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In vitro Antimicrobial, Cytotoxic and Radical Scavenging Activities and Chemical Constituents of the Endemic *Thymus laevigatus* (Vahl)

Mohamed Al-Fatimi[1*], Martina Wurster[2], Gudrun Schröder[3] and Ulrike Lindequist[2]

[1]*Faculty of Pharmacy , Department of Pharmacognosy, Aden University, P.O. Box 5411, Maalla, Aden, Yemen*

[2]*Institute of Pharmacy, Department of Pharmaceutical Biology, Ernst-Moritz-Arndt-University Greifswald, 17487 Greifswald, Germany*

[3]*Friedrich Löffler Institute of Medical Microbiology, Ernst-Moritz-Arndt-University Greifswald, Germany*

Abstract: The leaves of *Thymus laevigatus* (Vahl), Lamiaceae (Labiatae), an endemic species of Yemen, are traditionally used in the treatment of various disorders including stomach and respiratory system. In a first biological and chemical study of this endemic species we investigated antimicrobial, cytotoxic and antioxidant activities of different extracts of the leaves of this plant. The preliminary phytochemical screening of extracts composition was performed by TLC while the composition of the essential oil was determined by GC-MS. Twelve constituents were detected from the essential oil, which constituted 99.6 % of the total amount. The major constituents of the oil were: carvacrol (84.3 %), p-cymene (4.1 %) p-mentha-1, 4-diene (4.0 %) and *trans*-anethole (3.6%). The main active components were identified by TLC as carvacrol and anethole for dichloromethane extract and as non-volatile phenols and flavonoids for the methanol extract. The methanol, dichloromethane and aqueous extracts were tested for their antimicrobial activities against five bacteria strains and six human pathogenic fungi. Both methanol and dichloromethane showed strong activities against most human pathogenic strains. In the contrast, methanol extract showed broader and stronger antibacterial activities than the dichloromethane extract, especially against the Gram-negative bacterium *Pseudomonas aeruginosa.* The methanol extract showed the same strong radical scavenging activity in the DPPH assay (14.9µg/ml), when compared to the standard antioxidant, ascorbic acid. In contrast, the cytotoxic activity of the methanol against FL cells, a human amniotic epithelial cell line, was only moderate (IC_{50} 298, 8 µg/ml). On the contrary, the water extract did not show any biological activity. Results presented here suggest that the essential oil and extracts of *Thymus laevigatus* possess strong antimicrobial and antioxidant properties, and therefore, they can be used as a natural preservative ingredient in food and/or pharmaceutical industry.

Keywords: *Thymus laevigatus*; Essential oil; Phytochemical; Antimicrobial activity; Antioxidant activity; Cytotoxicity.

* Corresponding author: E-Mail: alfatimi@web.de

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1. Introduction

The genus *Thymus* which belongs to the family Lamiaceae (Labiatae) includes 350 species widespread all around the world. Several studies investigated earlier the chemical compositions of the essential oils of numerous species of the *Thymus* genus and focused on their antimicrobial activities [1-5]. Recently, the essential oils of various species of the *Thymus* genus have been screened for their traditional indigenous uses and investigated intensive as promise sources of antibacterial, antifungal, antioxidant and other natural products. Therefore, several EOs of the *Thymus* species have been studied to investigate their chemical composition and antimicrobial activities [6-21]. The most species of *thymus* contain phenolic monoterpenes, thymol and/or carvacrol. Therefore, various species studied for their antioxidant activities [16-27]. Comparison to the EOs, only few extracts from the used parts of the genus species was studied to investigate their biological and chemical properties [19-23, 28-31].

In the flora of Yemen, this genus is represented by only the species *Thymus laevigatus* (Vahl) (Lamiaceae, Labiatae), which is endemic to Yemen. It occurs in the higher mountains in North Yemen in Haggah (2500 m) and in Dhamar (2200 m) [32]. The Yemeni local name of the plant is Za'tar. In Yemeni folk medicine, the fresh and dried leaves of *Thymus laevigatus* are used as powder in warm milk, sesame oil or olive oil to treat different stomach diseases, cough, tonsillitis, pharangitis, and renal colic [33].

