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ANTIVIRAL AND ANTIMICROBIAL ACTIVITY OF *THYMUS TRANSCASPICUS* ESSENTIAL OIL

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Summary

In the present study, the antiviral and antimicrobial effects of *Thymus transcaspicus* essential oil were investigated. Plant essential oil was prepared by a hydro-distillation method using Clevenger-type apparatus. Antimicrobial activity of the EO was investigated by a broth microdilution method. The MIC of the essential oil for *Escherichia coli*, *Pseudomonas aeroginosa*, *Staphylococcus aureus*, *Bacillus subtilis*, *Klebsiella pneumonia* and *Candida albicans* were determined to be at the EO dilutions of 1:1600, 1:400, 1:2000, 1:5000, 1:2000 and 1:6000 (v/v), respectively. The antiviral activity of the oil was investigated using plaque reduction assay. After several enriching stages of phage CP51, phage titration was performed to determine the phage concentration in phage lysate. This was used to specify the dilution factor of the phage to be used as negative control for the next working stages. Then the IC_{50} of trifluridine (as a positive control) for phage CP51 was determined. To determine whether the oil had the ability to inhibit the adsorption of virus to the host cells, it was pre-incubated with phage CP51 for 30 min at 25 °C. The growth and reproduction of phage were inhibited (>50%) at 10^{-2}, 10^{-3} and 10^{-4} (v/v) EO dilutions. In order to test the effects of the oil on the transfection process, *Bacillus cereus*, phage CP51 and EO were incubated together. The growth and reproduction of phage were inhibited (>50%) at 10^{-2}, 10^{-3} and 10^{-4} (v/v) dilutions. These results indicated that the essential oil of *T. transcaspicus* had a moderate antimicrobial and antiviral activity.

Keywords: *Thymus transcaspicus*; essential oil; antiviral; antimicrobial; plaque reduction assay; microdilution method

Introduction

Treatment of infectious diseases continues to be problematic in modern time. With the available antiviral and antimicrobial drugs, the treatment often leads to the problem of resistance (1, 2). There is little likelihood that available orthodox antiviral drugs can eliminate all or even most viral diseases (3). The search for new antimicrobial substances exhibiting minimal side effects is warranted because of the severe side effects of some drugs currently in use (2, 4). So, there is an increasing need for new substances with antiviral and antimicrobial activity. Medicinal plants have been traditionally used for different kinds of ailments including infectious diseases (1). Therefore, the development of new medicinal plant products is vital in controlling the threats posed by some pathogenic microorganisms (3).

Lamiaceae (formerly Labiatae) is one of the most important plant families in which *Thymus* with about 215 species, is a significant genus (2). *Thymus* species are well known as medicinal plants because of their biological and pharmacological properties. In traditional medicine, the extracts of different species of *Thymus* have been widely used for the treatment of gastritis, athma, diarrhea and enuresis in children, bronchitis and whooping cough (Pertussis) (5).

Several studies have been shown that *Thymus* species have antibacterial (2, 6, 7), antifungal (6, 8-10), cytotoxic (9), analgesic (11), antiparasitic (9), topical anti-inflammatory (12), antispasmodic (13), mosquitocidal (14) and antioxidant (15, 16) activities. Antiviral effect of extracts from some plants of the Lamiaceae family against HSV-1 (1, 17) and HSV-2 (17) has been reported and the extract of *Thymus vulgaris* has been shown antiviral activity (17). Antioxidant (15) and antiemetic (18) effects of *Thymus transcaspicus* have been reported in some studies. Therefore, the present work was carried out to investigate the antimicrobial and antiviral activities of *Thymus transcaspicus* which grows wild in different parts of Iran.

Materials and Methods

Plant material

Thymus transcaspicus was collected from Tondure area in Dargaz road (Khorasan Province, Iran). It was identified in the Herbarium of the Mashhad School of Pharmacy (Iran) and a voucher specimen was deposited in the Herbarium of the Mashhad School of Pharmacy (Iran) with reference number: 153-2020-18. The aerial parts of the plant (leaves, stems and flowers) were air-dried and finally ground to a fine powder.

