

Antimicrobial activities of extracts from stem bark of *Tagetes minuta*^{1,*}Pillai, M.K., ¹Santi, L.I. and ²Mekbib, S.B.¹Department of Chemistry and Chemical Technology, Faculty of Science and Technology, National University of Lesotho, Roma Campus, P. O. Roma 180, Kingdom of Lesotho, Southern Africa²Department of Biology, Faculty of Science and Technology, National University of Lesotho, Roma Campus, P. O. Roma 180, Kingdom of Lesotho, Southern Africa**Article history:**

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Tagetes minuta hexane stem bark extract (TMHESB), chloroform stem bark extract (TMCHSB), ethyl acetate stem bark extract (TMEASB) and methanolic stem bark extract (TMMESB) were evaluated for their antimicrobial activities using hole-plate diffusion method. Six bacterial isolates viz. *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli* (wild), *Escherichia coli* (O157:H7), *Pseudomonas aeruginosa* and *Serratia marcescens* and two fungal isolates viz. *Candida albicans* and *Penicillium digitatum* were used for this study. The inhibition zones were found to be in the ranges of 10.0±1.6 to 15.5±1.9 mm against bacterial isolates and 11.3±2.1 to 13.4±1.2 mm against *P. digitatum*. However, these extracts did not exhibit any visible inhibition zone against *C. albicans*. Additionally, the minimum inhibitory concentrations (MICs) of these extracts were also determined and was found to be in the range of <31.25 to 1000 µg/mL. From this study, we concluded that extracts of the stem bark of *T. minuta* showed a moderate to significant antimicrobial activities. *T. minuta* has been used in food and beverage industries as preservative, coloring and flavoring agents. *T. minuta* also finds therapeutic applications in the traditional medicine.

1. Introduction

Known by other names such as khaki bush, khakhi weed, African marigold and Kakiebos, *Tagetes minuta* belongs to the Asteraceae family (Ndou and Koekemoer, 2017). *T. minuta* is native to South America but it is commonly found as widespread weed in Africa, South Europe, South Asia and Australia. *T. minuta* grows in dry or moist soil and found in areas such as abandoned gardens, farms, roadsides and waste places. *T. minuta* is an annual herb and has a pleasant smell with stems up to 2-meter tall. *T. minuta* has stalked light green leaves, which are 7-15cm long and pinnately dissected into 4-6 pairs of pinnae. The leaves bear sunken oil glands with liquorice-like aroma. *T. minuta* flowers in February, June and November and fruits in December-January, March-April and July-September (Ofori *et al.*, 2013). *T. minuta* is rich in orange-yellow carotenoids (Nerio *et al.*, 2010; Rahimi *et al.*, 2010) and therefore, it has been used as a colorant to make foodstuffs such as pasta, margarine, mayonnaise, salad dressing, confectionery, baked goods, poultry feed and dairy products (Nerio *et al.*, 2010; Rahimi *et al.*, 2010). *T. minuta* is a good source of

essential oil and this essential oil has been used as a flavoring agent in food industries (Nerio *et al.*, 2010; Rahimi *et al.*, 2010). Additionally, *T. minuta* has been used as a preservative for a wide range of foodstuffs and beverages. *T. minuta* has several medicinal benefits, which include remedy for colds, respiratory inflammations and stomach problems (Parastoo *et al.*, 2014). *T. minuta* has also been used as anti-spasmodic, anti-septic and anti-parasitic (Parastoo *et al.*, 2014). *T. minuta* has been used to cure skin infections, for dilating the bronchi, facilitating the flow of mucus and dislodging congestion (Wang *et al.*, 2006; Govindarajan, 2011; Maity *et al.*, 2011; Nikkon *et al.*, 2011; Aristatle *et al.*, 2013). The decoction from *T. minuta* has been used as effective control of intestinal parasites in domestic livestock. Roots of *T. minuta* are effective against nematodes, worms, fungi and perennial weeds. *T. minuta* has extensively been used in traditional and complementary medicines. A few articles on the antimicrobial studies of essential oils obtained from the aerial parts of *T. minuta* have previously been reported (Senatore *et al.*, 2004; Gakuubi *et al.*, 2016). However, the antimicrobial activities of extracts from other parts of

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this plant have not been explored well, particularly, the species from the Kingdom of Lesotho. The aim of the present study was to evaluate the antimicrobial activities of hexane, chloroform, ethyl acetate and methanolic extracts from stem bark of *T. minuta* against six bacterial strains viz. *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli* (wild), *Escherichia coli* (O157:H7), *Pseudomonas aeruginosa* and *Serratia marcescens* and two fungal isolates viz. *Candida albicans* and *Penicillium digitatum*. The results are communicated in this article.