In continuation of our research program to investigate the properties of ethnopharmacological used Yemeni plants [34-35], we report here for the first time some chemical and biological properties of the Yemeni endemic plant, *T. laevigatus*. In this study, the essential oil obtained from *T. laevigatus* was analyzed for its chemical composition was investigated. In addition, the extracts of the dried leaves of this plant were studied for their antimicrobial, antioxidant and cyotoxicty activities and screened for their phytochemical characters using TLC method. To the best of our knowledge, no literature information is available on the chemical compositions and biological activities of the EOs or extracts of the Yemeni endemic *Thymus laevigatus*. Therefore, this work is the first report about the chemical compositions of the essential oil and the antimicrobial, antioxidant and cytotoxic activities of the leaves extracts of the plant.

2. Materials and Methods

2.1. Plant Material

The leaves of *Thymus laevigatus* were collected in March 2009 from Haggah, North Yemen, (Mountain, altitude 2500 m). The collected plant was taxonomically identified at the Pharmacognosy Department Aden University, Aden, Yemen. A voucher specimen (MAF-H 001) of the plant material has been deposited at the Pharmacognosy Department, Aden University, Yemen.

2.2. Extraction

2.2.1. Dried leaves extraction

The *T. laevigatus* leaves were allowed to air dry and afterwards pulverized in grinder. Thirty grams of the fine pulverized materials were successively extracted with 300 ml of dichloromethane, 300 ml of methanol and 300 ml of water at room temperature for 8 h. After that the extracts were concentrated under reduced pressure at 40 °C, freeze dried and stored in exsiccator until use.

2.2.2. Essential oil extraction

Dried leaves (15 g) of *T. laevigatus* were hydrodistilled for 3 h in a Clevenger type apparatus according to European Pharmacopoeia. The oil yield was calculated on a dry weight basis by moisture free. The obtained essential oil was subsequently dried over anhydrous Na_2SO_4 and stored at 4 °C before analysis. The essential oil was subjected to GC-MS analysis.

2.3. Determination of chemical compositions

2.3.1. Gas Chromatography-Mass Spectrometry for essential oil

Analytical GC-MS system consisted of an Agilent® G1530 N gas chromatograph and a mass selective detector (Agilent® G2588 A Network MSD). Injection was done with Agilent® G2613A, Series Injector (Split 1:20 at 250 C, 2.0 µL; carrier gas: helium 1.0 mL/min (60 kPa) at 110ºC; pressure rise: 6 kPa/min). The MS operated in the electron impact mode with ionization energy of 70eV. The oven program started with 1min at 70°C, the oven temperature was increased at 3°C/min to 220°C. Full scan mass spectra were acquired from 35-350 *m/z* at a rate of 4.51scans/s and with a 5.00 min solvent delay. Chromatography was performed using a 30 m DB-5MS column (J&W Scientific, Folsom, USA) with 0.25 mm i.d. and 0.25 µm film thickness. The detected compounds were identified by processing of the raw GC-MS data with ChemStation G1701CA software and comparing with NIST mass spectral database 2.0 d (National Institute of Standards and Technology, Gaithersburg, USA) and from retention times and mass spectra of standard compounds. Relative amounts of detected compounds were calculated based on the peak areas of the total ion chromatograms (TIC).

2.3.2. Phytochemical screening for the extracts

The screening of the chemical constituents was carried out with the extracts of the *Thymus laevigatus* leaves, using chemical reagents and thin layer chromatography (TLC) methods according to the methodology suggested by Wagner [36].

2.4. Determination of antimicrobial activities

2.4.1. Microorganisms

The following bacterial strains were employed in the screening: *Staphylococcus aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 6059), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Micrococcus flavus* (SBUG 16). As fungal strains *Candida maltosa* (SBUG 17), *Candida albicans* (ATCC 90028), *Candida krusei* (ATCC 90878), *Aspergillus fumigatus* (13550/99), *Trichophyton mentagrophytes* (05/2004) and *Absidia corymbifera* (100798) were used. The SBUG strains were obtained from the strain collection of the Institute of Microbiology (SBUG) and the strains 13550/99, 05/2004 and 100798 from the Friedrich-Loeffler-Institute of Medical Microbiology of the Ernst-Moritz-Arndt-University Greifswald, Germany.

