

PHYTOCHEMICAL SCREENING AND POTENTIAL ANTIBACTERIAL ACTIVITY OF *TAGESTES MINUTA* L. LEAVES

RIKISAHEDREW, J. J.¹ – NAIDOO, Y.² – DEWIR, Y. H.^{3*}

¹*Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Tartu 51014, Estonia*

²*School of Life Sciences, University of KwaZulu-Natal, Westville Campus, Private Bag X54001, Durban 4000, South Africa*

³*Plant Production Department, College of Food & Agriculture Sciences, King Saud University, Riyadh 11451, Saudi Arabia*

*Corresponding author
e-mail: ydewir@ksu.edu.sa

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Abstract. *Tagetes minuta* is an important species in the traditional medicine due to its essential oil from leaves. In this study we report on the qualitative and quantitative phytochemical analyses of leaves extract using organic solvents. Qualitative analysis indicated the presence of alkaloids, sterols, saponins, terpenoids, phenols, and lipids in the leaves extract. Gas-chromatography mass-spectrometry (GC-MS) analysis revealed constituents with the highest percentage were 9-octadecen-1-ol (4.51%), β -sitosterol (6.07%), olean-12-en-3-one (7.47%), and 3-methyl-1-butanol (14.77%). Leaves extract showed inhibitory activity against selected gram-positive and gram-negative bacteria (*P. aeruginosa*, *E. coli*, *S. aureus*, MRSA, and *B. subtilis*). This study confirmed that the leaves of *T. minuta* contain bioactive compounds with pharmacological and biological properties that can be further explored.

Keywords: antibacterial, bioactive compounds, GC-MS, methanol extract, traditional medicine

Introduction

Plant material has been used in traditional medicine for millennia in both developing and developed countries due to its ease of access and lesser risk of side effects on human health, and as such have been cultivated in many communities (Qazi Majaz and Molvi Khurshid, 2016; Mickymaray, 2019; McGaw et al., 2022). The use of ethnomedicine is becoming increasingly popular in modern societies as natural alternatives to synthetic products and has become a socio-cultural phenomenon of third world countries (Dixit et al., 2013; Agidew, 2022; Kola et al., 2022). While plant extracts and traditional medicines are colloquially deemed to be safer for use, it is important to note that safe usage typically depends on a good understanding of the biochemical effects of extracts in the context of treatment, as well as monitoring the dosage in which it is used (Nobahkt et al., 2022). In Southern Africa, healthcare is largely polarised between Western medical therapies and traditional African healthcare systems (Anywar et al., 2020; McGaw et al., 2022). Although concurrent usage of traditional healers and allopathic providers of medicine is common, allopathic healthcare facilities are often limited and not easily accessible to most in South Africa (Bisi-Johnson et al., 2017). This has implemented the medicinal plant industry in South Africa being supported by approximately 30 million local consumers with almost 70 thousand species of plants being used for medicinal purposes, many of which are threatened by overharvesting and international trade (Street and Prinsloo, 2013; Xego et al., 2016; Khumalo et al., 2022). The indiscriminate

overharvesting coupled with extensive land degradation has affected the availability of medicinal plants that can be naturally foraged, resulting in an increasing demand for medicinal plant material (Phondani et al., 2016; Tanga et al., 2018). In order to establish strategies to protect endemic medicinal plants, it is vital that they are screened for the bioactive phytochemicals responsible for their therapeutic properties. This will ensure regulation for use of wild populations of medicinal plants by putting into place mitigative measures such as systematic plant cultivation for the production and monitoring of this underutilised resource (Tanga et al., 2018; Khumalo et al., 2022). Moreover, many isolated phytometabolites from medicinal plants have led to the development of novel drugs and therapies to treat several human diseases such as cancer, diabetes, and gastrointestinal diseases (Hosseinzadeh et al., 2015; Shakya, 2016; Kudumela and Masoko, 2017; Sulaiman et al., 2022; Abubakar et al., 2022).

