

Chemical compounds, *in vitro* antitumor and antibacterial activities of *Trachyspermum copticum* L essential oil

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ABSTRACT

Trachyspermum copticum is one of the most important Iranian Apiaceae species which is widely distributed in the northern region of Iran. In this study, chemical compounds, anti-tumor and antibacterial activities of *T. copticum* essential oil were evaluated. The essential oil obtained by Clevenger apparatus and then the chemical composition was analyzed by GC-MS. Antibacterial activity of essential oil was evaluated by well diffusion method. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by the macrodilution method. An MTT cytotoxicity assay was employed to test effects of the essential oil on each human breast cancer cell lines. The GC-MS spectrums showed 9 major compounds, in which the highest chemical composition was related to thymol (42.16%), γ -Terpinene (31.49%) and *p*-cymen (23.29%) compounds. *T. copticum* essential oil showed good antitumor activity on SKOV3 and MDA-MB-231 cell lines (IC_{50} of 208.13.6 μ g/ml and IC_{50} 236.16 μ g/ml, respectively). It also showed good activity against tested bacteria. The MIC and MBC values of the bacterial strains sensitive to the essential oil were in the ranges of 0.4 to 3.1 mg/ml and 0.4 to 6.25 mg/ml, respectively. Because of the high concentration of phenolic compounds, the essential oil from *T. copticum* exhibited antimicrobial and antitumor activities, which deserve further research into the chemoprevention and treatment of human ovary and breast cancers as well as infectious diseases.

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Keywords

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INTRODUCTION

The use of medicinal plants as antimicrobial and anti-inflammatory drugs in folk medicine is a practice common in Iran, although in most cases the active principles of the plants are unknown [1]. Essential oils are the odorous, volatile products of aromatic plants secondary metabolism, normally formed in special cells or groups of cells, found in many leaves and stems [2]. Due to the presence of antimicrobial compounds, they have a potential as natural

agents for the food preservation [3]. The antimicrobial activity of essential oils is assigned to a number of small terpenoid and phenolic compounds, which also in pure form have been shown to exhibit antibacterial or antifungal activity [2, 4]. However, there are often large differences in the reported antibacterial activity of oils from the same essence. The reasons for this variability can be due to the geographical sources, the harvesting seasons, the genotype,

the climate, the drying and the distilled part of the plant which are significant factors influencing the chemical composition and relative proportions of the individual constituents in the essential oils of the plant [5, 6].

In recent years, multiple drug resistance in human pathogenic microorganisms has been developed due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases. This situation forced scientists to search for new antimicrobial substances from various sources like medicinal plants [7]. *Trachyspermum copticum* L is a genus of the well-known medicinal plant of *Apiaceae* family and growing wild in the Mediterranean area [8]. It has aromatic and medicinal characteristics and has been also used to treat various ailments such as cramps, muscle pains, nausea, indigestion, diarrhea, and infectious diseases [9, 10]. *T. copticum* is one of the most important of Iranian *Apiaceae* species which is widely distributed in the northern region of Iran [11]. Recently, many studies have focused on antibacterial and antifungal activity of the essential oil or extracts of *T. copticum* [12, 13].

In the present work, a wide range of potentially pathogenic and multidrug resistant bacteria was used to evaluate antibacterial activity of essential oil of the *T. copticum*. Antimicrobial activity was determined by the disc diffusion method. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of tested essential oils were determined using the agar dilution method. Furthermore, human breast tumor cell lines were used to determine anticancer activity of *S. bakhtiarica* leaf essential oil.

MATERIALS AND METHODS

Samples collection

Apparently healthy leaves of *Trachyspermum copticum* were collected during the flowering period, from the mountain region of Mazandaran Province in Iran and identified by the Department of Botany of the Sari Agricultural University. A voucher specimen was deposited in the Herbarium of Faculty of Agriculture.

Extraction of essential oil

Air-dried plant material (100 g) was hydrodistilled for 3 h using a Clevenger type apparatus. The essential oils were collected over water, separated and dried over anhydrous sodium sulfate. They were stored in sealed vials at 4–6°C prior to antimicrobial screening.