2.4.2. Antimicrobial assays

2.4.2.1. Agar diffusion method

Modified agar diffusion method [37] was used to determine antibacterial and antifungal activities. The bacterial cell suspension was prepared from a 24 h culture and adjusted to an inoculation of 1×10^6 colony forming units per ml. Sterile nutrient agar (Immunpräparate, Berlin, D, 26 g agar/l distilled water) was inoculated with bacterial cells (200 µL of bacterial cell suspension in 20 mL medium) and poured into dishes to give a solid medium. Yeasts and hyphomycetes (1×105 colony forming units per mL) were inoculated into sterile Mueller-Hinton-agar (Becton Dickinson, Heidelberg) according to DIN E 58940-3 [38] for the agar disc-diffusion assay. Forty microliters of test material (equivalent to 2 mg of the dried extract), dissolved in the same solvent which was used to prepare the tested extract, were applied on sterile paper discs (6 mm diameter, Schleicher and Schuell, D, ref. no. 321860). Ampicillin, gentamicin and nystatin were used as positive control, and the solvents dichloromethane and methanol as negative control. The solvents were allowed to evaporate in a stream of air. The discs were deposited on the surface of inoculated agar plates. Plates were kept for 3 h in refrigerator to enable prediffusion of the substances into the agar. Plates with bacteria were incubated for 24 h at 37 °C, plates with yeasts for 48 h at 36 °C and plates with hyphomycetes for 72 h at 30 °C. Inhibition zone diameters around each of the disc (diameter of inhibition zone plus diameter of the disc) were measured and recorded at the end of the incubation time. An average zone of inhibition was calculated for the three replicates.

2.4.2.2. Broth micro-dilution assay for minimum inhibitory concentrations (MIC)

The broth micro-dilution method described by Mann and Markham [39] with modifications was used to determine the MIC of extracts against the five standard bacteria strains. With sterile round-bottom 96-well plates, duplicate two-fold serial dilutions of extract (100 µL/well) were prepared in the appropriate broth containing 5 % (v/v) DMSO to produce a concentration range of 1000 to 50 µg of extract/mL. Two-fold dilutions of ampicillin were used as a positive control. A bacterial cell suspension (prepared in the appropriate broth) of 100 µL, corresponding to 1 × 106 CFU/mL, was added in all wells except those in columns 10, 11 and 12, which served as saline, extract and media sterility controls, respectively. Controls for bacterial growth without plant extract were also included on each plate. The final concentration of bacteria in the assay was 5 × 105 CFU/mL. The final concentration of extracts ranged between 1000 to 50 µg/mL. Plates were then incubated at 37 °C for 18 h overnight. After incubation, the MIC of each extract was determined as the lowest concentration at which no growth was observed in the duplicate wells. Twenty microliters of a *p*-iodonitro-tetrazolium violet solution (0.04 %, w/v) (Sigma, USA) was then added to each well. The plates were incubated for a further 30 min, and estimated visually for any change in color from yellow to pink indicating reduction of the dye due to bacterial growth. The highest dilution (lowest concentration) that remained yellow corresponded to the MIC. Experiments were performed in duplicate.

2.5. Determination of radical scavenging activity

The free radical scavenging activity was measured by using 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) assay. Qualitative determination was done as described in [40]. Quantitative estimation was carried out according to the method of [41]. The reaction mixture contained 500 µL of test extract and 125 µL of DPPH in ethanol. Different concentrations of test samples (10, 50, 100, 500 and 1000 µg/mL) were prepared while the concentration of DPPH was 1mM in the reaction mixture. These reaction mixtures were taken into Eppendorf tubes and incubated at 37 °C for 30 min, the absorbance was measured at 517 nm. Percent radical scavenging activity by sample treatment was determined by comparison with ethanol treated control group. Ascorbic acid was used as positive control. The DPPH radical concentration was calculated using the following equation:

Radical scavenging activity (%) = $Abs_{control}$ - $Abs_{sample}/Abs_{control} \times 100$

2.6. Cytotoxicity assay

The cytotoxicity was measured by the neutral red uptake assay [42] using FL-cells, a human amniotic epithelial cell line [43]. Only living cells are able to take up and accumulate neutral red. FL-cells were cultivated in a 96-well microtiter plate (105 cell/mL EAGLE-MEM, Sifin, Berlin, D, 150 µL/well) at 37 °C in a humidified 5 % carbon dioxide atmosphere. The EAGLE-MEM was completed by l-glutamine (0.10 g/L), HEPES (2.38 g/L), penicillin G (105 IE/L), streptomycin sulphate (0.10 g/L) and FCS (Gibco, 80.0 mL/L). After 24 h 50 µL of the extract solution (extract dissolved in ethanol under stirring in ultrasonic bath for 5 min and then diluted with MEM: test mixture) or medium with equal amounts of ethanol (control) were added. After a further incubation for 72 h cells were washed three times with phosphate buffered saline solution. One hundred microliters of neutral red solution (SERVA, 0.3% in EGLE-MEM) were added per well. The cells were then incubated for 3 h at 37 °C, followed by another three times washing with PBS. One hundred microliters of a solution of acetic acid (1 %, v/v) and ethanol (50 %, v/v) in distilled water were added. After shaking for 15 min, the optical density was measured at 540 nm with an ELISA-Reader HT II (Anthos Labtec Instruments Salzburg, A). The mean of four well measurements for each concentration was determined (n = 3).