Composition of the essential oil

Water distilled essential oil from aerial parts of *T. transcaspicus* have been analysed by means of GC and GC/MS. Forty-seven compounds were identified, representing 99.5% of the total oil. The main compounds were thymol (56.4%), γ-terpinene (7.7%), carvacrol (7.6%) and *p*-cymene (6.3%) (19).

Extraction of the essential oil

The essential oil (EO) of the plant dried powder (100 g) was isolated by hydro-distillation for 4 h, using a Clevenger-type apparatus according to the method recommended in British Pharmacopoeia (20). The distillated oil was dried over anhydrous sodium sulfate and stored in tightly closed dark vials at 4 °C until use. The total oil was 3 mL (3% v/w) and yellow in color.

Media and buffers

Mueller Hinton Broth (Himedia, India) was used for preparation of microbial suspentions. Nutrient Agar was used for one night microbial cultures. Sabourated Dextrose Broth was used for preparation of *Candida albicans* suspention. Phage Assay Broth (PA Broth): Nutrient Agar 13 g L^{-1}, NaCl 5 g L^{-1}, at pH 5.6-6.0. Phage Assay Agar, consisted of PA Broth with the addition of 15 g L^{-1} agar, was used for *Bacillus cereus* culture to produce the phage. Phage assay top agar (PA Top Agar): consisted of PA Broth with the addition of 7 g L^{-1} agar, was used for plaque assay as the soft layer agar. 5 mL of the solution consisting of 40 g L^{-1} Mg(SO$_4$). 7H$_2$O, 10 g L^{-1} MnSO$_4$.H$_2$O and 30 g L^{-1} CaCl$_2$.2H$_2$O, was added to per litre of PA Broth, PA Agar and PA Top Agar. Soybean casein digest agar (SCDA): casein enzymatic hydrolysate 15 g L^{-1}, papaic digest of soybean meal 5 g L^{-1}, sodium chloride 5 g L^{-1}, agar 15 g L^{-1}. 5 μg L^{-1} gentamycin and 250 μg mL^{-1} ketoconazole solutions were used as positive controls for antimicrobial tests (21). All reagents and media except Muller Hinton Broth, were purchased from Merck, Germany.

Test organisms and growth conditions

Various Gram-positive and Gram-negative Standard bacterial strains including *Escherichia coli* (PTCC 0331), *Pseudomonas aeroginosa* (PTCC 7401), *Staphylococcus aureus* (PTCC 7331), *Bacillus subtilis* (PTCC 3201), *Klebsiella pneumonia* (PTCC 3501) and one fungi (*Candida albicans* PTCC 7205) were used for antimicrobial tests. Strains were obtained from Persian Type Culture Collection, Iranian Research Organization for Science and Technology (PTCC, Iran). In order to investigate antiviral effects, *Bacillus* phage CP51, and *Bacillus cereus* (ATCC 10876) as the host were used. *Bacillus cereus* Cultures were stored at -20°C in 15% glycerol (22) and a stock culture of the bacteria was maintained on SCDA plate.

Microbial cultures for antimicrobial testing were prepared by picking a single colony from 24-h-old Nutrient Agar plates and it was suspended in an appropriate medium (Mueller Hinton Broth for bacteria and Sabourated Dextrose Broth for fungi). 1 mL of each freshly grown suspension was diluted with proper growth medium to 10^6 cfu/ml (23).