2. Materials and methods

2.1 Plant materials

The stem bark of *T. minuta* was collected in August 2018 from inside the National University of Lesotho, Roma campus, Lesotho, Southern Africa. A voucher specimen viz. Santi/TMSB/2018 was kept in the Organic Research laboratory, Department of Chemistry and Chemical Technology, Faculty of Science and Technology, National University of Lesotho, Roma campus, Maseru district, Kingdom of Lesotho, Southern Africa.

2.2 Processing of materials

The chopped stem bark was allowed to air dry at room temperature (about 22-25°C) for two weeks and ground into powder using a commercial blender (Waring Blender, Blender 80119, Model HGB2WT93, 240V AC, 50-80Hz, 3.6AMPs, Laboratory and Analytical Supplies).

2.3 Preparation of plant extracts

The powdered stem bark (150.172 g) of *T. minuta* was macerated with methanol at room temperature (about 22-25°C) for 3 days with occasional shaking. The solution was filtered using a filter paper (Boeco, Germany). The solvent was removed in *vacuo*. The procedure was repeated once again. Finally, the same plant material was extracted with methanol under reflux condition for 6 hrs. A mass of 5.732 g of combined methanolic extract was obtained after removal of solvent. The same extraction procedure was followed to get 0.9271 g of hexane extract, 1.4158 g of chloroform extract and 4.3107 g of ethyl acetate extract, respectively from 150.025, 150.158 and 150.421 g of powdered stem bark.

2.4 Microorganisms

The microorganisms used in this study were six bacterial isolates viz. *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Escherichia coli* (wild), *Escherichia coli* (O157:H7), *Staphylococcus aureus* and

Serratia marcescens and two fungal isolates viz. *Candida albicans* and *Penicillium digitatum*. All these microorganisms were obtained from the culture collection at the Department of Biology, Faculty of Science and Technology, National University of Lesotho, Roma campus, Maseru district, Kingdom of Lesotho, Southern Africa.

2.5 Evaluation of antimicrobial activity

The antimicrobial activities of various extracts from *T. minuta* were evaluated by *in vitro* hole-plate diffusion method as described in literature (Manilal et al., 2009; Alghazeer et al., 2012). Briefly, solutions of various extracts were prepared separately at a concentration of 100 mg of extract in 1 mL of DMSO. The solutions were filtered separately using a 0.20 µm filter paper and then used for both antibacterial and antifungal activities. For antibacterial activity, 0.1 mL of the broth culture was evenly seeded on the Nutrient agar (NA) plates. The agar wells of sizes 4.00mm height and 6.00mm diameter were punched on the agar plate using a sterile cork-borer. The wells were filled with 30 µL aliquots of the extracts. The petri plates were then incubated at 37°C for 24 hrs. Tetracycline served as a positive control for *E.coli* (wild), *E. coli* (HO157), *S. aureus* and *L. monocytogenes*. Amoxicillin served as a positive control for *P. aeruginosa* and *S. marcescens*. DMSO served as a negative control. For the antifungal assay, the petri plates containing 25 mL of Potato Dextrose Agar (PDA) were used. The agar plates were evenly seeded with 0.1 mL of the fungi. The holes of sizes 4.00 mm height and 6.00 mm diameter were punched using a sterile cork-borer and filled with 30 µL aliquots of the extracts. The plates were incubated at 24°C for 48 hrs. Miconazole nitrate served as a positive control for *C. albicans*. DMSO served as a negative control. However, positive control for *P. digitatum* was not available. The diameter of inhibition zones on the agar surface around the holes was measured to determine the sensitivity of tested microorganisms to the various extracts of *T. minuta*. All experiments were performed in duplicate and results were reported as the mean of two experiments. A clear zones >10 mm are considered as positive results (Lima-Filho et al., 2002). Inhibition zones of <12, 12-20 and ≥20 mm diameter are expressed as weak, moderate and strong activities, respectively.