Gas chromatography mass spectrometry Analysis

GC-MS analysis of the oil was conducted using a Hewlett Packard 6890 instrument operating on EI mode and equipped with a 5MS-HP fused silica column (30 m × 0.25 mm × 0.25 µm film thickness capillary columns). Helium (99.99%) was used as the carrier gas at a constant flow of 1 mL/min. The oven temperature was held at 60°C for 1 min, then programmed to 210°C at a rate of 6°C/min, and then held for 10 min. The injector and detector (FID)

temperatures were kept at 250°C and 280°C, respectively. The components of the oil were identified by comparison of their MS with those obtained from authentic samples and/or the NIST/NBS and Wiley mass spectral database. They were also confirmed by comparison of their retention indices (RI) [14] and retention times (RT), either with those of authentic compounds or with published data [15].

Strains of pathogens

The microorganisms used in this study were *Staphylococcus aureus* ATcc 25923, *E. Coli* ATcc 25922, *Pseudomonasa aeruginosa* ATcc 27853, *Klebsiella pneumoniae* ATcc7881, *Serratia marcesens* ATcc 35668, *Shigella dysenteriae* ATcc 25923, *Shigella flexneri* ATcc 15305, *Enterobacter aerogenes* ATcc 25933 and *Proteus vulgaris* ATcc 25922 were provided from the Institute pasture, Tehran- Iran.

Test for Antibacterial Activity

The antimicrobial activity of essential oils was tested by the agar well diffusion method on Muller Hinton Agar (MHA). Using a cork borer, five wells (6 mm in diameter) were made in the agar medium (one in center and four wells were at corner) and inoculums containing 1.5×10^6 CFU/ml of the test bacteria were spread onto the surface of the medium with a sterile swab. In the case of essential oil, 10 µl, 20 µl, 30 µl, 40 µl and 50 µl of the essence was pipette into the wells, whilst 50 µl of DMSO served as a control. Gentamycin disk used as positive control. The agar plates were incubated for 24h at 37°C and the diameter of the zone of inhibition surrounding the wells was measured. Assays were performed in triplicate and the data are shown as the mean ± standard deviation (SD).

Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC of the essential oil against the test microorganisms was determined by the broth macrodilution method. An eight-fold serial dilution of the essence in DMSO, including 100, 50, 25, 12.5, 6.25, 3.12, 1.6, 0.8 and 0.4 mg/ml, was prepared in sterile test tubes. These dilutions were added to 1ml Muller Hinton Agar medium containing 1.5×10^6 CFU/ml bacteria. Two test tubes served as a positive and negative control, respectively. The test tubes were incubated at 35°C for 24h. The concentration at which complete inhibition of the growth was observed was recorded as MIC. To determine MBC, broth was taken from each well and inoculated in Mueller Hinton agar (MHA) for 24 h at 37 °C. The MIC and MBC for each of the test bacteria was determined in triplicate assays and the data are shown as mean ±SD.

Cell lines and cell culture medium

A normal cell line (human embryonic kidney cells, HEK) and cancerous cell lines (human breast cell MDA-MB-231 and human ovary cancer cell SKOV3) were used in this

study. All cell lines, which obtained from Pasteur Institute, Tehran, Iran, were cultured in RPMI 1640 medium (Sigma), supplemented with 10% inactivated fetal bovine serum (FBS) (Gibco), penicillin (100 U/ml) and streptomycin (100 mg/ml). The medium was then sterilized by filtering through 0.22 mm microbiological filters and kept at 4°C before use. Cells were grown at 37°C, under a 5% CO₂ atmosphere and at 90% humidity for 24h. Cell counts were determined.

