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Original Article

Evaluation of Antibacterial Activity and Total Phenolic and Flavonoid Content of *Tanacetum fruticulosum* Ledeb Against 6 Bacterial Strains

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ABSTRACT

Following previous investigations on different species of *Tanacetum,* as a novel survey, this study was conducted with the aim to evaluation of the antibacterial effect of the total methanol extract *Tanacetum frutoculosum* Lebeb and its Sub-fractions against 6 bacterial strains including: *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, Methicillin resistant *Staphylococcus aureus* ATCC 33591, *Salmonella typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 9027 and *Escherichia Coli* ATCC 8739. Primarily antibacterial effects were investigated by the Agar well diffusion method and then Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) were determined using broth Macro dilution method. The total phenolic and flavonoid contents of the extract and its fractions were measured based on gallic acid and rutin equivalents (GAE and RE) respectively.
The total methanol extract showed the highest phenolic and flavonoid contents (1.858mg GEA/g and 21mg RE/g respectively) and had the highest antibacterial effect against *S. epidermidis* (MIC 15.62 mg/ml).
The petroleum ether fraction showed the lowest phenolic and flavonoid contents (0.760mg GAE/g and 11.6 mg RE/g respectively) and had the lowest antibacterial activity against *P. aueroginosa* (MIC 250 mg/ml). These findings demonstrated the plant potential antibacterial effect against various human pathogenic bacteria.

Keywords: Flavonoid, Compositae, Minimum inhibitory concentration, Minimum bactericidal concentration.

INTRODUCTION

Bacteria could acquire resistance to therapeutic agents by the ability of genetic transmission. Infections caused by multi-drug resistant (MDR) bacteria lead to death for about 25,000 people in the European Union and 63,000 in the United States annually[1]. Indiscriminate use of antibiotics has produced drug resistance among common microorganisms. Beside these facts the adverse effects of chemical antimicrobial agents induce an increasing need to develop new alternative natural antibacterial agents.

The genus *Tanacetum* with approximately 200 species is spread throughout Europe, Iran, Iraq, Siberia, Afghanistan, Turkmenistan, Himalaya, Tibet and Turkey[2]. Previous identification of the compositions of different species of *Tanacetum* reported such compounds as acetylenes[3], flavonoides[4], sesquiterpene lactones[5].

The essential oil of *Tanacetum fruticulosum* from Iran was investigated by GC, GC/MS and NMR spectroscope, identified monoterpenes, 1,8 cineole, Ester, lavandolol and lavandolil acetate[6].

Herbs of *Tanacetum* species in the family of Compositae include perennials and herbaceous plants and are covered with plain corks. All the aerial members of the plant give off a strong odor like that of spearmint[7].

T. fruticulosum which is a subfamily of Anthamidea and genus of *Tanacetum* known as an aromatic herb[8] and there are 26 herbaceous and shrub species of this plant in Iran, 12 species of which are indigenous[7].

In this study the antibacterial effect of the total methanol extract and Sub-fractions of *T. fruticulosum* against 6 bacterial strains including: *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, Methicillin resistant *Staphylococcus aureus* ATCC 33591, *Salmonella typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 9027 and *Escherichia Coli* ATCC 8739 were investigated. The total phenolic and flavonoid contents of methanol extract and its fractions were also determined.

MATERIALS AND METHODS

Plant material

Shade dried *T. frutoculosum* (Figure.1)were obtained from Hormozgan Province, Iran in September 2012 and were identified by R. Asadpour in Department of Pharmacognosy, Pharmaceutical Sciences Branch, Islamic Azad University, Tehran, Iran. A sample was deposited in the herbarium of the university with voucher specimen No.1038. Plants were then ground down to fine powder by a mechanical miller.

Preparation of total methanol extract and sub-fractions

Approximately 1000 g of the fine powder was added to percolator with methanol for 70 hours[9].

The extract was concentrated by rotary evaporator apparatus (Heidolph laborota 4000) to produce the total methanol extract (TME). Liquid-liquid fraction method was used for fractioning of TME by petroleum ether (PEF) and chloroform (CF)[9]. The yield of extract and fractions were determined.