Tagetes minuta belongs to the Asteraceae family, and its essential oils are renowned for its medicinal, ornamental, and therapeutic values (Dixit et al., 2013; Rajvanshi and Dwivedi, 2017; Walia et al., 2020; Abdoul-Latif et al., 2022). The common name for *T. minuta* is Mexican marigold as it is native to South America, but has since been naturalised in Africa, Europe, North America, and Asia (Meshkatsadat et al., 2010). The essential oils of *Tagetes* have been researched for its nematocidal (Gutierrez et al., 2006), cosmetic (Farjana et al., 2009), food additive (Nandita et al., 2012), and antimalarial (Kyarimpa et al., 2014) properties. Phytochemical analyses of *Tagetes* essential oil show that the plant is rich in alkaloids, phenolic compounds, flavonoids, and thiophenes which are responsible for its therapeutic properties (Chamorro et al., 2008; Meshkatsadat et al., 2010; Devika and Koilpillai, 2012). While *Tagetes minuta* is used medicinally in Southern America, this plant is considered invasive in South Africa; a better understanding of phytochemical constituents and uses for this plant can allow for reassessment of its classification (Jinsheng et al., 2023; Ngondya and Munishi, 2023). The oils of *T. minuta* have been reported as having a high inhibitory effect on both gram positive and gram-negative bacteria and fungi (Hethelyi et al., 1986), however leaf infusions and solvent extracts have not been analysed despite these being the most common preparation methods. Therapeutically, leaf infusions of *T. minuta* have several reported medicinal benefits such as remedies for respiratory inflammations, stomach pains, chest infections, coughs, and congestion (Shirazi et al., 2014; Abdoul-Latif et al., 2022). It also has a healing effect on wounds, cuts, and calluses (Rahimi et al., 2010; Maity et al., 2011). This study was undertaken to examine the qualitative phytochemical composition of three solvent extracts of *Tagetes minuta* using phytochemical tests; to determine the relative quantitative chemical compositions using GC-MS, and to investigate the antibacterial potential of the leaf extracts against both gram-positive and gram-negative bacteria.

Materials and Methods

Plant collection and preparation of leaf extract

Fresh mature leaves were collected from a population (approximately 50 individuals) of *T. minuta* at the University of KwaZulu-Natal (UKZN) Westville campus (29.817°S, 30.940°E) in Durban, South Africa. A voucher specimen was deposited into the UKZN Westville Herbarium (accession number 18216, voucher number 01). The leaves were cleaned with distilled water and air-dried for 4 weeks at 24 °C. The dried leaves were then powdered using a mortar and pestle to form a fine powder. Ten grams of *T. minuta*

powdered leaf material was placed in a round bottom flask containing 100 ml of hexane. The flask was attached to a Soxhlet apparatus and boiled for three 3-hour sessions to obtain the crude plant extract. This procedure was then repeated using the same leaf material for chloroform and methanol, respectively. The crude extracts were filtered twice through Whatman No. 1 filter paper and then stored in airtight jars at 4 °C for further analysis. All analyses were completed within 4 weeks of extraction.

Phytochemical analyses

a) Detection of carbohydrates

Molisch's test was performed according to Deepthi Yadav Chappidi et al. (2013). Two drops of α -naphthol were added to 2 ml of each solvent extract and gently shaken. One ml of concentrated sulphuric acid was slowly poured along the side of the test tube and allowed to stand. The formation of a red-purple ring at the junction of the two liquids was indicative of the presence of carbohydrates in the sample. Benedict's test and Fehling's test were performed according to Deepthi Yadav Chappidi et al. (2013). One ml of Benedict's reagent was added to 1 ml of filtrate and boiled for 2 min in a water bath at 100°C. An orange-red precipitate indicated the presence of reducing sugars. For Fehling's test, 1 ml each of Fehling's solutions A and B were mixed into 1 ml of extract and boiled in a water bath (100 °C). The formation of a red precipitate indicated the presence of carbohydrates.

b) Detection of amino acids

Ninhydrin test – two drops of ninhydrin reagent were mixed into 2 ml of extract. A purple colour indicated the presence of amino acids and proteins (Minj et al., 2015).