In vitro cytotoxicity assay

The cytotoxic effects of the essential oils against tumor and normal cell lines were determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The cell suspension (1×10⁴ cells) was placed in a 96-well flat-bottomed tissue culture plate. After that the cells were treated with different volumes of essential oils. After determining the dry weight of essential oil (12 mg/ml), different concentrations were prepared in 1% DMSO-PBS solution. One hundred microliters of each concentration of essential oil (10, 25, 50, 100 and 200 µg/ml diluted in RPMI-1640 medium), were added into the plate. Cells treated with a cytotoxic drug, methotrexate, were used as a positive control, whereas untreated cells (DMSO) were used as a negative control. After 24 h incubation at 37°C, cell viability was evaluated using MTT assay. The assay was performed in triplicate for each of the concentrations. Briefly, the supernatants were removed from the wells and 200 µl MTT (Sigma) solution (2 mg/ml in PBS) was replaced. The plates were incubated for 2 h at 37°C, and 125 µl of DMSO was added to the wells to dissolve the MTT crystals. The plates were placed on a shaker for 15 min and the absorbance at 595 nm was read by a multi-well spectrophotometer. The mean of the cell viability values was compared to the control to determine the effect of the

essential oil on cells and percentage of cytotoxicity was plotted against concentrations of the extract. The percentage of cytotoxicity was calculated as following:

$$\% \text{ Cytotoxicity} = 1 - \frac{[(\text{OD extract treated} - \text{OD blank}) / (\text{OD control} - \text{OD blank})] \times 100}$$

Statistical analysis

Statistical analysis of the data obtained in the present study was carried out in a completely randomized design layout with three replicates using Stat graphics plus 2.0. All data are reported as means ±SD. Data were analyzed using SPSS (version 16.5). A probability of less than 0.05 was considered as statistically significant.

RESULTS

The qualitative and quantitative analyses of the *T. copticum* essential oil are depicted in Table 1. We found nine main constituents in which *thymol* (42.16%), *γ-Terpinene* (31.49%) and *p-cymen* (23.29%) were prominent.

The results regarding the preliminary tests on antibacterial activity of the essential oil from *T. copticum* against various bacteria using well diffusion are indicated in Table 2. The results show that bacterial growth was suppressed by *T. copticum* L. The inhibitory effect of the oils increased in proportion to their concentrations. It showed good activity against to all test bacteria, except for *Ps. aeruginosa*, compared to Gentamicin antibiotic (as positive control). At 10 µl of the essential oils, *T. copticum* showed good inhibitory effect on *S. marcesens*, *S. dysenteriae*, *K. pneumoniae* and *S. flexneri* rather than Gentamicin (G.M). At 50 µl of the essential oils, *T. copticum* showed stronger inhibitory effects. *S. marcesens*, *S. dysenteriae* and *S. flexneri* were the most sensitive bacteria to essence. Control treatment (DMSO) did not show an inhibitory effect on any

Table 1. The composition of the essential oil from *T. copticum*

	Compounds	<i>T. copticum</i> (%)	RI
1	β-Myrcene	2.7%	988
2	α-Terpinene	31.49%	1017
3	thymol	42.16%	1268
4	p-Cymene	23.29%	1010
5	γ-Terpinene	29.4%	1060
6	β-Terpinene	0.6%	919
7	β-Phellandrene	2.3%	985
8	α-Terpinolene	0.3%	927
9	Sabinene	0.4%	1280

Table 2. Antibacterial activity of *T. copticum* essential oil

Bacteria	Inhibition zone (mm)					
	10µl	20µl	30µl	40µl	50µl	G.M
<i>E. Coli</i>	17.22±0.65	22.73±0.15	24.15±0.55	27.75±0.65	33.66±0.15	20.23±0.15
<i>K. pneumoniae</i>	20.15±0.15	25.65±0.2	28.35±0.15	30.15±0.25	33.25±0.14	13.23±0.25
<i>S. aureus</i>	20.73±0.73	26.13±0.15	29.13±0.13	33.73±0.35	35±0.13	21.35±0.35
<i>S. marcesens</i>	24.15±0.3	30.15±0.73	33.23±0.5	36.15±0.15	44.13±0.23	20.15±0.13
<i>S. dysenteriae</i>	22-23±0.73	28.5±0.93	31.15±0.23	35.13±0.23	42.13±0.13	17.73±0.25
<i>S. flexneri</i>	21.13±0.13	28.15±0.13	30.73±0.75	33.93±0.5	40.15±0.23	18.13±0.13
<i>E. aerogenes</i>	19.73±0.13	23.65±0.2	25.14±0.25	28.13±0.3	31±0.18	20.13±0.2
<i>P. vulgaris</i>	21.13±0.5	27.13±0.25	30.13±0.23	33.92±0.15	36.1±0.16	21.15±0.35
<i>P. aeruginosa</i>	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	13.15±0.14