2.6 Determination of minimum inhibitory concentrations (MICs)

The MIC value is the minimum concentration of the sample needed to inhibit the growth of the microorganisms (Alghazeer et al., 2012; Alghazeer et al., 2017). The MIC values of <100 µg/mL, 100 to ≤625 µg/mL and >625 µg/mL were considered as significantly

active, moderately active and weakly active, respectively (Emmanuel et al., 2012; Njimoh et al., 2015; Matela et al., 2018). The MIC values were determined as described in the literature (Daud et al., 2005; Alghazeer et al., 2017; Matela et al., 2018). Briefly, a stock solution at a concentration of 1000 µg/mL of various extracts of *T. minuta* was prepared separately. Two-fold serial dilutions such as 1000, 500, 250, 125, 62.5 and 31.25 µg/mL were made from the stock solutions. A suspension of the microorganism was prepared at a concentration of 1×10^6 to 2×10^6 colony-forming units (CFU) per mL by growing the strains in nutrient broth in an incubator with continuous shaking and then used against various extracts as per the method described in literature (Daud et al., 2005). The cylindrical cavities of sizes 4.00 mm height and 6.00 mm diameter were punched on the agar plate using a sterile cork-borer and filled with 30 µL aliquots of the extracts. The plates were then incubated at 37°C for 24 hrs for bacterial species and at 24°C for 7-14 days for fungal species.

2.7 Statistical analysis

The statistical analysis was performed using SPSS (ANOVA) statistics program. The differences were considered statistically significant when $p \leq 0.05$.

3. Results and discussion

The antibacterial and antifungal activities of *T. minuta* hexane stem bark extract (TMHESB), *T. minuta* chloroform stem bark extract (TMCHSB), *T. minuta* ethyl acetate stem bark extract (TMEASB) and *T. minuta* methanol stem bark extract (TMMESB) are summarized

in Table 1. Against *S. aureus*, TMHESB, TMCHSB, TMEASB and TMMESB showed inhibition zones of 13.5 ± 2.1 , 13.4 ± 2.0 , 12.5 ± 1.9 and 12.9 ± 1.0 mm, respectively. These results showed that all extracts were moderately active and showed relatively weak activity compared to the positive control. The positive control, tetracycline, showed an inhibition zone of 24.5 ± 0.7 mm. Against *L. monocytogenes*, TMHESB, TMCHSB, TMEASB and TMMESB showed inhibition zones of 10.7 ± 1.4 , 13.0 ± 1.9 , 12.7 ± 1.5 and 13.4 ± 2.1 mm, respectively. THHESB exhibited weak activity with an inhibition zone of 10.7 ± 1.4 mm and all other extracts showed moderate activity with inhibition zones greater than 12.0 mm. However, all four extracts showed relatively weak activity compared to the positive control. The positive control, tetracycline, showed an inhibition zone of 29.5 ± 0.6 mm. Against *E. coli* (wild), TMHESB, TMCHSB, TMEASB and TMMESB showed inhibition zones of 15.5 ± 1.9 , 15.4 ± 2.1 , 12.3 ± 2.7 and 13.3 ± 1.0 mm, respectively. This result showed that all extracts were moderately active. Here again, all four extracts showed relatively weak activity compared to the positive control. The positive control, tetracycline, showed an inhibition zone of 20.5 ± 3.3 mm against the same bacteria. Against *E. coli* (O157:H7), TMHESB, TMCHSB, TMEASB and TMMESB showed inhibition zones of 13.3 ± 2.0 , 10.0 ± 1.6 , 14.3 ± 1.7 and 12.3 ± 2.8 mm, respectively. TMCHSB was weakly active with an inhibition zone of 10.0 ± 1.6 while other extracts were moderately active with inhibition zones greater than 12.0 mm. The positive control, tetracycline, showed an inhibition zone of 24.0 ± 2.0 mm against the same bacteria. Against *P. aeruginosa*, TMHESB, TMCHSB, TMEASB and

Table 1. Inhibitory effect of hexane, chloroform, ethyl acetate and methanolic extracts from stem bark of *T. minuta* on selected bacterial and fungal isolates

Microorganisms	Extracts/Zones of inhibition (mm) (diameter)				
	TMHESB	TMCHSB	TMEASB	TMMESB	Positive Controls
Bacterial isolates					
<i>S. aureus</i>	13.5 ± 2.1^a	13.4 ± 2.0^a	12.5 ± 1.9^a	12.9 ± 1.0^a	24.5 ± 0.7^a
<i>L. monocytogenes</i>	10.7 ± 1.4^a	13.0 ± 1.9^a	12.7 ± 1.5^a	13.4 ± 2.1^a	29.5 ± 0.6^a
<i>E. coli</i> (wild)	15.5 ± 1.9^a	15.4 ± 2.1^a	12.3 ± 2.7^c	13.3 ± 0.9^a	20.5 ± 3.3^b
<i>E. coli</i> (O157:H7)	13.3 ± 2.0^a	10.0 ± 1.6^b	14.3 ± 1.7^a	12.3 ± 2.8^b	24.0 ± 2.0^a
<i>P. aeruginosa</i>	12.3 ± 1.6^a	14.2 ± 2.2^a	13.4 ± 2.0^a	11.7 ± 2.4^b	22.5 ± 2.1^a
<i>S. marcescens</i>	13.6 ± 1.6^a	13.7 ± 1.4^a	11.4 ± 1.0^a	10.9 ± 1.6^a	09.0 ± 0.0^a
Fungal isolates					
<i>P. digitatum</i>	11.3 ± 2.1^b	13.4 ± 1.2^a	12.0 ± 1.5^a	11.9 ± 1.4^a	NT
<i>C. albicans</i>	-	-	-	-	24.5 ± 1.8^a

