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EVALUATION OF ANTIMICROBIAL ACTIVITY İN EXTRACTS OF DIFFERENT PARTS OF THREE *TAGETES* SPECIES

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ABSTRACT

Indiscriminate use of antibiotics often results in the development of resistant microbial strains. The unprecedented increase in cases of antimicrobial drug resistance, the discovery of newer agents, particularly from natural plant metabolites, are required for the control of such prevalent and recurring infectious diseases worldwide. This study aimed to evaluate antibacterial and antifungal activities of extracts from different parts of *Tagetes patula, Tagetes erecta,* and *Tagetes minuta,* which are important medicinal plants. Five grams of air-dried flower, leaf, and bud (only for *T. patula* and *T. erecta*) samples were extracted three times with methanol: water (4:1) at room temperature in the dark for 24 h. The methanol: water extracts were combined, filtered, and concentrated to dryness using a rotary evaporator at 40 °C. The extracts were screened for their *in vitro* antimicrobial activities against nine indicator strains [three Gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis,* and *Enterococcus faecalis*), three Gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa*) and three fungi (*Candida albicans, Aspergillus niger,* and *Phytophthora erythroseptica*)] by Agar well diffusion assay. Broth microdilution method was used to determine minimum inhibition concentrations (MIC) of the extracts that showed antimicrobial activity against Gram-positive bacteria and *P. erythroseptica*. The *T. patula* leaf extracts led to the highest antibacterial activity against *B. subtilis* ATCC 6633 with an inhibition zone of 17 mm and a MIC value of 256 µg/ml. The bud and flower extracts of *T. patula* were potent against the same strain at an intermediary level. Also, the *T. erecta* bud extracts inhibited the growth of *E. faecalis* ATCC 29212 at a moderate level. In this study, only the *T. patula* flower extracts showed antifungal activity against *P. erythroseptica* strain with a MIC value of 426.7 µg/ml. Our findings make an excellent contribution to revealing the antimicrobial activity of *T. erecta, T. patula* and *T. minuta* by comparing the methanol-extracted leaf, bud, and flower parts at a single experimental setup.

Keywords: Antimicrobial activity, Extraction, *Tagetes patula, Tagetes erecta, Tagetes minuta*

INTRODUCTION

Tagetes sp. contains about 56 species belonging to the Asteraceae family, many of which have different chemical and biochemical properties, and can produce high pharmaceutical and nutritional value compounds. Extracts of *Tagetes* sp. has vast amount of orange-yellow carotenoids (Ibrahim et al., 2018). *Tagetes minuta* L., known as marigold, is a single or perennial plant and can spread from tropical to temperate climate under wide climatic conditions. *T. minuta* extracts are ingredients of medicinal drogs used to treat common cold, inflammation, bowel and stomach illnesses, skin infections, cough, cold and, wound (Ali et al., 2014).

In addition, Obongoyai et al. (2010) reported inhibition of the *Fusarium oxysporum* conidium, a soilborne pathogen, by *T. minuta*. Antimicrobial studies with this species were reviewed in detail (dos Santos et al., 2017). In particular, the literature mainly focused on antimicrobial activities of essential oil extracts. *T. minuta* has a high larvicidal effect and is used as a housefly repellent in Kenya. *Tagetes patula,* also known as France marigold, is a one-year plant with a wide distribution. It synthesizes similar ingredients like *T. minuta* but also has patuletin and patulitrin, rare antimicrobial and anti-inflammatory chemicals. It was reported that this species synthesizes different chemicals like benzofuran, flavonoid and carotenoid. The effects of different organic fractions, different plant parts, and their several combinations in *T. patula* were investigated in different studies (Politi et al., 2016). *Tagetes erecta* is another type of marigold with high pigment content, and several studies evaluated metabolite production, phytoremediation potential, and stress tolerance strength. Like the other two species, it

contains the active substance used in the treatment of diseases such as ulcers, eye diseases, rheumatism, and bronchitis. The flower extract contains lutein, which can be used as a nutrient and food dye (Feng et al., 2018).