Table 3. MIC of essential oil of *T. copticum*

Bacteria	MIC (mg/ml)								
	100	50	25	12.5	6.25	3.1	1.6	0.8	0.4
<i>E. Coli</i>	-	-	-	-	-	+	+	+	+
<i>K. pneumoniae</i>	-	-	-	-	-	+	+	+	+
<i>S. aureus</i>	-	-	-	-	-	-	+	+	+
<i>S. marcesens</i>	-	-	-	-	-	-	-	-	+
<i>S. dysenteriae</i>	-	-	-	-	-	-	-	-	+
<i>S. flexneri</i>	-	-	-	-	-	-	-	+	+
<i>E. aerogenes</i>	-	-	-	-	-	+	+	+	+
<i>P. vulgaris</i>	-	-	-	-	-	-	+	+	+
<i>P. aeruginosa</i>	+	+	+	+	+	+	+	+	+

Table 4. MBC of essential oil of *T. copticum*

Bacteria	MBC (mg/ml)								
	100	50	25	12.5	6.25	3.1	1.6	0.8	0.4
<i>E. Coli</i>	-	-	-	-	+	+	+	+	+
<i>K. pneumonia</i>	-	-	-	-	-	+	+	+	+
<i>S. aureus</i>	-	-	-	-	-	+	+	+	+
<i>S. marcesens</i>	-	-	-	-	-	-	-	-	+
<i>S. dysenteriae</i>	-	-	-	-	-	-	-	+	+
<i>S. flexneri</i>	-	-	-	-	-	-	-	+	+
<i>E. Aerogenes</i>	-	-	-	-	+	+	+	+	+
<i>P. Vulgaris</i>	-	-	-	-	-	-	+	+	+
<i>P. Aeruginosa</i>	+	+	+	+	+	+	+	+	+

of the bacteria. Antibacterial activity of essential oil in all concentrations (from 10 to 50 µl) was found for tested bacteria.

Minimum inhibitory concentrations (MIC) of essential oil of *T. copticum* were determined against several strains of Gram-positive and Gram-negative bacteria (Table 3). As shown in Table 3, the MIC value of the sensitive bacteria was in the range of 0.4 mg/ml to 25 mg/ml. *Serrashia marcesens* and *Shigella dysenteriae* were found to be the most sensitive bacteria to essential oil showing the MIC of 0.4 mg/ml while *S. flexneri* ranked next with 0.8 mg/ml followed by *P. vulgaris* and *S. aureus* (1.6 mg/ml), *E. aerogenes*, *E. coli* and *K. pneumoniae* with MIC of 3.1 mg/ml. *Pseudomonasa aeruginosa* does not showed a sensitive to essential oil even in range of 100 mg/ml.

Minimum bactericidal concentrations (MBC) of essential oil of *Trachyspermum copticum* were determined against several strains of Gram-positive and Gram-negative bacteria (Table 4). *Serrashia marcesens* was found to be the most sensitive bacteria to essential oil showing the MBC of 0.4 mg/ml. While *S. flexneri* and *Shigella dysenteriae* were ranked next with 0.8 mg/ml followed by *P. vulgaris* (1.6 mg/ml), *S. aureus* and *K. pneumoniae* (3.1 mg/ml), *E. coli* and *E. aerogenes* with MBC of 6.25 mg/ml. *Pseudomonas aeruginosa* didn't show a sensitive to essential oil even in range of 100 mg/ml.