Test Organisms

Gram-positive bacteria including *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228 and Methicillin resistant *Staphylococcus aureus* ATCC 33591, and Gram-negative bacteria including *Escherichia coli* ATCC 8739, *Salmonella typhimurium* ATCC 14028 and *Pseudomonas aeruginosa* ATCC 9027 were obtained from Iranian Research

Organization for Science and Technology, Persian type culture collection (PTCC).

Antibacterial susceptibility test

Primarily antibacterial activities of the Total methanol extract and sub-fractions of *T. fruticulosum* were investigated by the agar well diffusion method[10,11]. The total methanol extract and sub-fractions were serially diluted by sterile Tween20 (20% v/v) from 1000 to 31.25 mg/ml.

Cups with 6mm diameter were made in Muller Hinton agar (MHA) plates (Merck, Germany) and which streaked by the microorganism saline suspension from overnight bacterial agar culture with turbidity equal to a 0.5 Mc Farland[12].100 µliters of each dilution was added in each cup and solvent as negative control were added and incubated at 37°c for 24 hours.

Minimum inhibitory concentration (MIC)

The MIC of the extract and its fractions were determined by macro broth dilution method[11,13].

Briefly the total methanol extract and sub-fractions were serially diluted and dilutions of the total methanol extract or its fractions were added to each of the tubes containing Muller Hinton Broth (Merck, Germany) with the final inoculums count of 5×10^5 CFU/ml.

After 24 h incubation at 37 °C, the test tubes were evaluated for possible growth. The lowest concentration which inhibits the visible growth of bacteria in liquid medium is defined as MIC[13].

Minimum bactericidal concentration (MBC)

50 µliter from each tube with no visible growth in MIC test was collected and sub cultured in MHA plates and incubated for 24 hours at 37°C. MBC was defined as the lowest concentration with no growth of bacteria[13].

Total phenolic content

Folin Ciocalteau reagent was used for the analysis of total phenolic content of the extract and the fractions[14]. Stock solutions of the total extract and the fractions in ethanol (0.8 mg/ml) were prepared and 1 ml of each stock solution was added to 60 ml of distilled water in a volumetric flask. Then, 5 ml of diluted Folin Ciocalteau reagent was added to the volumetric flask. The mixture was kept at room temperature for 3 min and then, 15 ml Na_2CO_3 20% solution was added and increase distilled water volume up to 100 ml. After 30 min, absorbance of the mixture was measured at 760 nm by UV-spectrophotometer (Multispec-1501 Shimadzu). A standard curve was prepared using gallic acid (Merck, Germany). The determinations were carried out in triplicate and the total phenolic content was expressed as gallic acid equivalents (mg of GAE /g of sample)[14].

Total flavonoid content

The total flavonoid contents were measured by a colorimetric assay[15].The dried extract was dissolved in 80% methanol to obtain a final concentration of 1 mg/ml. The calibration curve was prepared using 0.1-1 ml aliquots of Rutin solution, 500 µL of the acetic acid solution, 2 ml of the pyridine solution and 1 ml of the aluminum chloride solution. The final volume was adjusted to 10 ml with 80% methanol and the final Rutin concentration was 1-10 µg/ml. To quantify the flavonoids, 0.5 ml of the total extract or the fractions was transferred to a test tube and 0.5 ml of the acetic acid solution, 2 ml of the pyridine solution, 1 ml of the reagent aluminum chloride solution and 6 ml of 80% methanol were added. The samples remained at room temperature for 30 min and the absorbances of the mixtures were measured in 420 nm. The test was performed three times and the flavonoid content was expressed as milligrams of Rutin equivalents (RE) per gram of sample of extracts (mg RE/g)[15].

RESULTS

Yield of the extract and fractions

The results of the extraction and fractionation yields are shown in Table1.

Evaluation of antibacterial effect by the Agar well Diffusion Method

The diameter of inhibition zones made by the total methanol extract (TME) and its fractions (PEF, CF) are shown in Table 2. Tween 20 (20% V/V), as negative control, showed no inhibitory effect against the tested strains. As shown in Table 2, the total methanol extract and its fractions showed antibacterial activity against all of the tested microorganisms (diameters of zone of inhibition ranging between 10 to 24 mm). The largest inhibition zone made by TME extract was against *S. aureus, S. epidermidis, S. typhimurium*. The chloroform fractions reflected larger inhibition zones while petroleum ether fractions showed smaller zones.