Nowadays, scientific studies have returned to ''Natural'' products (Santos et al., 2017). Researches have focused intensively on finding pharmaceutical equivalents of traditional uses of medicinal plants, as well as research on the discovery of new antimicrobial compounds from a wide variety of plants (Chandra et al., 2017; Chootip et al., 2017). In this context, the discovery of natural plant metabolites as well as the production of high amounts of existing ones is of great industrial importance. However, increasing multidrug resistance in pathogenic microorganisms necessitates screening new antibiotic sources. In contrast to synthetic drugs, plant-derived antimicrobials have no side effects and they have known potential in treatment of infectious disease (Doughari and Manzara, 2008). So far, many plant species have been tested for their antimicrobial properties, but the vast majority have not been adequately evaluated. Studies in the literature of *Tagetes* sp. belong only to a certain organ or a single period of development and even to examples taken from herbalists. Therefore, this study makes a good contribution to the related literature by comparatively evaluating antimicrobial activities of the samples extracted from leaf, bud and flower parts of three different *Tagetes* species.

MATERIALS AND METHODS

Plant material

Three species (*T. erecta*, *T. minuta* and *T. patula*) were planted on 30 April-1 May, 2019 in the Field Crops Experiment Area of Faculty of Agriculture, Ondokuz Mayıs University. The leaves, buds, and flowers from *T. erecta* and *T. patula* were collected on 31 May, 2019. For *T. minuta*, the leaves and buds were collected on 2 October, 2019 while the flower parts were harvested on 14 October, 2019. All the materials collected, were dried in an oven at 35 °C for 48 h.

Extraction

An extraction procedure reported in Hamza et al. (2006) was used with slight modifications Firstly, 5 g of the dried plant materials were extracted by maceration within 75 ml of a 4:1 ratio of methanol: water mixture under the dark conditions and at room temperature for 24 h (Hamza et al., 2006). This step was repeated three times during 72 h. The extracts from each step were collected, filtered, and concentrated at 40 ℃ using a rotary evaporator to give a final dry weight of 300 mg. The extracts were stored at -86 °C and resuspended with 1 ml of 80% methanol before antimicrobial testing.

Antimicrobial activity

Indicator microorganisms:

Indicator strains used in this study are *Bacillus subtilis* ATCC6633, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 10231, *Aspergillus niger* ATCC 16404 and *Phytophthora erythroseptica*. All microorganisms except for *P. erythroseptica* were purchased from Refik Saydam National Type Culture Collection (Ankara, Turkey). *P. erythroseptica* was kindly provided from Dr. Sezer Okay (Hacettepe University, Ankara, Turkey).

Agar well diffusion method:

The extracts were tested against indicator strains using the Agar well diffusion method described in Romero et al. (1984). The results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) recommendations (CLSI M100-S24, CLSI M44-A2, CLSI M51-A). Amikacin (30 µg) and ketoconazole (300 µg) were used as standards for bacteria and fungi, respectively. Indicator strains were grown in Mueller Hinton Broth (MHB, for bacteria) and Sabouraud Dextrose Broth (SDB, for fungi) up to an OD_{600} value of 1.0. Then, 3.3 ml of the cultures were mixed gently with 100 ml of molten (at 45 °C) MHA/SDA. Media-microorganism mixtures were poured into Petri dishes and allowed to solidify. A 50 µl of extracts were added to the wells generated in the solid media by using a cup borer (6 mm). All Petri dishes were kept at +4 °C for 2 h. Then, bacterial cultures were incubated at 37°C for 12-15 h; fungal cultures were incubated at 26°C for 24 h. Inhibition zones that occurred around the wells were measured as diameter (mm). The experiment was repeated two times, and two technical replicates were used. Methanol control was also included to the experiments.