Values with different superscripts in the same column are statistically different at $p < 0.05$. TMHESB = *T. minuta* hexane stem bark extract; TMCHSB = *T. minuta* chloroform stem bark extract; TMEASB = *T. minuta* ethyl acetate stem bark extract; TMMESB = *T. minuta* methanolic stem bark extract. Tetracycline served as a positive control for *E. coli* (wild), *E. coli* (O157:H7), *S. aureus* and *L. monocytogenes*. Amoxicillin served as a positive control for *P. aeruginosa* and *S. marcescens*. Miconazole nitrate served as a positive control for *C. albicans*. Positive control was not maintained for *P. digitatum*. N/T = Not Tested. DMSO served as negative control. (-) = inactive.

TMMESB showed inhibition zones of 12.3±1.6, 14.2±2.2, 13.4±2.0 and 11.7±2.4 mm, respectively. These results showed that TMMESB was weakly active with an inhibition zone of 11.7±2.4 mm while all other extracts were moderately active. The positive control, amoxicillin, showed an inhibition zone of 22.5±2.1 mm. Against *S. marcescens*, TMHESB, TMCHSB, TMEASB and TMMESB showed inhibition zones of 13.6±1.6, 13.7±1.4, 11.4±1.0 and 10.9±1.6 mm, respectively. TMEASB and TMMESB exhibited weak activity with inhibition zones of 11.4±1.0 and 10.9±1.6 mm, respectively while TMHESB and TMCHSB were moderately active with inhibition zones greater than 12mm. The positive control, amoxicillin, showed an inhibition zone of 9.0±0.0 mm. In general, all extracts exhibited activity against all six bacterial isolates but their relative activity varied from one extract to another as shown in Table 1. Against *P. digitatum*, TMHESB, TMCHSB, TMEASB and TMMESB showed inhibition zones of 11.3±2.1, 13.4±1.2, 12.0±1.5 and 11.9±1.4 mm, respectively. This result showed that TMHESB and TMMESB were weakly active with inhibition zones of 11.3±2.1 and 11.9±1.4 mm, respectively while TMCHSB and TMEASB showed moderate activity with inhibition zones of 13.4±1.2 and 12.0±1.5 mm, respectively. Against *C. albicans*, these four extracts did not exhibit any visible inhibition zones while the positive control, miconazole nitrate showed an inhibition zone of 25.8±1.8 mm.

The minimum inhibition concentrations (MICs) of various extracts of *T. minuta* are summarized in Table 2. The MIC values of TMHESB and TMCHSB were found to be 62.5 µg/mL for each extract against *S. aureus*. However, TMEASB and TMMESB exhibited MIC values of 125 and <31.25 µg/mL, respectively against the same bacterial isolates. The MIC value of TMCHSB and TMEASB was found to be <31.25 µg/mL for each

extract while the MIC values of TMHESB and TMMESB were found to be >1000 and 125 µg/mL, respectively against *L. monocytogenes*. The MIC value of TMHESB, TMCHSB and TMMESB was found to be <31.25 µg/mL for each extract against *E. coli* (wild) while TMEASB exhibited MIC value of 62.5 µg/mL against the same bacterial isolates. The MIC values of TMHESB, TMCHSB, TMEASB and TMMESB were found to be 125, 1000, <31.25 and 250 µg/mL, respectively against *E. coli* (O157:H7). The MIC values of TMHESB, TMCHSB, TMEASB and TMMESB were found to be 250, 62.5, 125 and 250 µg/mL, respectively against *P. aeruginosa*. The MIC value of <31.25 µg/mL was found for TMHESB and TMCHSB for each extract against *S. marcescens* while TMEASB and TMMESB exhibited MIC value of 500 µg/mL for each extract. The MIC values of TMHESB, TMCHSB, TMEASB and TMMESB were found to be 500, <31.25, 250 and 125 µg/mL, respectively against *P. digitatum*. The MIC assay against *C. albicans* was omitted since all extracts showed no visible inhibition zone in the preliminary study (Table 1).