Determination of minimum inhibitory concentration (MIC)

Dilutions of the essential oil were prepared in proper growth medium with addition of tween 80 (at a rate of less than 0.5%) (24). Antimicrobial activity of *T. transcaspicus* essential oil was investigated by broth microdilution method (25) as follows: 100 μl of each standarized microbial suspension (10^6 cfu/ml) was added to the wells of a 96-well microtitre plate already containing 100 μL of essential oil dilutions in proper medium. Final dilutions of the essential oil in each well were 1:400, 1:1600, 1:2000, 1:4000, 1:8000 and 1:12000 (v/v). Negative control wells were prepared with bacterial suspension and culture medium but no oil. Positive controls were wells with a bacterial suspension and dilutions of antimicrobial agent (gentamycine and ketokonazole,at the specified concentrations, for bacteria and fungi respectively). The plates were incubated either at 37 °C for bacteria or at 30 °C for fungi for 24 h. 40 μL of MTT solution (2 mg mL^{-1}) in water was added to each well and the plates were incubated at 37°C for 30 min. The lowest concentration of the plate without color change was determined as the MIC.

Antiphage activity assay

The antiviral activity of *Thymus transcaspicus* essential oil was investigated by using *Bacillus* phage CP51 (bacterial virus) and plaque reduction assay (26, 27).

Bacteriophage titration

The phage was propagated using the plate method in its host *Bacillus cereus* (22). Briefly, 100 µL of an aliquot phage sample (10-fold serially diluted with PA broth) was mixed with 100 µL of *Bacillus cereus* suspention (10^8 cfu ml^{-1}) in PA broth (prepared from an overnight SCDA culture), in a sterile 1.5 mL tube and incubated for 15 min at 37°C to facilitate attachment of the phage to the host cells. The mixture was added to 2.3 ml of soft layer agar (PA Top Agar) cooled to 45 °C, then well mixed by swirling and poured over the PA Agar plate and allowed to sit for 15 min at room temperature. The plates were incubated for 18 h at 37°C and a plate showing almost confluent plaques was used to prepare a concentrated phage suspension by overlaying with 5 mL of PA broth. The over layer medium containing the phage CP51 was decanted and filtered through a 0.22 µm syringe filter. The filtrate was used as a phage stock solution. Several dilutions of phage solution were made.

Phage inactivation assays

Either pre-incubation or no pre-incubation antiphage activity assay was done (26, 27).

Pre-incubation protocol Different dilutions of the essential oil in PA broth medium with addition of 0.5% tween 80 (24) were prepared and filter sterilized. One loopful of *B. cereus* from overnight culture was taken and inoculated into a PA Broth medium (10 mL). The medium was mixed thoroughly and incubated at 37°C for 5 h. 100 µL of phage in proper dilution was added to 500 µL sterile solution of extract and the mixture was incubated at 25°C for 30 min. Then, 500 µL of bacterial suspension and 1.9 mL of PA Top Agar medium were added. This mixture was overlaid onto a PA agar plate and incubated overnight at 37°C. The negative control contained all above except the extract solution which was replaced with PA Top Agar. In the positive control plate the extract was replaced with 500 µL trifluridine (Sina Darou Co.) in the IC_{50} concentration. Antiphage activity of the essential oil was noted by the absence of plaque formation in comparison with control where typical bacterial lysis accurs .

No pre-incubation protocol To a 500 µL sterile solution of extracts, 100 µL of phage in proper dilution, 500 µL of bacterial suspension and 1.9 mL of PA Top agar were added and the mixture was overlaid onto a PA agar plate and incubated overnight at 37°C. The negative and positive controls were like Pre-incubation protocol.

Statistical analysis

Experiments were performed in triplicate. The arithmetic mean ± SD of control and experimental results were calculated using the Student`s t-test. $P < 0.05$ was considered statistically significant.

Results

MIC values of *Thymus transcaspicus* essential oil were determined as an evaluation of its antimicrobial activity against selected bacteria strains and fungi. The essential oil showed the MIC values of 1:400 (v/v) and 1:1600 (v/v) agains *P. aeroginosa* and *E. coli*, respectively. *S. aureus* and *K. pneumonia* were inhibited by the essential oil with a MIC value of 1:2000 (v/v). The MIC for *B.subtilis* and the pathogenic yeast *C. albicans* were determined as dilutions of 1:5000 (v/v) and

1:6000 (v/v), respectively. There was no growth inhibition due to the effect of the solubelizer (Tween 80) control.