Broth microdilution method:

The extracts that show remarkable inhibition zones against indicator strains by agar well diffusion assay were further analyzed quantitatively to determine Minimum Inhibitory Concentration (MIC) values. MIC; refers to the minimum concentration of substance required to inhibit microbial growth. The results were evaluated according to CLSI recommendations (CLSI M07-A10 for bacteria, CLSI M27-A3 and CLSI M27-S4 for yeast, CLSI M38-A2 for filamentous fungi). Firstly, 100 µl of Cation adjusted Mueller-Hinton Broth (CA-MHB) (Oxoid) were added to the 96-well round-bottomed microtiter plates. Double dilution series of extracts/ standard antibiotics (Amikacin for bacteria, ketoconazole for fungi) were added into the wells in a concentration scale ranging from 512 to 0.125 µg/ml. Microbial cultures were diluted to 0.5 McFarland solution (≅ 1-4 × 10^8 CFU/ml) at OD_{625}. A hundred µl of the ten-fold diluted microbial cultures were added to each well. The microdilution plates were first kept at 4 °C for 2 h and incubated at 37°C for 12-15 h for bacteria. For fungal strains, the incubation temperature and time were 26°C and 24-48 h. The MIC values were evaluated by turbidity observation in the wells. The experiments were performed as three technical replicates. The control reactions were also included (medium and extract without inoculum, medium and inoculum without

extract, medium and inoculum with methanol instead of extract).

RESULTS AND DISCUSSION

Following maceration, the extracts were collected, filtered with blotting paper, and then kept in -86 °C (Figure 1).

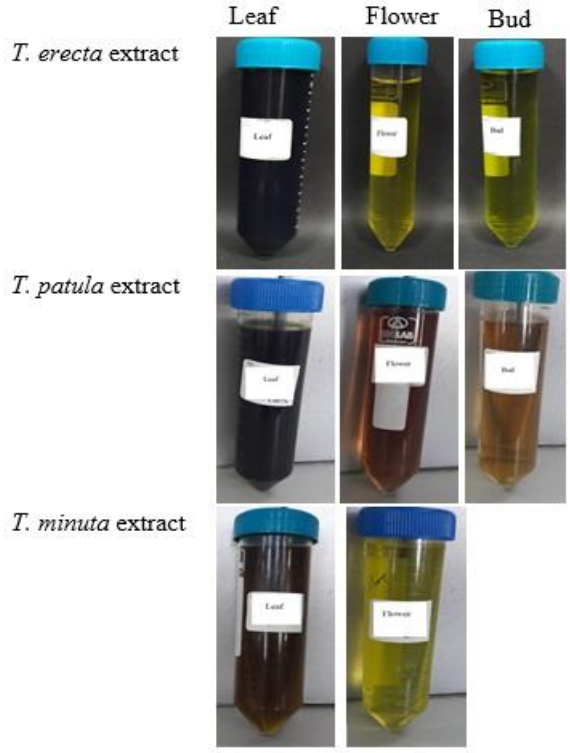

Figure 1. The methanol extracts obtained following maceration of different parts of *Tagetes* species.

Then, 1.8 ml of extracts were transferred to 2 ml Eppendorf tubes and were flown in evaporator at 40°C for 9 h. 300 mg/ml extracts were dissolved with 1 ml of 80% methanol. By using six different pathogenic bacteria, two fungi and a yeast as indicator organisms, antimicrobial and antifungal activity of the extracts were determined by Agar well diffusion method (Table 1).

Bioassay results showed that the *T. erecta* leaf and bud extracts, the *T. patula* leaf, flower and bud extracts, and the leaf and flower extracts of *T. minuta* contain phytoconstituents that have antimicrobial activity against Gram-positive bacteria. The extracts obtained from *T. erecta* buds showed antibacterial activity against *E. faecalis* ATCC 29212 at an intermediary level. In particular, the antimicrobial activity results of *T. patula* extracts were found more prominent. All extracts obtained from different parts of *T. patula* resulted in inhibition zone formation against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923 and *E. faecalis* ATCC 29212, all Gram-positive bacteria tested. Indeed, the leaf and bud extracts of *T. patula* provided the highest antimicrobial activity in this study and led to inhibition of *B. subtilis* ATCC 6633 at a high level (inhibition zone with 17 mm). *B. subtilis* ATCC 6633 was also susceptible to *T. patula* flower and bud extracts at an intermediary level (15-15.5 mm). Only, the extracts obtained from *T. patula* flowers showed antifungal acitivity against *P. erythroseptica* among the tested fungi by causing the same inhibitory effect as that of ketoconazole (10.5 mm). None of the extracts showed antibacterial activity on the Gram-negative microorganisms tested.