3. Results and Discussion

3.1. Chemical composition of the essential oil

In the course of our screening for the biological and pharmacological activities of natural products from Yemen, the endemic plant *Thymus laevigatus*, used in Yemeni traditional medicine, was evaluated. Hydrodistillation of the dried leaves of *Thymus laevigatus* offered the essential oil in a yield of 0.41 % on dry weight basis. The obtained oil had a yellow colour and a pleasant smell. The composition of the EO is presented in Table 1. The GC-MS analysis of the oil allowed the identification of twelve compounds which accounted for 99.6 % of the oil (Table 1). There have been no reports on GC-MS analysis of the EO.

Table 1. Chemical composition of essential oil of the leaves of *Thymus laevigatus*

RI	Compounds[a]	Content %
987	β-Pinene	0.1
1007	p-Mentha-1,3-diene	0.2
1015	p-Cymene	4.1
1041	p-Mentha-1,4-diene	4.0
1146	Borneol	0.8
1271	p-(1-Propenyl) anisole (=Anethole)	3.6
1295	Carvacrol	84.3
1358	Carvacryl acetate	1.1
1462	α-Caryophyllene	0.6
1541	α-Bisabolene	0.3
1581	Spathulenol	0.4
1690	(Z,E)-Farnesol	0.1
	Total identified	99.6

[a]Compounds listed in order to their elution on the DB-5 column
Retention indices on the DB-5 column relative to C10-C20 *n*-alkanes

The essential oil from *T. laevigatus* showed a high content of oxygenated monoterpenes (86.2%) and low contents of monoterpene hydrocarbons (8.4%), sesquiterpene hydrocarbons (0.9%), and oxygenated sesquiterpenes (0.5%). Also worth noting, we detected one phenylpropanoid derivative p-(1-propenyl) anisole (anethole) (3.6 %), which found for the first time in the EOs of the species of *Thymus* genus (Table 2). The sesquiterpenes fraction attained lower amount (1.4%) in the oil, two sesquiterpene hydrocarbon compounds α-caryophyllene (0.6 %), α-bisabolene (0.3 %) and two alcohol sesquiterpenes spathulenol (0.4) and farnesol (0.1%). The essential oil was characterized by the content of the hydrocarbone monocyclic monoterpenes, p-cymene (4.1 %), the precursor of carvacrol, p-mentha-1,4-diene, (4.0 %) and p-mentha-1,3-diene (0.2 %) and of hydrocarbone bicyclic monoterpene, β-pinene (0.1 %). It contains the oxygenated monoterpenes (stearoptenes) carvacrol (84.3%), borneol (0.8 %) and carvacryl acetate (1.1 %). The predominant compound among the EO components was carvacrol (84.3%) while the amount of all other components of the oil was less than (16%) (Table 1).

Table 2. Class composition of *T. laevigatus* composition

Class of compounds	**Content%**
Monoterpenes hydrocarbon	8.4
Monoterpenes oxygenated	86.2
Sesquiterpenes hydrocarbon	0.9
Sesquiterpenes oxygenated	0.5
Phenylpropanoid	3.6
Total	99.6

The comparison of our results with those of the literature showed that the chemical composition of the endemic Yemeni species *Thymus laevigatus* is markedly different from that of other *Thymus* species. Different species of *Thymus* genus were reported for their extracts and EOs constituents, from those, with presence of phenolics monoterpenes thymol and/or carvacrol as main or minor constituents. Most reported species EOs contained thymol beside its isomer carvacrol with average of (3.3-66.3%) for thymol and (3-58.9%) for carvarol, from those were *T. persicus*, *T. eriocalyx* [11], *T. tosevii*, *T. caramanicus,T. vulgaris, T. pallescens* and *T. pulegioides* [6, 22, 44-46]. Certain reported species characterized by the presence of thymol without or with small amout of its isomer carvacrol, such as *T. ciliatus, T. transcaspicus, T. carnosus, T. daenensis* and *T. dreatensis* [47-49, 31, 45]. Some reported species contain only trace amount of thymol and no carvacrol such as *T. serpyllum* and *T. aureopunctatus* [50-51]. On the contrary, few *thymus* species contain high amount of carvacroal and small amount of thymol, such as *T. Caramanicus* and *T. Pubescens* [22,52]. To the best of the knowledge, our results found that the EO of *T. laevigatus* contains carvacrol without presence of thymol. On the other hand, very few EOs of *Thymus* species contain neither thymol nor carvacrol, such as *T. camphorates* and *T. mastichina* [24]. In GC-MS analysis we could show that *T. laevigatus* contained carvacrol (84.3 %) as main constituent and higher than in other *Thymus* species except *T. caramanicus* (85.9) [22]. Additionally, thymol or other phenol monoterpenes could not be found in the oil. Another new result is the detection of anethole (3.6 %) as a new component in the essential oil of a *Thymus* species. This finding suggests the occurrence of a new chemotype for the *Thymus* genus.