Figure 1 illustrates the cytotoxicity effects of *T. copticum* essential oil at different concentrations ranging from 10 µg/ml to 400 µg/ml. Two cancerous cell lines, (human breast cell MDA-MB-231 and human ovary cancer cell SKOV3), were used to determine anticancer properties of *T. copticum* leaf essential oil. To determine the antitumor activity of essential oil against cancer cells, cytotoxicity

MTT assay was carried out. At maximum concentration (400 µg/ml), essential oil showed the highest activity on cancer cells. No significant difference was shown in the cytotoxicity activity of essential oil between both cancerous cells at the lowest concentration; however, SKOV3 cell lines (*IC*₅₀ of 208.13.6 µg/ml) were more sensitive than MDA-MB-231 cells (*IC*₅₀ 236.16 µg/ml). Its activity on normal HEK cell lines was also interesting compared with tumor cell lines. Normal HEK cell lines were least sensitive to

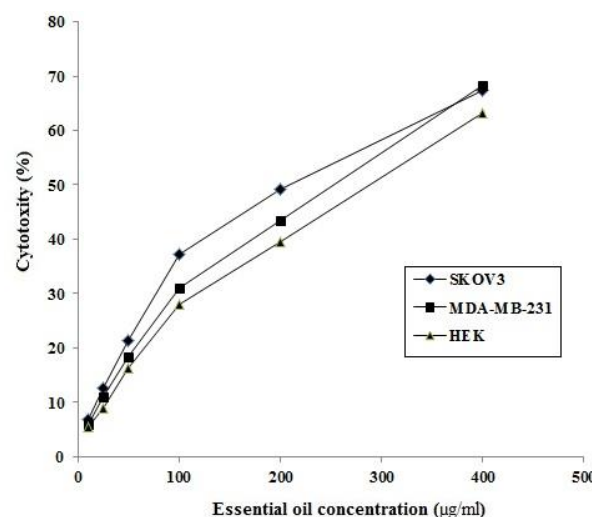


Figure 1. The dose-dependent cytotoxic effect on SKOV3, MDA-MB-231 and HEK cell lines of the essential oils (EO) of *T. copticum*. Cytotoxicity was measured as the reduced change in absorbance in cultures containing EO at 595 nm as compared with control untreated cultures. Each point represents the average from three separate measurements, each done in triplicate, and the standard deviation of the mean.

cytotoxicity of essential oil particularly at high concentrations (IC_{50} of 283.12 μ g/ml).

DISCUSSION

Recently, the acceptance of traditional medicine as an alternative form for health care and the development of microbial resistance to the available antibiotics have led authors to investigate the antimicrobial activity of medicinal plants [16, 17]. Plant extracts are extensively used in the traditional medicine of Iran. The essential oils have also been used as flavouring agents in food and beverages and, due to the presence of antimicrobial compounds, they have a potential as natural agents for the food preservation [3]. Moreover, researchers have been interested in biologically active compounds isolated from plant species for the elimination of pathogenic microorganisms because of the resistance that they have developed to antibiotics.