c) Detection of alkaloids

Mayer's test – three drops of potassium mercuric iodide solution was added to 2 ml of filtrate. A yellow-orange precipitate confirmed the presence of alkaloids (Surendra et al., 2016). Wagner's test – two drops of Wagner's reagent was added to 1 ml of extract. A brown precipitate indicated the presence of alkaloids (Surendra et al., 2016). Dragendorff's test – two drops of Dragendorff's reagent were added to 1 ml of extract. An orange-red precipitate indicated the presence of alkaloids (Surendra et al., 2016).

d) Detection of saponins

Foam test – two ml of extract was diluted using 5 ml of distilled water and vigorously shaken for 5 min. A persistent foam layer above the mixture indicated the presence of saponins (Bargah, 2015).

e) Detection of sterols

Salkowski's test – three ml of chloroform and 2 drops of concentrated sulphuric acid was added to 2 ml of extract and gently shaken. A red ring in the chloroform layer and green precipitate in the extract indicated the presence of sterols (Bargah, 2015).

f) Detection of terpenoids

Sulphuric acid test – two ml of chloroform was added to 5 ml of extract and shaken gently. Three ml of sulphuric acid was poured gently along the side of the test tube. A red-brown ring indicated the presence of terpenes (Bargah, 2015).

g) Detection of phenolic compounds

Ferric chloride test – two drops of 5% ferric chloride was added to 2 ml of filtrate. A green-black precipitate indicated the presence of phenolic compounds (Surendra et al., 2016).

h) Detection of fixed fats and oils

Filter paper test – One drop of extract was placed on Whatman No. 1 filter paper and left to dry. A persistent oily residue present on the filter paper indicated a positive test for fixed fats and oils (Minj et al., 2015).

Gas chromatography-mass spectrometry (GC-MS)

Five grams of powdered leaf material was submerged in 50 ml of methanol in a round bottom flask and boiled for two 3 hr sessions. The crude extracts were filtered twice through Whatman No. 1 filter paper and stored in an airtight jar at 4°C until analysed. The extract was analysed by GC-MS using a QP-2010 Ultra Shimadzu system with a Rx-5SilMS fused silica column of length 30 m (0.25 µm internal diameter and 0.1 µm film thickness). Helium was used as the carrier gas at a constant pressure of 69 kPa. The flow rate was 0.96 ml/min with a total flow of 4.9 ml/min, along with a linear velocity of 36.7 cm/s at purge flow of 3.0 ml/min. The injection port temperature was set at 250 °C. The temperature of the oven was initially set to 50 °C for 1 min. The temperature was then increased to 310 °C at a rate of 5 °C/min and was maintained for 10 min. The MS was taken at 70 eV. The mass selective detector was operated in the scan mode between 50 and 800 m/z. Peak identification was carried out by comparison of the mass spectra with mass spectra data available on database of NIST and WILEY libraries (Kataria et al., 2016). The chemical compounds present in the crude methanolic extract of *T. minuta* were expressed as percentages based on peak area.

Antibacterial activity screening

The crude methanolic extract was first dried to a powder and re-suspended in sterile deionized water to avoid antibacterial activity from the methanol (Valle et al., 2016). The extract was subsequently adjusted to a final concentration of 1 mg/ml. The preliminary antibacterial activity of the crude aqueous extract was evaluated against *Escherichia coli* (ATCC 25218), *Staphylococcus aureus* (ATCC 29213), methicillin-resistant *Staphylococcus aureus* (ATCC BAA-1683), *Bacillus subtilis* and *Pseudomonas aeruginosa* (ATCC 25215). Agar plates were prepared using Mueller Hilton agar (Biolab, Merck) which was poured into sterile petri dishes to set at room temperature. The bacterial strains were cultured in a nutrient broth for 18 hrs at 37°C before being standardised using the 0.5 McFarland turbidity standard (OD 0.08–0.1 λ 625 nm). The bacterial cultures were then swabbed uniformly onto the plates using sterile cotton swabs and left to dry. The wells were aseptically punched using an agar corer (gel puncture – 5 mm in diameter). The samples were pipetted into the wells (90 µl), and the plates were incubated at 37 °C. The antibacterial activity was assessed after 24 hrs by measuring the diameter of the zone

of inhibition (mm). The experiment was conducted in triplicate, with gentamicin (for gram-negative) and streptomycin (for gram-positive) being used as standard bacterial positive controls, and sterile deionized water as the negative control.