3.2. Phytochemical screening of the extracts

The results of the phytochemical screening of the different extracts are presented in Table 3. The phytochemical screening of the leave extracts of *Thymus laevigatus* indicated that the non-polar compounds are removed from the plant material during extraction into the dichloromethane extract, which contained most essential oils components, such as hydrocarbons terpenes, oxygenated terpenes

(carvacrol) and phenylpropanoid derivative (anethole) besides non-volatile components like triterpenoids. The methanol extract contained non-volatile compounds like flavonoids and phenolic compounds. On the other hand, the water extract lacks phenolic compounds. In generally, TLC analysis of the dichloromethane extract revealed the presence of volatile components with carvacrol and anethole as main active constituents while the analysis of the methanol extract revealed the presence of the non-volatile components with flavonoids and phenols as its main active constituents (Table 3).

Table 3. Phytochemical screening of the extracts

Extracts	**Phytochemical constituents**
Dichloromethane	volatile oils, terpenoids, sesquiterpenoids, triterpenoids, phenylpropanoids, tannins
Methanol	flavonoids, tannins, anthraquinones, phenolic compounds
water	saponins, reducing sugar

To the best of our knowledge, the essential oil and extracts composition and the antimicrobial and antioxidant activities of *T. laevigatus,* have been not reported before and therefore our results can be evaluated as the first report about the antimicrobial and antioxidant properties in respect to the chemical composition. The investigation of the different extracts of the *Thymus* species increased during the last years to obtain non-volatile constituents, such as flavonoids [26-28] besides the investigation of the volatile constituents, such phenol terpenes and to study their biological activities.

3.3. Antimicrobial activities of the extracts

The results of the antibacterial and antifungal screening of dichloromethane, methanol and water extracts of the dried leaves of *T. laevigatus* against five bacteria and against six human fungi species are summarized in Table 4 (inhibition zones in the agar diffusion assay) and Table 5 (MIC values). An inhibition zone > 15 mm in the agar diffusion assay was considered as a high antimicrobial activity. The controls utilized to evaluate the antimicrobial activities of plant extracts are standard antibiotics. Both dichloromethane and methanol extracts showed stronger antibacterial activity against *S. aureus* (50µg/mL) in comparison with reference drug. In the contrast, both of extracts showed only moderate activities against *B. subtilis* and *E. coli*. Dichloromethane extract showed weak activity against *M. flavus* and moderate activity against *P. aeruginosa* while methanol extract showed very strong activity against *M. flavus* (250 µg/mL) and against *P. aeruginosa* (125 µg/mL). Interestingly, the specific antibacterial activity of methanol extract activity against Gram-negative bacterium *P. aeruginosa* (125 µg/mL) that was better and stronger in comparison with the reference drug Gentamicin (Table 4 and 5).

On other hand, both methanol and dichloromethane extracts showed better and stronger antifungal activity than of the reference drug, against *T. mentogrophytes* (inhibition zones 40 mm and 42 mm, 25 mm resp.) and *A. fumigatus* (inhibition zone 40 mm and 30 mm, 25 mm resp.), but nystatin has better antifungal activity against *A. corymbifera*. In the contrast, the methanol extract showed better antifungal activity against all three *Candida* species in comparison to dichloromethane extract and to the reference drug. To the best of the knowledge it is the first time to identify the human antifungal activities of *Thymus* species against the fungi strains *A. corymbifera, T. mentogrophytes and T. Mentagrophytes* (Table 4). On the contrary, water extract has no antimicrobial activity.