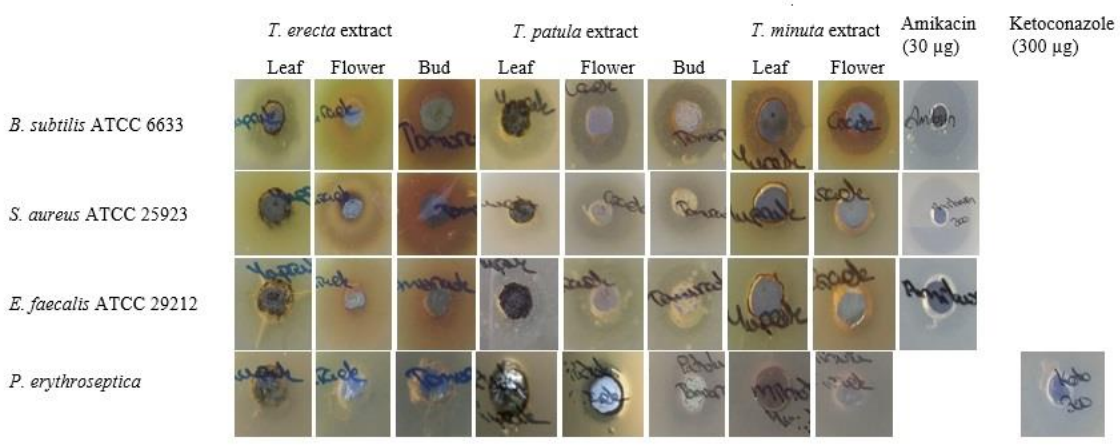

Figure 2. Inhibition zones obtained from flower, bud, and leaf extracts from *T. erecta*, *T. minuta* and *T. patula* against indicator strains.

Followingly, MIC values of the extracts having remarkable antimicrobial activity as shown by bioassay, were determined by the broth microdilution method (Table 2). The highest MIC value was recorded as 256 µg/ml and obtained against *B. subtilis* ATCC 6633 with the use of the *T. erecta* bud extracts and the leaf extracts of both *T. patula* and *T. minuta*. In addition, the *T. erecta* leaf extracts and the *T. patula* flower extracts resulted in a MIC value of 341.3 µg/ml against *B. subtilis* ATCC 6633. The higher MIC value of 426.7 µg/ml was obtained against *S. aureus* ATCC 25923 when the *T. patula* leaf extracts were used. In this study, the only *T. patula* flower extracts exerted antifungal activity against *P. erythroseptica* with a MIC value of 426.7 µg/ml. The remaining MIC results were recorded as 512 µg/ml or more (indicated as Not determined) against other methanol extracts tested in the broth microdilution method.