In order to determine the antiviral activity of the essential oil, the effect of different concentrations of trifluridin (TFT) on reduction of phage CP51 was investigated. IC_{50} for the Pre-incubation method was 138 µg mL^{-1} and for the No Pre-incubation protocol was 264 µg mL^{-1} (Fig. 1, 2). After examination of tween 80, no significant reduction of plaques was observed in both protocols.

After pre-incubation of different dilutions of *Thymus transcaspicus* essential oil with phage CP51 for 30 min, a significant reduction (>50%) in plaque forming unit was observed for dilutions of 10^{-2}, 10^{-3} and 10^{-4} (v/v). At highest dilution (10^{-5} v/v) still significant reduction of plaque forming units was observed while at lowest dilution (10^{-2} v/v) no plaque was formed ndicating a 100% inhibition (Fig. 3).

When no pre-incubation protocol was used, a significant reduction (>50%) in plaque forming unit was observed for dilutions of 10^{-2}, 10^{-3} and 10^{-4} (v/v) while at highest dilution (10^{-5} v/v) still significant reduction of plaque was observed. At lowest dilution (10^{-2} v/v) no plaque was formed indicating a 100% inhibition (Fig. 3).
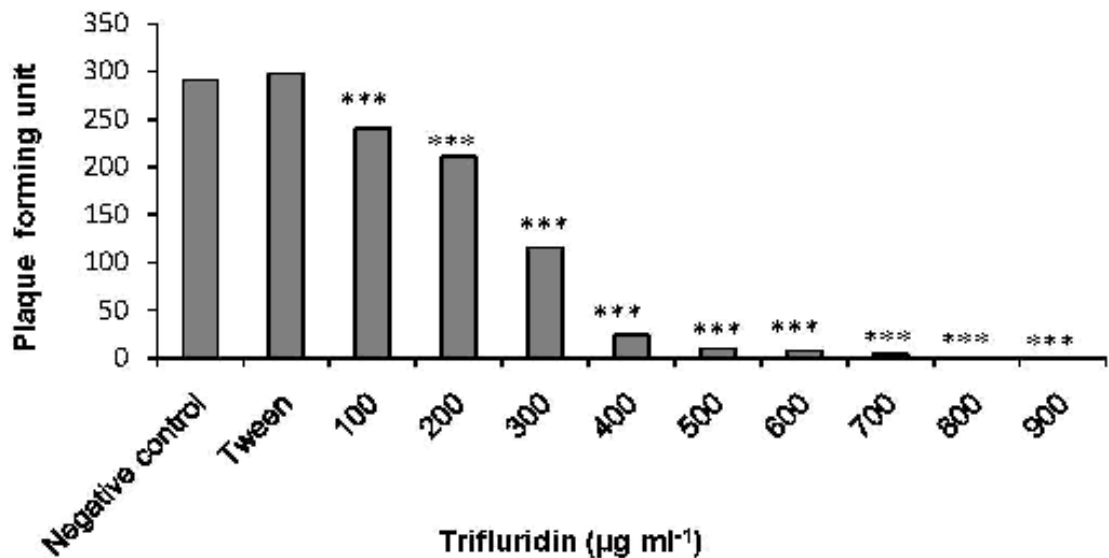