Our results showed that the dichloromethane extract was more active against Gram-positive than against Gram-negative bacteria. This result confirmed the evidence that Gram-positive bacteria

are more susceptible to essential oils contents than Gram-negative bacteria [4-8, 53-60]. It seems that the responsible non-polar antibacterial components of the EO of the plant could be isolated with the solvent dichloromethane. Several studies have identified the volatile phenol, carvacrol, as responsible compound for the antimicrobial activity of the essential oils of *Thymus* species [54-61]. Anethole was also, reported as main antimicrobial component of EOs of other species belongs to Lamaiceae [29, 62-65]. Both active compounds indicated through TLC analysis in DCM extract. Therefore, the strong antibacterial activity of DCM against *S. aureus* and its strong narrow antifungal activity thus are related to the major predominant volatile phenol component, carvacrol and to the phenylpropanoid, anethole. The finding, that anethole was reported for the first time in the *Thymus* genus, indicated its specific synergic antifungal activities in the DCM extract. On other hand, volatile terpenes alcohols, such as borneol [54-55,66] and momonterpene hydrocarbons, such as p-cymene [54-56] and pinene [18] were reported to exhibited synergic antimicrobial effects. Therefore, the monoterpene alcohols and hydrocarbons that indicated in the DCM extract can be support additive antimicrobial effects.

Table 4. Antimicrobial activity of the extracts of *Thymus laevigatus* leaves, investigated with agar diffusion test.

Microorganisms strains	**Diameter of Inhibition zone (mm)** [a]					
Bacterial strains	**Extracts**			**Standard**		
	D	**M**	**W**	**Nys.**	**Amp.**	**Gen.**
Bacillus subtilis (ATCC 6059)	15	15	8	NT	28	NT
Escherichia coli (ATCC 25922)	15	15	8	NT	NT	15
Micrococcus flavus (SBUG 16)	8	20	8	NT	31	NT
Pseudomonas aeruginosa (ATCC 27853)	15	20	8	NT	NT	18
Staphylococcus aureus (ATCC 29213)	35	30	10	NT	26	NT
Fungal strains						
Absidia corymbifera (100798)	25	26	8	28	NT	NT
Aspergillus fumigatus (13550/99)	40	30	8	25	NT	NT
Candida albicans (ATCC 90028)	20	28	8	26	NT	NT
Candida krusei (ATCC 90878)	21	29	8	25	NT	NT
Candida maltosa (SBUG 17)	10	25	8	25	NT	NT
Trichophyton mentagrophytes (05/2004)	40	42	8	25	NT	NT

[a]Values are inhibition zone diameter (mm); D, dichloromethane; M, methanol; W, water; (conc, 2 mg/disc); Amp, ampicillin (10 µg/disc); Gen, gentamicin (10 mg/disc); Nys, nystatin (100 µg/disc); NT: not tested; negative controls did not show any activity.

In the case of methanol extract, TLC analysis identified the presence of the non-volatile components of the plant, phenols and flavonoids. Our results indicated that the combination of non-volatile phenols and flavonoids in methanol extract insert better and stronger antibacterial and antifungal activities compared with the combination of the volatile components of DCM extract. Interestingly, our results showed that the combination effects of methanol extracts had the best synergetic effect against all tested bacteria strains, especially against Gram-negative bacterium *Pseudomonas aeruginosa*, which was reported as antibiotic-resistant and weak or moderate sensitive to the other *Thymus* species oils or extracts, such as *Thymus revolutus* [15]. This suggests that the non-volatile polar compounds of *T. Laevigatus* leaves, such as flavonoids, were soluble in methanol could be the main active compounds in this plant. Our previous study reported that methanol is a better solvent for extraction of antimicrobial natural compounds from some Yemeni medicinal plants, than DCM and water [34-35]. This observation was evidenced in our present study. On the other hand, the lack of antimicrobial activity of the water extract is, probably, due to the absence of the phenolic components.

Table 5. MIC values of the antibacterial activity of the extracts of *Thymus laevigatus* leaves

Bacteria strains	**MIC in µg/mL**				
	Plant Extracts			**Standard**	
Gram-positive	**D**	**M**	**W**	**Ampicilin**	**Gentamicin**
Bacillus subtilis	>1000	>1000	>1000	NT	NT
Micrococcus flavus	>1000	250	>1000	0.25	NT
Staphylococcus aureus	50	50	>1000	0.05	NT
Gram-negative					
Escherichia coli	>1000	>1000	>1000	NT	NT
Pseudomonas aeruginosa	>1000	250	>1000	NT	NT

D, dichloromethane; M: methanol; W: water; NT: Not tested.