Recently, several studies have focused on the antimicrobial activity of the essential oil and extracts of *T. copticum*. For instance, Souri *et al.*, [13] evaluate the antioxidant activity of methanolic extracts of 24 selected plant materials (seeds or fruits), which are used by Iranian people as folk remedies and/or food supplements. The *T. copticum* extract had the most phenolic contents (919.12 \pm 34.67 mg/100g dry) compared with the other studied plants [13]. In another study, Rasooli *et al.*, [12] investigated the chemical compositions, antifungal activities and aflatoxin inhibition of the essential oils from *T. copticum* L. Chemical analysis of the components of the oils led to identification of 9 components in *T. copticum* L. The major components of *T. copticum* L. essential oil were Thymol (37.2%), *p*-Cymene (32.3%) and γ -Terpinene (27.3%) which was comparable with our study. In our study thymol (42.16%), γ -Terpinene (31.49%) and *p*-cymen (23.29%) were the major compounds in essential oil of *T. copticum* L. In addition to the antifungal activity, they have reported *T. copticum* had a good fungicidal activity. It killed more than 50% of spore population in 30 min and 90–100% lethal effects were observed within 210 min of the exposure to the oil. In our study, the *in vitro* antimicrobial activity of *T. copticum* L essential oil against some human pathogenic bacteria and its activity potentials were qualitatively and quantitatively assessed by the presence or absence of inhibition zones, zone diameters, MIC and MBC values. The results of the present study indicated that essential oil of *T. copticum* L have good antimicrobial effect on human pathogenic bacteria, which could be attributed to the high content of the essence, of compounds with known antimicrobial activity, such as thymol and *p*-cymene. However, the essential oil extract did not display any antibacterial activity against *Ps. Aeruginosa*. The basis of varying degree of sensitivity of test organisms of bacteria may to be due to the intrinsic tolerance of microorganisms and the nature and combinations of phytochemicals present in the essential oil [16].

Investigation the antitumor activity of *T. copticum* was another part of our study. Several recent studies have also reported the cytotoxicity properties of some plant essential

oils. For instance, in study by Yousefzade *et al.*, [18] they reported that *S. khuzistanica* essential oil significantly reduce cell viability of Vero, SW480, MCF7, and JET 3 cancer cells in a dose-dependent manner, with the IC_{50} values calculated for each cell type being, respectively, 31.2 μ g/ml, 62.5 μ g/ml, 125 μ g/ml, and 125 μ g/ml. Here, the *in vitro* anti-tumor activity of *T. copticum* essential oil against several human cancer cell lines was evaluated. Our results demonstrated that the essential oil from *T. copticum* has a good antitumor activity on human tested tumor cell lines. Approximately 50% of tumor cells growth was inhibited at 200–250 μ g/ml; however, it was lower than to other types particularly *Satureja* species. This evidence suggested *T. copticum* essential oil has a moderate antitumor activity against tested cancer cell lines. The antitumor property of the oils of *T. copticum* is most likely attributable to the phenolic compounds thymol, γ -Terpinene and *p*-cymene. Therefore, further study is needed in order to obtain information regarding the practical effectiveness of this essential oil to prevent the growth of tumor cells, and its use as an antitumor agent for cancer diseases in humans.

Activity of *T. copticum* is probably due to phenolic compounds. The volatile terpenes, γ -Terpinene and *p*-Cymene are seemed to be responsible for the antimicrobial activity of some essential oils. Antibacterial and antifungal activities of these substances have also been reported in previous studies [19, 20]. Some studies have concluded that whole essential oils have a greater antibacterial activity than the major components mixed [21, 22], which suggests that the minor components are critical to the activity and may have a synergistic effect or potentiating influence. In earlier investigations, *T. copticum* L have been studied with respect to essential oil composition and show to be rich in components such as carvacrol, γ -terpinene, thymol, and *p*-cymene [23–25]. Essential oils rich in phenolic compounds such as carvacrol are widely reported to possess high levels of antimicrobial activity [26, 27], which has been confirmed and extended in the present studies. This is the first study to provide data that the essence of *T. copticum* L plants evaluated against a wide range of microorganisms possess potential antibacterial. These results indicate potential of essential oils of *T. copticum* L. as natural preservatives in against to some multidrug human bacteria.

Based on these results, it is possible to conclude that the using the essential oil of *T. copticum* L as natural antibacterial, have a strong and broad spectrum of antibacterial activity against many human pathogenic bacteria, because the oil possesses strong antibacterial activity. The antibacterial property of the essences of the *T. copticum* L is probably attributable to the phenolic compounds such as thymol and to the *p*-cymene. Further study is needed in order to obtain information regarding the practical effectiveness of *T. copticum* L essential oil to prevent the growth of human pathogenic bacteria as antimicrobial agents in new drugs for therapy of infectious diseases in human.

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CONFLICT OF INTEREST

The authors declare that this research does not have any conflict of interest with anyone or any Institute.

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