Results and Discussion

The phytochemical screening of *T. minuta* extracts were performed qualitatively for the three organic solvents, viz. hexane, chloroform, and methanol, and the results are presented in *Table 1*. The hexane extract tested positively for the presence of alkaloids, saponins, sterols, terpenoids, and fixed fats and oils. The chloroform extract tested positively for the presence of carbohydrates, alkaloids, sterols, terpenoids and fixed fats and oils. The methanolic extract tested positively for the presence of carbohydrates, alkaloids, amino acids, terpenoids, phenols, and fixed fats and oils. In our previous study on *T. minuta*, histochemical analysis revealed the presence of alkaloids and phenolic compounds in non-glandular trichomes (Naidoo et al., 2021).

Table 1. Qualitative phytochemical analysis of *Tagetes minuta* leaf extracts (n = 3)

Phytochemical compound	Test	Hexane extract	Chloroform extract	Methanol extract
Carbohydrates	Molisch's	–	+	+
	Benedict's	–	+	++
	Fehling's	–	+	–
Alkaloids	Mayer's	+	+	++
	Wagner's	++	+	+
	Dragendorff's	+	++	+
Amino acids	Ninhydrin	–	–	+
Saponins	Foam	+	–	–
Sterols	Salkowski's	+	+	–
Terpenoids	Sulphuric acid	+	+	+
Phenols	Ferric chloride	–	–	+
Fixed fats & oils	Filter paper	+	+	+

– Absent, + Present (reaction occurred within minutes), ++ Intense positive reaction (immediate reaction)

These bioactive compounds are known to provide the plant with protection against pathogens, predators, diseases, and abiotic factors (Saxena et al., 2013; Aloo et al., 2019) which ties in with our previous findings of these compounds accumulating specifically in the surface-level structures of the leaves on *T. minuta* (Naidoo et al., 2021). A similar phytochemical profile was identified for the extract of *T. erecta* (Rajvanshi and Dwivedi, 2017; Kumar and Upadhyay, 2022) where it is shown that these classes of compound are responsible for antifungal, antibacterial, and anti-inflammatory properties in this genus. According to Shakya (2016), medicinal plants that contain sterols and saponins exhibit antimicrobial and anticancer properties, and it is documented that the essential oils from whole, flowering *T. minuta* plants have antimicrobial, antioxidant, and antitumour activity (Ickesh et al., 1973; Upadhyay et al., 2010). Alkaloids, flavonoids, terpenes, saponins, and phenolic compounds have been reported to possess various pharmacological and therapeutic effects, including antimicrobial, antioxidant, anti-diabetic, and anti-inflammatory activity (Abdul et al., 2018).

The chemical constituents of the methanolic extract of *T. minuta* were analysed using GC-MS (Figure 1). The separation of all 108 bioactive compounds were identified by retention time and height percentage. Twenty-four out of the 108 compounds displayed a peak percentage greater than 1% (Table 2). Compounds with a peak area less than 1% were considered as low-level compounds (Pakkirisamy et al., 2017) and thus not included in this analysis. The compounds with the highest composition percentage were 3-methyl-1-butanol (14.77%), olean-12-en-3-one (7.47%), β -sitosterol (6.07%) and 9-octadecen-1-ol (4.51%). These results are surprising in that they differ greatly as compared to the chemical profiling of the essential oils of *T. minuta*, where tagetones are often the most abundant compounds (Shahzadi et al., 2010; Shirazi et al., 2014; Rezaei et al., 2018). The difference in chemical constituents can be attributed to the use of methanol as an extracting agent, increasing the likelihood of phenolic compounds in solution (Babaei et al., 2021).