Our results indicate a broad spectrum of antimicrobial activity for 80% methanol extracts of different organ parts belonging to *T. erecta*, *T patula* and *T. minuta*, and accorded well with the previous reports. For instance, Verma and Verma (2012) investigated the antimicrobial activity of five different parts (flower, root, leaves, bud, stem) of *T. erecta* exracted with ethanol (by using soxhlet apparatus) against five Gram-positive strains by Agar disc diffusion assay. The ethanols extracts exerted different levels of antibacterial activity. Thereby, their bioassay results showed that both leaf and flower extracts provided remarkable sensitivity by *Staphlococcus lutea* and *Bacillus circulence*. In another study, Ali et al. (2019) obtained chloroform extract of *T. erecta* flowers and tested its antibacterial activity against nine bacteria using Agar disc diffusion and Agar well diffusion methods. They found that the Agar well diffusion method provided better antibacterial activity results compared to the Agar disc diffusion method. In addition, they showed that *Salmonela* spp. was the most sensitive indicator strain providing 15 mm inhibition zone when exposed to 62.5 mg/ml of the *T. erecta* flower extract. Padalia and Chanda (2015) evaluated antimicrobial efficacy of different solvent extracts of *T. erecta* flower, both alone and in combination with antibiotics. They obtained maximum inhibition against indicator strains when polar organic solvents especially acetone and methanol were used in the extraction process by cold percolation method. The combination of the flower extract and the antibiotics provided a synergistict effect against human pathogens.

As shown in Table 1 and 2, our *T. patula* extracts exerted more efficient antimicrobial activity against the indicator strains. In the literature, there are different approaches indicating antimicrobial potebtial of *T. patula* extracts. For example, Ayub et al. (2017) evaluated antimicrobial properties of *T. patula* ant *T. erecta* petals extracted with methanol and n-hexane, respectively, through the Agar disc diffusion method and MIC determination. The *T. patula* petal extracts showed better antibacterial activity than that of *T. erecta* by leading to larger inhibition zones (12.4 -20.2 mm) and smaller MIC values (0.19 - 4.05 mg/ml). In addition, Jain et al. (2012) investigated *in vitro* antibacterial potential of methanol extracts of *T. erecta* and *T. patula* flowers against different pathogens by using the Agar disc diffusion method and test tube dilution method. Their methanol extracts of *T. erecta* and *T. patula* flowers provided MIC values ranging from 20 to 160 mg/ml for most of the tested bacteria. It was previously reported that the use of methanol as organic solvent was found to be effective for extraction of phenolic compounds (Hussain et al., 2011). Antifungal activity of methanol-treated *T. patula* thiophene extracts were tested against two phytopathogenic fungi, *Botrytis cinerea* and *Fusarium moniliformin*, in an earlier study by Mares et al. (2002). Accordingly, they observed growth of fungal strains in SDA including different concentrations of extracts (5-10-50 µg/ml). The results showed that *B. cinerea* had a high dose-dependent inhibition with a remarkable difference upon to light regimen. Later, Faizi et al. (2008) performed a bioassay-guided isolation study performing antimicrobial activity experiments on different parts of *T. patula*, especially on flowers at the same time. In another study, ethanol extracts of *T. patula* flowers and aerial parts were assayed on *Microsporum canis* and *Trichophyton rubrum* to indicate it potential acaricide activity. Therefore, they found strong inhibition of both strain by the flower extracts (MIC: 193.3 µg/ml and 253.9 µg/ml, respectively, for *M. canis* and *T. rubrum*) and by the arerial parts against *T. rubrum* (312.5 µg/ml). Recently, Faraz et al. (2020) have reported pharmacognostic, antimicrobial and toxicological activities of *T. patula* L. In that study, ethanol extracted *T. patula* flowers provided good antimicrobial and larvacidal activities against human pathogens and brine shrimps, respectively.

Table 1. Evaluation of antimicrobial activity of different *Tagetes* extracts by Agar well diffusion method

Zone diameter (mm)											
	***T. erecta* extract (15 mg)**			***T. patula* extract (15 mg)**			***T. minuta* extract (15 mg)**		**Amikacin (30 µg)**	**Ketoconazole (300 µg)**	**CLSI (S/I/R)****
	Leaf	**Flower**	**Bud**	**Leaf**	**Flower**	**Bud**	**Leaf**	**Flower**			
B. subtilis ATCC 6633	11	ND*	11	17±2.1 (**S**)	15 (**I**)	15.5±0.7 (**I**)	11.5±0.7	13	29.5±2.1	ND	≥16/15-16≤15
S. aureus ATCC 25923	ND	ND	12	10	12	10	ND	ND	26.5±2.1	ND	≥17/15-16≤14
E. faecalis ATCC 29212	ND	ND	13 (**I**)	10	11.5±0.7	12	ND	ND	256	ND	≥16/13-15≤12
P. erythroseptica	ND	ND	ND	ND	10.5±0.7	ND	ND	ND	ND	10.5±0.7	ND

*ND: Not Determined**S/I/R: S, refers to sensitivity of the strains against the extract; I, refers to the susceptibility of the strains against the extract at an intermediary level; R, refers to resistance of indicator strains against the extract.