Fig. 1 Effect of different concentration of trifluridin (TFT) on reduction of phage CP51 using no pre-incubation protocol. Data are mean±SEM of three independent experiments.***p<0.001, Tukey-Kramer test
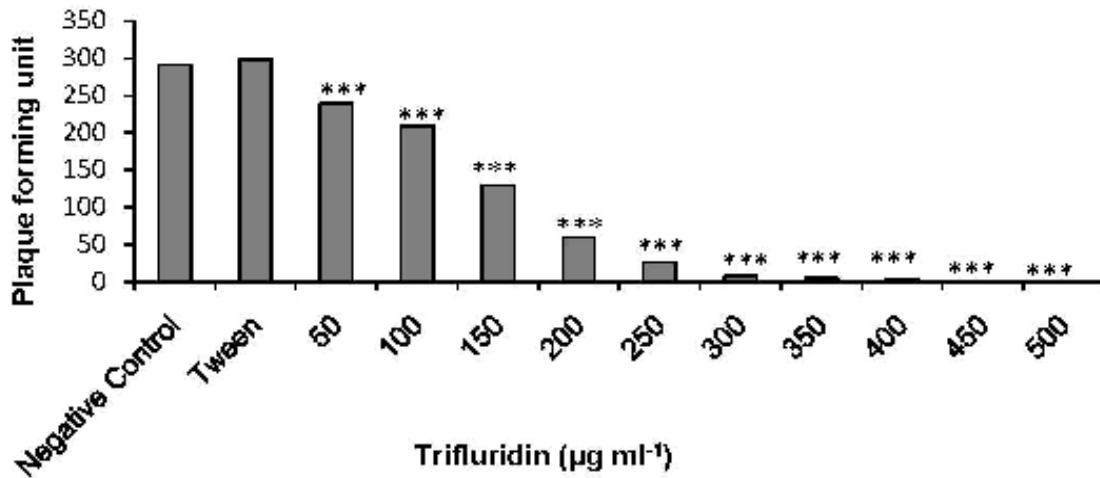
Fig. 2 Effect of different concentration of trifluridin (TFT) on reduction of phage CP51 using pre-incubation protocol. Data are mean±SEM of three independent experiments.***p<0.001, Tukey-Kramer test
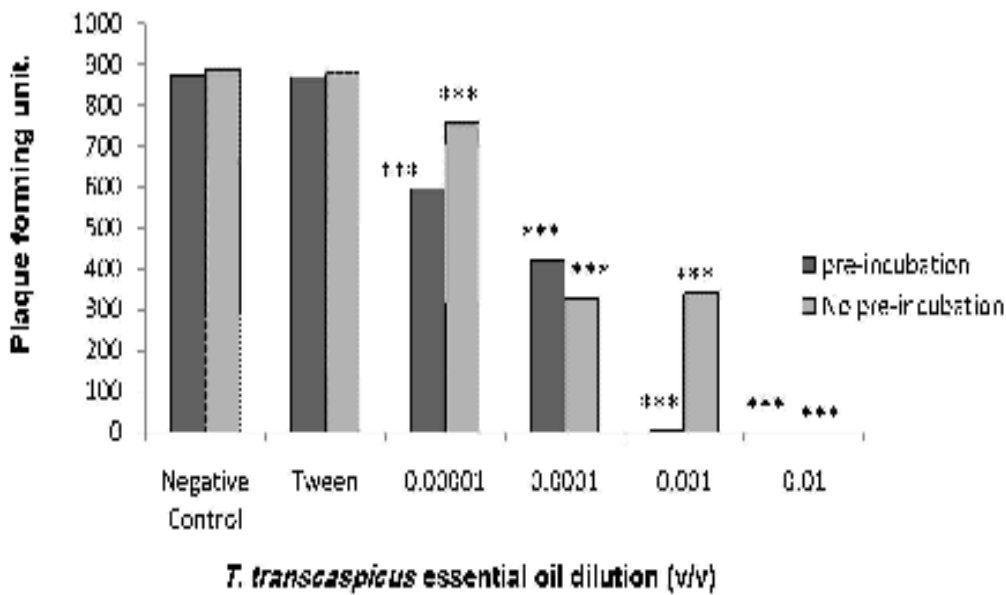

Fig. 3 Effect of different concentration of *T. transcaspicus* essential oil on reduction of phage CP51 using pre-incubation protocol   and no pre-incubation protocol. Data are mean±SEM of three independent experiments.***p<0.001, Tukey-Kramer test