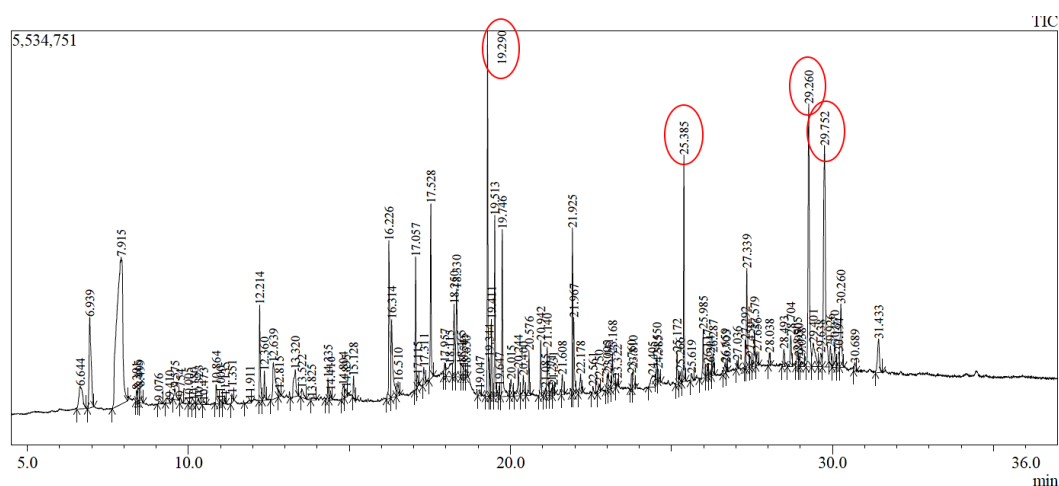


Figure 1. GC-MS chromatogram of the methanolic extract of *Tagetes minuta* leaves

The compound 9-octadecen-1-ol is a long-chain aliphatic alcohol that is known to decrease low-density lipoprotein (LDL) cholesterol and increase high-density lipoprotein (HDL) cholesterol and is most commonly synthetically produced as a lipid-lowering drug for high cholesterol patients (Santos et al., 2015). Additionally, Shen et al. (2018) has shown that 9-octadecen-1-ol is also a large constituent of *Bidens pilosa*. This becomes important when paired with the findings of Cid et al. (2016) who studied the effects of co-cropping *B. pilosa* with *T. minuta*, claiming that their similar chemical profile aids their ability to bioaccumulate metals and act as an herbicide. Beta-sitosterol is a nontoxic isoprenoid that has displayed anticancer effects against breast cancer, prostate cancer, colon cancer, lung cancer, stomach cancer, ovarian cancer, and leukaemia by interference of multiple cell-signalling pathways (Bin Sayeed and Ameen, 2015).

The effects of β -sitosterol and olean-12-en-3-one on cancer cells in vitro are weak as compared to other oleanane-type triterpenoids, but still exhibit antibacterial, antiviral, and gastroprotective properties (Ge et al., 2018; Oladosu et al., 2018). Kyarimpa et al. (2014) and Athuman et al. (2016) studied the repellence of the malaria-carrying mosquito, *Anopheles gambiae*, using essential oils from *T. minuta*. Both studies concluded that some constituents in the essential oil causes 100% mortality of mosquito larvae as well as adult mosquitoes, but the compound was not identified. Zohdy et al. (2015) established the use

of 3-methyl-1-butanol as an odour-bait for *Anopheles* mosquitoes. This chemical compound produced by both plants and animals acts a lure for mosquitoes. This implies that *T. minuta* has the potential to act as lure for malaria-carrying mosquitoes, as well as acting as an insecticide. The presence of these phytochemicals makes *T. minuta* a considerable candidate for the production and optimisation of valuable compounds with pharmacological and therapeutic benefits.

Table 2. Phytochemical compounds with % peak area >1 in the methanolic extract of *T. minuta* by GC-MS