Determination of MIC

Table 3 shows MIC values of the total extract and its fractions against all tested microorganism. The MIC values obtained in this study ranged from 15.62 to 250 mg/ml. The total methanol extract and the chloroform fractions showed the lowest MIC (15.62 mg/ml) against *S. epidermidis* and *MRSA*. This indicates the highest sensitivity of *S. epidermidis* for the TME and *MRSA* for the chloroform fraction among the six microorganisms tested in this study. *P. auerоginosa* was the most resistant microorganism against all of the fractions (MIC equal to 250 mg/ml for the petroleum ether fraction)

Determination of MBC

The MBC values obtained in this study (Table 3) ranged from 31.25 to 500 mg/ml. The lowest MBC value of 31.25 mg/ml was observed from TME extract against *S. epidermidis* and from chloroform fraction against *MRSA*. Similar to results of MIC determination, *P. auerоginosa* was the most resistant microorganism against all the fractions and the extract (MBC equal to 500 mg/ml for petroleum ether fraction).

Total phenolic content

After making a standard calibration curve by gallic acid ($y = 1.46x - 0.04$, $r^2 = 0.99$), the total phenolic content of the extract and the fractions were measured. The total phenolic content was ranged from to mg GAE/g of dry powder. As shown in Table 4, the petroleum-ether fraction (PEF) had the lowest and the total extract (TME) had the highest total phenolic contents.

Total flavonoid content

Standard calibration curve of Rutin was used to evaluate the content of flavonoid in the extract and the fractions ($y = 0.02x – 0.04$, $r^2 = 0.97$). Results are shown in Table 4. The lowest flavonoid content was seen in the petroleum-ether fraction (mg RE/g of dry powder) and the highest was seen in the total extract (mg RE/g of dry powder)

DISCUSSION

The scientists have been focused on finding new plant originated antibacterial substances because worldwide resistance of microbial pathogens to chemical antibiotics and their side effects such as hypersensitivity and allergic reaction[16]. Previous investigations on different species of *Tanacetum* have shown the presence of antibacterial effect of this family[17-20] and In this study the antibacterial effect of *T. fruticulosum* total methanol extract and its fractions against 6 bacterial strains were evaluated. The antibacterial activity of the extract was primarily evaluated by the agar well diffusion method[12]. The results indicated a broad spectrum activity against both Gram positive and Gram negative bacteria. The

largest inhibition zone (24 mm) was seen for TE against *S. epidermidis*, *S. typhimurium* and *MRSA* at the concentration of 500 mg/ml. The smallest inhibition zone at this concentration was seen for petroleum ether fraction against *P. auerоginosa* and *E. coli* with the diameter of 12 mm. The results showed that *S. epidermidis* and *MRSA* (Gram positive bacteria) and *S. typhimurium* (Gram negative bacteria) could be more sensitive based on their larger inhibition zone. TME showed the minimum value of the MIC and MBC against *S. epidermidis* at the concentration of 15.62, 31.25 mg/ml while the chloroform fraction showed the best effect against *MRSA* at the concentration of 15.62, 31.25 mg/ml. Gram negative bacteria were more resistant to the total methanol extract and its fractions. Presence of an outer membrane in Gram negative bacteria may explain this resistance effect[21]. *P. auerоginosa* was found to be the most resistant bacteria with the MIC of 250 mg/ml for petroleum ether fraction. In fact the total methanol extract and chloroform fractions generally showed better antibacterial activity, which can be related to the total phenolic and flavonoid content of the fractions[22,23]. The highest phenolic and flavonoid content was measured in the total methanol extract compared with the chloroform and the petroleum ether fraction, in which the most antibacterial action was also observed. It has been shown that the antimicrobial efficacy of the herbal extracts correlates with their phenolic and flavonoid contents[22,23]. The total phenolic and flavonoid content of the extract and its fractions were expressed in term of Gallic acid and Rutin standard equivalent. According to the results, TME contains 1.858 mg GAE/g of phenolic content while chloroform and petroleum ether fractions contain 1.266 mg GAE/g and 0.760 mg GAE/g. and TME contains 21 mg Rutin/g of flavonoid content while chloroform and petroleum ether fraction contain 19.9 mg Rutin /g and 11.6 mg Rutin/g.

Considering the antibacterial effects of *T. frutoculosum*, there is a potential benefit of using the extract in combination with classic antibacterial agents to improve the antibacterial effects of the classic compound and consequently reduce the side effects of the compound.