Discussion

Antiviral or antimicrobial activity of some *Thymus* species have been shown in other previous studies. The extract of *Thymus vulgaris* has been shown a high antiviral activity against HSV-1 and HSV-2 (17). *Thymus pubescens* and *Thymus vulgaris extract demonstrated* good *antibacterial activity against some* drug resistant *Gram-positive bacteria (2, 7).* The essential oil of the *Thymus caramanicus* showed high inhibitory activity against *Helicobacter pylori* (28). Several compounds derived from Thymus species have been shown to have antimicrobial or antiviral activity for example, carvacrol, thymol and *p*-cymene isolated from *Thymus x viciosoi* displayed antifungal activity against *Candida, Cryptococcus, Aspergillus* and *dermatophyte* species. Amoung these compounds, *P*-cymene showed weaker activity (29), Monoterpene compounds such as γ-terpinene and *P*-cymene and thymol from thyme oil interacted in a dose-dependent manner with HSV-1 particles thereby inactivating viral infection (30). In a study, an antimicrobial activity test carried out with fractions of the essential oil of Thymus pectinatus Fisch. et Mey. var. pectinatus, showed that the activity was mainly observed in those fractions containing thymol, particular, and carvacrol (31). Therefore, the present study was carried out to investigate the antiviral and antimicrobial activity of *T. transcaspicus*.

T. transcaspicus EO was tested for its antibacterial activity against various Gram-positive and Gram-negative bacteria Standard strains and one fungi. All the bacteria and fungi were inhibited by the EO but in variable degrees.

The analysis of the essential oil composition indicated that thymol (64%) was the main component in the oil of *T. transcaspicus*. Other major components were identified as γ-terpinene (7.7%), carvacrol (7.6%) and *p*-cymene (6.3%). These results show a high content of phenolic compounds (64% thymol and carvacrol) (19). Thymol and carvacrol (from essential oils) have been shown fungitoxic effects *against* Cryptococcus neoformans opportunistic fungus (32). Inhibition of *Staphylococcus aureus* (33) and antibacterial effects against *E. coli* (34) by thymol and carvacrol have been reported. Carvacrol also has been reported to exhibit a dose dependent inhibitory effect on *Vibrio cholerae* in food (35). Thymol, which is the main component of many *Thymus* spp. and also in the oil of *T. transcaspicus* (64%), is known as an antiseptic agent (19). The antimicrobial activity of *T. transcaspicus* EO was, therefore, attributed to the presence of Thymol. Other constituents of the essential oil such as gamma-terpinene and p-cymene, could be also taken into account for their possible synergistic or antagonistic effects effects.

Most extracts or phytochemicals act as antiviral via two mechanisms including exhibiting their effects on viral particles prior to attachment to host cell or after the virus enters the host cell for example, flavonoids were reported to exhibit antiviral effects via inhibiting the RNA synthesis of viruses and polyphenols act principally by binding to the protein coat and thus arrest absorption of the virus, they also reported to inhibit viral replication enzymes (such as RT for HIV and RNA polymerase for influenza virus) (3). Therefore, to differentiate between these two mechanisms we used either protocol where phage was pre-incubated with the EO prior to its exposure to *B. cereus* or without any pre-incubation with phage. The results indicated that the EO exerted its antiphage activity in either method but slitely higher in Pre-incubation protocol suggesting that the EO approximately alike inactivate the phage before attachment of phage to the host cells and after it.

Inactivating viral infection of HSV-1 particles by monoterpene compounds such as γ-terpinene and *P*-cymene and thymol from thyme oil have been reported (30). With respect to preliminary phytochemical screening of *T. transcaspicus* EO, the antiviral activity of the oil could be attributed to its monoterpene compounds especially thymol which is the main component of the oil (64%).

As a conclusion, this study confirms that the essential oil of *T. transcaspicus* has a moderate antimicrobial and antiviral activity. It is a good candidate for further antiviral testing using human viruses. The present study together with previous analysis supports the antibacterial properties of the *T. transcaspicus* EO. Additional clinical trials of this oil has to be performed if it is to be used for medicinal purposes.

Acknowledgments

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