Several studies have demonstrated the antimicrobial activity the essential oils and/or the extracts of many species of the genus *Thymus* rich in volatile phenols and/or volatile alcohols [4-31,66-71]. The comparison of our results for the endemic species *Thymus laevigatus* with those from the literature for other *Thymus* species revealed that the antimicrobial activities of the extracts of the leaves of the Yemeni endemic species were markedly broader and stronger than those of the essential oils or extracts of other *Thymus* species. *T. vulgaris* [3,44], *T. persicus, T. eriocalyx* [11], *T. baeticus, T. kotschyanus, T. pectinatus, T. eigii,* and *T. daenensis* [5,8,20-21,31] showed antibacterial activity less than of *Thymus laevigatus*. The essential oils of *Thymus vulgaris* L., *Thymus tosevii* L. [6], *Thymus pulegioides* [46] showed antifungal activities against other fungi strains. The most of the species of *Thymus* genus have been investigated for their antimicrobial properties against some bacteria and fungi, depending on thymol and its isomer carvacrol [8,11,22,44] and other species depending on thymol as main constituents [3,14,31,51,45]. We report here for the first time the antimicrobial activities of the endemic Yemeni plant *T. laevigatus* depending on its content of carvacroal as main constituent. Otherwise the content of carvacrol (84.3 %) in the Yemeni endemic plant *T. laevigatus* is higher than in other *Thymus* species. The antimicrobial effects of carvacrol are described already for carvacrol as component of oregano [72]. We assume that mainly carvacrol is responsible for the observed antimicrobial activities of *T. laevigatus* and that anethole causes synergic effects. There are no reports on the antimicrobial activity of essential oils or their major constituents towards human pathogenic fungi in comparison to their activities against plant pathogenic fungi [4, 15, 46, 70]. The high amounts of carvacrol (84.3%) with low amounts of all other volatile components less than (16%) and the presence of anethole in the plant are responsible for its significant antimicrobial activities. Moreover the non-volatile phenols in methanol extract showed better and stronger antimicrobial activity than the reported *Thymus* species extracts, which showed different antimicrobial activities depended on their volatile contents.

3.4. Antioxidant activities of the extracts

The results of the antioxidant activities of the dichloromethane, methanol and water extracts were comparable with the effect of ascorbic acid in Table 6. The DCM extract showed an antioxidant activity (28.0 µg/mL); only two times lower than that of the reference drug ascorbic acid (14.7 µg/mL). In contrast, the methanol extract showed an antioxidant activity (14.9 µg/mL), two times stronger than that of the DCM extract. It showed similar antioxidant activity to the reference drug ascorbic acid (14.7µg/mL). On the contrary, the activity of the aqueous extract was very small (Table 6). The results of this study clearly indicate that *T. laevigatus* contains non-polar and polar antioxidants, which could be separated by the solvents DCM and methanol. These data were further supported by phytochemical screening, indicating that the strong antioxidative potential of the DCM

extract was closely related to the high content of carvacrol [22, 73-76] and low content of to the anethole [62, 63, 77], which have been reported as antioxidants natural compounds. Anethole, which is reported in *Thymus* genus for the first time, was responsible with carvacroal for the antioxidant activities the in dichloromethane extract. The DCM extract was showed stronger antioxidant activities in comparison with the carvacrol and with EO of *T. caramanicus* which contains the highest carvacrol content (85.9%) [22]. This result guide to the observation, that the combination of one phenol monoterpene compound with one volatile phenylpropanoid compound, can insert stronger synergistic effect on the antioxidant potential than that of the combination of two different phenol monoterpenes. Additionally, our result supported the observation that the presence of carvacrol in high content more than (80%) and the other volatile components in small amount less than (16%) of oil showed better antioxidant [22]. The antioxidative capacity of the DCM extract of *T. laevigatus* could be attributed to the presence of high amount of carvacrol and low amount of anethole, which were better and stronger than that of the reported combination of carvacrol and its isomer thymol in other *Thymus* species [16-19]. We thought that the presence of other volatile phenolic compounds more other than carvacrol insert antagonist effect on the antioxidant of the oil.

Table 6. Cytotoxic activity against FL cells and free radical scavenging activity in the DPPH assay for the extracts of *Thymus laevigatus* leaves

Extracts	**IC_{50} (μg/mL)**	
	Free radical scavenging activity	**Cytotoxicity against FL-cells**
Dichloromethane	28.0	276.6
Methanol	14.9	298.8
water	>1000	>1000
Ascorbic acid	14.7	-

In the contrast, the active components of the methanol extract were identified through TLC method as non-volatile phenols and flavonoids. The antioxidant activities of non-volatile phenols and high polarity phenolic components and flavonoids have been reported in the literature [23,26,27,75]. The measured antioxidant activity of the methanol extracts of *T. laevigatus* was stronger than that of other *Thymus* species EOs [16-19, 24] and extracts [20-23]. These results suggest that the combination of non-volatile phenols and flavonoids in methanol extract exhibits stronger antioxidative activity than the combination of carvacrol and anethole in DCM extract.