CONCLUSION

In conclusion, among the methanol extract and its fractions, Total methanol extract showed the highest yield of phenolic content (1.858 mg of GAE/g) and flavonoid content (21 mg of Rutin/g) and highest antibacterial activity (with the MIC of 15/62 mg/ml against *S. epidermidis*). Based on these results, it could be suggested that the antibacterial activity of the herbal extract and fractions is related with their phenolic and flavonoid contents[22].

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Table 1. Yield of the total methanol extract and its fractions of *T. fruticulosum*

Extract& its fractions	Amount of *T. fruticulosum*(g)	Amount of solvent(ml)	Yield of extraction(g)	Yield of extraction %
TME	1000	5000	250	%25
CF	200	1000	130	%65
PEF	200	3000	40	%20

TME= Total methanol extract; PEF=Petroleum ether fraction; CF=chloroform fraction

Table 2. Mean diameter of growth inhibition zone (mm) of the total methanol extract of *T. fruticulosum* and its fractions (n=3)

Microorganism \ Extract concentration (mg/ml)		500	250	125	62.5	31.25	15.62
***S. aureus* ATCC 6538**	TME	24±0.00	20±0.40	17±0.00	15±0.00	-	-
	PEF	13±0.00	12±0.40	11±0.00	-	-	-
	CF	23±0.00	19±0.40	16±0.00	14±0.00	-	-
***S. epidermidis* ATCC 12228**	TME	24±0.00	21±0.00	17±0.40	14±0.00	-	-
	PEF	13±0.00	12±0.00	11±0.40	-	-	-
	CF	23±0.00	20±0.00	16±0.40	14±0.00	-	-
S. typhimurium ATCC 14028	TME	24±0.00	20±0.40	17±0.00	15±0.40	-	-
	PEF	12±0.00	11±0.00	10±0.00	-	-	-
	CF	23±0.40	19±0.00	16±0.00	14±0.00	-	-
Methicillin resistant *S. aureus* ATCC 33591	TME	22±0.00	20±0.40	17±0.00	15±0.00	-	-
	PEF	12±0.00	11±0.40	-	-	-	-
	CF	21±0.00	19±0.00	16±0.40	14±0.00	-	-
***P. aueroginosa* ATCC 9027**	TME	13±0.00	12±0.81	11±0,00	10±0.00	-	-
	PEF	12±0.40	11±0.00	10±0.00	-	-	-
	CF	13±0.00	12±0.40	11±0.00	10±0.00	-	-
***E. coli* ATCC 8739**	TME	12±0.00	11±0.00	10±0.40	-	-	-
	PEF	12±0.00	11±0.40	-	-	-	-
	CF	12±0.00	11±0.00	10±0.00	-	-	-

TME=Total methanol extract; PEF=Petroleum ether fraction; CF=chloroform fraction, respectively, Zone of Inhibition, including the diameter of the well (6 mm); mean ± SD value of three independent experiments.

Table 3. MIC and MBC of total methanol extract and Sub-fraction of *Tanacetum fruticulosum* against 6 bacterial strains (n=3)

Extract and its fractions / Microorganism	TME		CF		PEF	
	MIC mg/ml	MBC mg/ml	MIC mg/ml	MBC mg/ml	MIC mg/ml	MBC mg/ml
S. aureus ATCC 6538	31.25	62.50	31.25	62.50	125	250
S. epidermidis ATCC 12228	15.62	31.25	31.25	62.50	125	250
S. typhimurium ATCC 14028	31.25	62.50	31.25	62.50	125	250
Methicillin resistant *S. aureus* ATCC 33591	31.25	62.50	15.62	31.25	125	500
P. aueroginosa ATCC 9027	62.50	125	62.50	125	250	500
E. coli ATCC 8739	62.50	125	62.50	125	250	250

TME= Total methanol extract; PEF=Petroleum ether fraction; CF=chloroform fraction, all tests were done in triplicate.

Table 4. Total phenolic and flavonoid of the total methanol extract of *T. fruticulosum* and its fractions

Extract and its fractions	Phenolic content (mg GAE/g)	Flavonoid content (mg Rutin/g)
Total methanol extract	1.858	21
Chloroform fraction	1.266	19.9
Petroleum ether fraction	0.760	11.6

Fig. 1. Image of *Tanacetum fruticulosum.*