005	0.0055	0.0065	0.0073
S. aureus	0.0065	0.0066	0.007	0.0075	0.006	0.0066	0.006	0.0075

negative strains *E. aerogenes* as well as *E. coli*, but the growth inhibition was less strong than the inhibition observed with *P. aeruginosa*.

Our finding on the antibacterial activities of *T. capitatus* cultivated from south of Jordan is different from the antibacterial activities observed with other geographical species of this plant which either displayed weak or insignificant growth inhibition against *P. aeruginosa*[25, 30, 31]. Several studies pointed to possible differences in antibacterial and antioxidant activities among thymus species collected from various geographical regions[27, 32].

In present work, primarily thymol fraction had the strongest antimicrobial action against *P. aeruginosa*. The potency of thymol antibacterial

activities was indicated by the extremely low MIC and MBC values. A previous report showed that 96% of the *P. aeruginosa* inhibition by oregano oil can be attributed to the combined effect of hydroxylated monoterpenes while the remaining 4% of bacterial inhibition comes from the other oil components[33]. In fact, the general antibacterial activities of thyme oil are believed to be mediated by the combined actions of their thymol and carvacrol phenolic constituents[31, 34]. These two hydroxylated monoterpenes are capable of disintegrating the outer membrane of *P. aeruginosa*[33] as well as other bacterial strains[33, 35]. The hydrophobicity of the aromatic ring is an important characteristic of these phenolic components, which enable them to expand and partition the lipids of bacterial cell membrane[35-37] causing leakage of critical molecules and ions that eventually lead to cell death[38]. Also, the benzene hydroxyl group and the delocalized electrons of aromatic ring provide further support to their membrane permeating function[37].

Despite the similarity in mechanism of antibacterial action between thymol and carvacrol isomers, their modes of interaction with the bacterial membrane seem to be different. It was observed previously that the position of hydroxyl groups in the phenolic molecule causes distinctive influence on thymol and carvacrol inhibition power against acetylcholinesterase enzyme[39] or the growth of *Bacillus cereus*[37]. Considering that the interaction of these hydroxylated phenolic compounds with the bacterial cell membrane is protein mediated[14], it is feasible to emphasize the critical role that can be played by the monoterpene hydroxyl group in their affinity of interaction with the bacterial membrane. In accordance with this observation, we detected a difference in the inhibition strength between purified thymol and carvacrol against various bacterial strains including *P. aeruginosa*.

Very few crude essential oils that were reported to exhibit an effective antibacterial activities towards *P. aeruginosa*[11,28,34]. However, the majority of plant essential oils proved to have either negative or insignificant inhibition effect on this particular pathogenic bacteria though they showed broad spectrum effect against other bacterial strains like Gram positive *S. aureus* and Gram negative *E. coli* [5, 6, 31, 40-44]. Regardless, of their plant origin those positively active essential oils against *P. aeruginosa* are predominantly characterized by having rich thymol chemotype. In contrast those essential oils with little or negative *P. aeruginosa* inhibition seem to possess carvacrol as prevalent chemotype.

Our data showed that the multiresistant strain *P. aeruginosa* is more vulnerable to the purified thymol or carvacrol monoterpenes than to the *T. capitatus* extract. Nascimento *et al.*,[45] reported on eugenol as the principle component of lemon-palm extract, being 10 times more lethal against the growth of *K. pneumoniae* than the plant extract mixture. This lethal action of eugenol was abolished when it was mixed with cinnamic acid. Also, the inhibition of *S. aureus* by thymol and carvacrol oregano essential oil was found to be reduced due to interference from other components in the plant mixture[33]. Altogether these observations suggest a significant difference between the antibacterial efficiency of free individual thymol or carvacrol compounds compared with their *in situe* activity within the plant mixture, which seem to have more impact on carvacrol than on thymol actions. This limitation in antibacterial efficiency might be ascribed to the low affinity of carvacrol for interacting with the *P. aeruginosa* membrane, which was more pronounced when this monoterpene is present within the plant mixture than when it is present freely. Further work is still needed to verify the significance of this weak interaction between carvacrol and *P. aeruginosa* membrane**.** Also, to find out if the weakness is due to a decrease in the ratio of thymol to carvacrol in the plant mixture or the result of a major antagonistic in sensitization on carvacrol from other components in the mixture.

In conclusion, the *Thymus capitatus* active components thymol and carvacrol proved to have the potential as an effective alternative treatment against the multiresistant strain of *P. aeruginosa* that may cause opportunistic infection in thermal injury patients.

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