In summary, our results clearly demonstrated that dichloromethane and methanol extracts of *T. laevigatus* displayed a potent antimicrobial with antioxidant effect. Furthermore, the antimicrobial and antioxidant activity of *T. laevigatus* seems to depend on the carvacrol, anethole and the flavonoids, as main active components. The non-polar extract obtained from *Thymus laevigatus* was effective scavengers of free radicals. The use of polar solvent, such as methanol resulted in the isolation of more effective fractions, which contained higher concentration of phenolic components as compared with the some fractions isolated with lower polarity solvent (dichloromethane).

3.5. Cytotoxic activities of the extracts

The results of cytotoxicity of the extracts on the FL-cell lines were shown in Table 6. The cytotoxicity of the DCM extract was stronger than that of the methanol. Carvacrol and anethole are also known to possess modest cytotoxic activity. Phenylpropanoids were more studied for their cytoctoxicity than the monoterpenes [78-80]. Therefore, the cytotoxicity of the DCM extract could be due to the synergistic effects of anethole with the main constituent carvacrol. The other monoterpenes and sesquiterpene compounds present small percentage; therefore they could not be the main compounds responsible for the observed cytotoxic activity of the DCM extract. These results indicated

that the volatile phenol component of *T. laevigatus* showed more cytotoxic activity than the non-volatile components of methanol extract.

4. Conclusion

To our knowledge, this is the first study on the phytochemical investigation of the extracts and of the essential oil of dried leaves of *T. laevigatus*. The endemic plant oil contains no phenol terpenes except carvacrol with highest content (84.3%), while the other volatile components presented less than (16%). Moreover, the oil contains anethole, as a phenylpropanoid derivative. These results suggest the finding of new chemotype for the *Thymus* species.

Besides, it is the first report to provide data about the biological activities of the dichloromethane, methanol and water extracts obtained from the leaves of endemic *T. laevigatus* commonly used in Yemen as folk medicine. In summary, the antimicrobial and antioxidant activities of the methanol extract were better and stronger than of dichloromethane extract. But both of the extracts showed the same antibacterial and antifungal potency against some antimicrobial strains. On the contrary water extract was inactive. Interestingly, the methanol extract of *Thymus laevigatus* possess strong antifungal potential against all human pathogenic fungi and against most bacteria strains, specially against *Pseudomonas aeruginos*, which resists all single actions of synthetic drugs. These results indicate that the non-volatile components of the methanol extract are considered to represent low risk for resistance development by pathogenic microorganisms. Moreover, methanol extract showed stronger antioxidant and less cyotoxic activity than DCM extract. Our phytochemical results showed that the volatile active components carvacrol and *trans*-anethole in the EO could be isolated by the non-polar solvent DCM. The new finding of this chemotpe caravcrol/anethole explains the specific biological activities of DCM extract. On the contrast, the active components of the methanol were identified as non-volatile phenolic and flavonoids components.

In conclusion, the plant can also represent a useful source of antimicrobial and antioxidative volatile and non-volatile constituents, which can easily isolate by the non-polar solvent DCM and the polar solvent methanol. Therefore, it is suggested that further studies should be performed on the isolation and identification of the active non-volatile components of the methanol extract of the plant. These results may be providing a starting point for the investigations to exploit new natural antimicrobial and antioxidant substances.

In conclusion, our results corroborate the importance of ethnopharmacological surveys in the selection of plants for biological screening. Also, the extensive use of this plant by the local people in Yemen as a general antiseptic (mouth, throat, skin) to treat respiratory infections and gastro-intestinal disorders can be related to the antimicrobial activity. This study validates the Yemeni traditional preparations of this plant as oil and as leaves powder. The results showed that the use of polar solvent resulted in the isolation of more effective extracts. Therefore, it could be advantageous to use the leaves of the plant as a tincture as it possesses strong antimicrobial activity and to get the highest antioxidant activities. The use of the crude drug as leaves powder contains all the active volatile and non-volatile components of the plant. The plant can be considered as a promising natural source for nutraceuticals and herbal medicinal preparations and used as natural bactericidal and fungicides or as a synergic agent with antibiotics.

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