Essential oils obtained from aerial parts of *T. minuta* collected in Egypt, South Africa and the UK have previously been evaluated for their antimicrobial activity against eight bacterial isolates viz. *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus faecalis*, *E. coli*, *Proteus mirabilis*, *P. aeruginosa* and *Salmonella enterica* serovar Typhi (Senatore et al., 2004). The essential oils from plants of the UK showed higher inhibition zones than the essential oils from the plants of Egypt and South Africa (Senatore et al., 2004). Additionally, the MICs for the essential oils from plants of the UK were found to be 6.25-25.0 µg/mL for Gram-positive bacteria and 25.0-50.0 µg/mL for Gram-negative bacteria with the lowest MIC of 6.25 µg/mL against *S. faecalis*. However, the essential oils from

Table 2. The minimum inhibitory concentrations (MICs) of hexane, chloroform, ethyl acetate and methanolic extracts from stem bark of *T. minuta* on selected bacterial and fungal isolates.

Microorganisms	Extracts/Minimum inhibition concentrations (MICs) (µg/mL)			
	TMHESB	TMCHSB	TMEASB	TMMESB
Bacterial isolates				
<i>S. aureus</i>	62.5	62.5	125	<31.25
<i>L. monocytogenes</i>	>1000	<31.25	<31.25	125
<i>E. coli</i> (wild)	<31.25	<31.25	62.5	<31.25
<i>E. coli</i> (O157:H7)	125	1000	<31.25	250
<i>P. aeruginosa</i>	250	62.5	125	250
<i>S. marcescens</i>	<31.25	<31.25	500	500
Fungal isolates				
<i>P. digitatum</i>	500	<31.25	250	125
<i>C. albicans</i>	N/T	N/T	N/T	N/T

TMHESB = *T. minuta* hexane stem bark extract; TMCHSB = *T. minuta* chloroform stem bark extract; TMEASB = *T. minuta* ethyl acetate stem bark extract; TMMESB = *T. minuta* methanolic stem bark extract, N/T = Not Tested. DMSO served as negative control.

plants of South Africa showed MICs of 50.0-100 µg/mL against both Gram-positive and Gram-negative bacteria. The MICs of the essential oils from plants of Egypt were found to be 100 and 50.0 µg/mL, respectively against *P. aeruginosa* and *Salmonella enterica* serovar Typhi. Against the other six bacterial isolates, these essential oils from the plants of Egypt showed lower MIC values than essential oils from the plants of South Africa (Senatore *et al.*, 2004). The antimicrobial activity of aerial parts of essential oils of *T. minuta* collected from Maseno area of Kenya were also evaluated against *Pseudomonas savastanoi* pv. *Phaseolicola* (PSP), *Xanthomonas axonopodis* pv. *Phaseoli* (XAP) and *Xanthomonas axonopodis* pv. *Manihotis* (XAM) (Gakuubi *et al.*, 2016). The inhibition zones were found to be 26.83±0.60, 26.83±0.17 and 41.83±0.93 mm for XAP, XAM and PSP, respectively. The MICs values were found to be 12, 24 and 48 mg/mL for PSP, XAP and XAM, respectively. Our literature search showed that extracts from stem bark of *T. minuta* have not previously been reported. To the best of our knowledge, this is the first report of this kind, particularly, the species from the Kingdom of Lesotho.

4. Conclusion

We evaluated antibacterial and antifungal activities of hexane, chloroform, ethyl acetate and methanolic extracts from stem bark of *T. minuta*. Six bacterial isolates viz. *S. aureus*, *L. monocytogenes*, *E. coli* (wild), *E. coli* (O157: H7), *P. aeruginosa* and *S. marcescens* and two fungal isolates viz. *C. albicans* and *P. digitatum* were used in this study. The zones of inhibition were found to be in the range 10.0±1.6 to 15.5±1.9 mm against bacterial isolates and 11.3±2.1 to 13.4±1.2 mm against *P. digitatum*. However, these extracts did not exhibit any visible inhibition zones against *C. albicans*. Additionally, the minimum inhibitory concentrations (MICs) of these extracts were also evaluated and was found to be in the range of <31.25 to >1000 µg/mL. To conclude, *T. minuta* showed a moderate to significant antibacterial and antifungal activities. *T. minuta* has been used in food and beverage industries as preservative, coloring and flavoring agents. *T. minuta* also finds therapeutic applications in the traditional medicine.

Conflict of interest

The authors declare that there is no conflict of interests.

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