Peak	Retention time(min)	Compound name	Molecular formula	Height (%)
1	6.664	D-Limonene	C ₁₀ H ₁₆	1.43
2	6.939	Valeric acid	C ₅ H ₁₀ O ₂	2.99
3	7.915	3-methyl-1-butanol	C ₆ H ₁₂ O ₂	14.77
20	12.214	Pyrrolidine	C ₄ H ₉ N	1.37
22	12.639	Benzaldehyde	C ₇ H ₆ O	1.65
24	13.320	Diethyl pyrocarbonate	C ₆ H ₁₀ O ₅	1.04
32	16.226	5-pyrimidinol,2-methyl-	C ₆ H ₆ N ₂ OS	3.15
33	16.314	Supinine	C ₁₅ H ₂₅ NO ₄	2.22
35	17.057	Phetyl acetate	C ₂₂ H ₄₂ O ₂	1.77
38	17.528	Heptadecanol-1	C ₁₇ H ₃₆ O	2.48
41	18.250	Rivastigmine	C ₁₄ H ₂₂ N ₂ O ₂	1.41
42	18.330	Pentadecanoic acid	C ₁₅ H ₃₀ O ₂	2.71
47	19.920	9-octadecen-1-ol, (Z)-	C ₁₈ H ₃₆ O	4.51
49	19.411	Limonen-6-ol, pivalate	C ₁₅ H ₂₄ O ₂	1.02
50	19.513	Nonadecanol-1	C ₁₉ H ₄₀ O	2.35
52	19.746	Phytol	C ₂₀ H ₄₀ O	2.64
63	21.925	2-chloro-4,6-dimetoxypyrimidine	C ₆ H ₇ ClN ₂ O ₂	2.12
64	21.967	Oleamide	C ₁₈ H ₃₅ NO	1.23
79	25.385	Squalene	C ₃₀ H ₅₀	2.59
89	27.339	1-heptacosanol	C ₂₇ H ₅₆ O	1.83
99	29.260	β-sitosterol	C ₂₉ H ₅₀ O	6.07
100	29.401	Heptadecanoic acid	C ₁₇ H ₃₄ O ₂	1.20
102	29.752	Olean-12-en-3-one	C ₃₀ H ₄₈ O	7.47
106	30.260	α-amyrin	C ₃₀ H ₅₀ O	1.50

The antibacterial efficacy of the methanolic extract from *T. minuta* leaves at a concentration of 1 mg/ml (Table 3) were assessed by measuring the diameter of the inhibition zone of each well using callipers. The extract exhibited varying degrees of inhibition against 5 bacterial strains (MRSA, *E. coli*, *S. aureus*, *B. subtilis*, and *P. aeruginosa*). The antibiotic control used for the gram-positive bacteria was streptomycin, while gentamycin was used as the control for gram-negative bacteria. In general, the methanolic extract proved more effective against gram-positive than gram-negative bacteria. Previous studies reported antibacterial activities of the essential oil from other species of the genus *Tagetes* such as *T. patula* (Rondon et al., 2006) and *T. erecta* (Tripathi et al., 2012). These studies report flavonoids, alkaloids, and phenolic compounds are responsible for the antibacterial effects of extracts from *Tagetes* species

(Kumud et al., 2010; Tripathy et al., 2017). Certain flavanoids can only be extracted using ethanol as the carrying solvent for *Tagetes minuta* leaves, which has been shown to contain the active phytochemical myricetin glycoside that possess high antiviral properties and has been shown to be effective against Herpes Simplex virus type-1 and West Nile virus in *in vitro* studies (Martinez et al., 2020).

Table 3. Antibacterial activity of *T. minuta* leaves extract ($n = 3$)

Bacterial strains	Zone of Inhibition (mm)	
	Methanolic extract 1 mg/ml (mm)	Antibiotic 1 mg/ml (mm)
Methicillin-resistant <i>Staphylococcus aureus</i>	16±0.47	0±0.00
<i>Escherichia coli</i>	10±0.31	18±0.92
<i>Staphylococcus aureus</i>	10±0.12	0±0.00
<i>Bacillus subtilis</i>	12±0.30	6±0.12
<i>Pseudomonas aeruginosa</i>	13±0.42	9±0.40

Data presented are means ± standard error

Conclusions

Tagetes minuta is an important medicinal plant for human use as well as an insecticide and herbicide. Its applications extend pharmaceutically to include antidiabetic, anti-inflammatory, anticancer, and antimicrobial activities as evidenced from the phytochemicals found in abundance in the methanolic extract from the leaves of *T. minuta*. This study compared antibacterial efficacy against streptomycin and gentamycin, hence a more conclusive result could be made by increasing the number of antibiotics. The results of this study show potential for research regarding the regulation, use, and development of bioactive compounds sourced from medicinal plants. Additionally, the plethora of pharmacologically useful compounds sourced from *T. minuta* makes a convincing case for the status to be changed from invasive species to underutilised medicinal plant crop in South Africa.

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