Table 2. MIC (µg/ml) values of the *Tagetes* species extracts against indicator strains

MIC (µg/ml)										
	***T. erecta* extract**		***T. patula* extract**			***T. minuta* extract**		**Amikacin**	**Ketoconazol**	**CLSI for MICs (S/I/R)****
	Leaf	**Bud**	**Leaf**	**Flower**	**Bud**	**Leaf**	**Flower**			
B. subtilis ATCC 6633	341.3±147.8	256	256	341.3±147.8	512	256	426.7±147.8	1.5±0.7	ND*	≤16/32/≥64
S. aureus ATCC 25923	ND	512	426.7±147.8	512	512	ND	ND	5±2.7	ND	≤16/32/≥64
E. faecalis ATCC 29212	ND	512	512	512	512	ND	ND	256	ND	≤64/128/≥256
P. erythroseptica	ND	ND	ND	426.7±147.8	ND	ND	ND	ND	256	ND

*ND: Not determined

**S/I/R: S, refers to sensitivity of the strains against the extract; I, refers to the susceptibility of the strains against the extract at an intermediary level; R, refers to resistance of indicator strains against the extract.

Regarding to *T. minuta*, our results included the leaf and flower extracts as we haven't harvested the buds, because of the small size and low extract yield. Unlike *T. erecta* and *T. patula* extracts, the antimicrobial activity was only recorded against *B. subtilis* ATCC 6633 strain resulting in inhibition zones of 11-13 mm and MIC values of 256 and 426.7 µg/ml, respectively, for the leaf and flower extracts. In the literature, Ali et al. (2014) investigated essential oil composition of *T. minuta* leaves and evaluated its antimicrobial activities by the Agar disc diffusion assay. They showed that the essential oil exerted remarkable antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and *C. albicans* with the inhibition zones of 23 and 26 mm, respectively. Gakuubi et al. (2016) characterized essential oils of *T. minuta* and determined the antibacterial activity of essential oils against three phytopathogenic bacteria, namely, *Pseudomonas savastanoi pv. phaseolicola*, *Xanthomonas axonopodis pv. phaseoli*, and *Xanthomonas axonopodis pv. manihotisdos*. Among them, *P. savastanoi pv. phaseolicola* was the most sensitive strain by exhibiting 41.8-44.8 mm inhibition zones. The MIC values against the tested bacteria were recorded as 95–190 mg/ml at 48 h. Later, dos Santos et al. (2017) critically reviewed the related studies on antimicrobial activity of *T. minuta*. They indicated that the methanol was the most frequently used organic solvent in the extraction process and this organic solvent leads to extraction of some metabolites such as polyphenols, glycosides and flavonoids (Rauha et al., 2000).

In conclusion, we obtained a broad range of antibacterial activity against human pathogen Gram-positive bacteria by using methanol extracts of three different *Tagetes* species. In particular, the all *T. patula* extracts exhibited remarkable antibacterial activity, while a mild level of antifungal activity was recorded against *P. erythroseptica*, a phytopathogenic fungus with the use of only the flower extracts. Among the tested strains the most susceptible one was *B. subtilis* ATCC 6633. Overall, our results are comparable with the literature data as the comparison of antimicrobial efficacy of three different organ parts from the three *Tagetes* sp., make a good contribution in this area. Our future studies will be focused on characterization of chemical composition of our extracts especially, the ones that showed the antimicrobial activity in the current study.

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