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IN VITRO ANTIFUNGAL, ANTIBACTERIAL, PHYTOTOXIC, BRINE SHRIMP, INSECTICIDAL ACTIVITIES AND COMPOSITION OF ESSENTIAL OIL OF *TAGETES MINUTA* FROM DIR-KOHISTAN, PAKISTAN

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Abstract

The essential oil of the flowering shoot of *Tagetes minuta* L., collected from Dir Kohistan of Pakistan, was investigated for biological activities and chemical composition. The GC and GC/MS analysis data showed that among twenty two compounds, verbenone (25%), Unknown(11.6%),4-(5-methyl-2-furanyl)-2-butanone (8.8%) and D-limonene (8.4%) were the major constituents. The biological activity results showed that out of six bacteria stains, the oil possessed inhibitory activities against *S. flexenari* and *S. aureus* with a zone of inhibition 22mm and 20mm respectively. Among five fungal strains, only *Aspergillus flavus* and *Candida albicans* were susceptible to oil, with 60% and 30% inhibition respectively. In insecticidal tests, the oil was noted for its effectiveness toward *Sitophilus oryzae*, with 50% mortality. Although the oil showed no cytotoxicity against *Artemia Salina*, while moderate phytotoxic activity was observed for *Lemna minor*.

Key words: Essential oil; *Tagetes minuta* L., verbenone; D-limonene, Antibacterial, Antifungal cytotoxicity, Phytotoxicity, Insecticidal activities.

Introduction

The term of medicinal plants incorporates different kinds of plants utilized as a part of Herbalism and a portion of these plants have a therapeutic exercise. These restorative plants, consider as a rich asset of fixings which can be utilized as a part of medication advancement and blend. Other than that these plants assume a basic part in the advancement of human societies around the entire world. In addition These plants incorporate ginger, green tea, walnuts and some other Medicinal plants used in different cultures, traditions and folklore medicine for the treatment of various ailments (Khalil *et al.,* 2014. The synthetic antibiotics that have been using in the recently, are accociated with several side effects, high cost and increasing resistance, therefor further investigation on the clinical findings and traditional medicines is needed to obtain new sources of therapeutic agents. Recently most of the scientists are focusing of the natural source of drugs for curing various ailments and have keen interest in the clinical trails of such medicines to investigate its potency and side effects. One of such traditional remidies is the essential oils (EOs) that has recently attracted the focus of practioners and researchers (Aneta *et al.,* 2018; Cowan 1999; Jan *et al.,* 2018; Jan *et al.,* 2017; Kalemba *et al.,* 2003; Noor *et al.,* 2018). Recent literature reveals about 3000 essential oils (Thosar *et al.,* 2013), and it was found that EOs have various biological activities (Baratta *et al.,* 1999; Hammer *et al.,* 1999; Guleria *et al.,* 2013).Our country has a greater potential of indigenous herbal resources and most of the medicinal plants of the country are yet to be explored (Shinwari, 1996; Shinwari, 2010) for its essential oil and bioactivities.

The present research is based on the biological activities and components of the essential of *Tagetes minuta* L. it is locally known as Zangali Dambar Guley an annual plant of the Asteraceae family, distributed in northern areas of Pakistan in a wide range of climatic conditions (Sadia *et al.,* 2013). Marigold (*Tagetes minuta L.*) oil has a good market in perfumery and flavor industry in India, cultivated in main growing areas like Himachal Pradesh, Jammu and Kashmir and Uttar Pradesh, producing the net profit estimated as 30-40 thousand rupees per hectare (Kumari *et al.,* 2000). It has been found that limonene, which is the main component of essential oil is responsible for the mosquito repellent effects (Gillij *et al.,* 2008). It has been reported that the essential oil of *T. minuta L. contains* z-β-ocimene and Dihydroxyacetone that is the active compounds for the control of nematode i.e. *Meloidogyne incognita* (Adekunle *et al.,* 2007). Tagetes has been used as a source of carotenoids, also used as a colorant in poultry feed, citrus juices, dairy products, confectionery, pasta, margarine, salad dressing, ice cream, vegetable oil and baked materials. *T. minuta* essential oil has been found to have antimicrobial, cytotoxic (Mohsen *et al.,* 2014*)*, antioxidant, anti-inflammatory (Karimian *et al.,* 2014) and diuretic activities (Ambasta SP 1986).

The literature survey reveals the differences in the composition and qualities of the essential oil of the *T. minuta L*. depending on the various locations, growth stages and environmental conditions (Gili *et al.,* 2000; Omidbaigi *et al.,* 2008; Meshkatalsadat *et al.,* 2010; Craveiro *et al.,* 1988; Rao *et al.,* 2006; Moghaddam *et al.,* 2007; Chamorro *et al.,* 2008). Due to variation in the composition from locality to locality and expected variation in medicinal potential, the composition and biological activities of the essential oil of *Tagetes minuta L*., was selected for investigation.

Materials and Methods

Plant material: Flowering shoot biomass of *Tagetes minuta* L. (Asteraceae) was collected from the vicinity of Shaheed Benazir Bhutto University Sheringal, Pakistan (26th November 2013). The Plant materials were

identified and voucher specimens (SHPC-AKJ-40) were preserved in the Herbarium of the Shaheed Benazir Bhutto University.

Isolation of oils: Fresh flowering shoot biomass (200g) of *Tagetes minuta* L. subjected to steam distillation in Clevenger apparatus. The oil (yellow color), extracted from *T. minuta* L., and yielded 2.48655% oils (4.9731g of oil/200 g of biomass).

Gas chromatography: Shimadzu-17-A, with Nitrogen as a carrier gas, was used to obtain GC spectrum. FID detector was used for the detection of the given compounds.

GC/MS analysis: "GCMS and JEOL MS-Route system (JMS-600H) was applied and used carrier gas with HP-5 column (30 m x 0.33 mm, with a film depth of 0.25 µm). The temp (°C) of the oven was maintained at 60°C for 10 mints. and adjusted to 220°C at the rate of 4°C per mints and then kept fixed at 220°C for 10 mints to 240°C @ of a 1°C per mints. The Injector temp was kept at 250°C and the split flow was adjusted at the 1 ml per min. The EI was the ionization mode and the MS were recorded at 70 eV. The MS range was recorded from 35-425 m/z. The compounds of essential oils in the study were identified by JEOL GC/MS computer library by reviewing matching factor of the corresponding mass spectra and confirmed their details by Peak Index of Masses and dictionary of natural products (DNP). Relative percentage amounts were calculated with the help of GC and TIC by using a computer."The essential oil composition is listed in Table 1.

Biological activities: "Six bacterial strain and five of fungal reference strains were used to test the microbial activity. Bacterial types were *B. subtilis* (ATCC 6633), *E. coli* (ATCC-25922), *Shigella flexenari (clinical isolate), Salmonella typhi (*ATCC-19430), *S. aureus (*ATCC-25923) and *Pseudomonas aeruginosa (*ATCC-27853). Fungal strains included *are i.e., Candida albicans* (ATCC-2091), *Candida glabrata* (ATCC-90030), *Aspergillus flavus* (ATCC-32611), *Microsporum canis* (ATCC-11622) and *Fusarium solani* (ATCC-11712). Antimicrobial tests were carried out by hole diffusion method using standard procedures detailed in the literature and our previous reports (Atta-ur-Rahman *et al.,* 2001; Jan 2009), following Mac Farland turbidity standard No.0.5 for the cell suspension of about 1.5×10^6 CFU/mL (Berghe *et al.,* 1991). The suspension concentration was standardized by using the wavelength of 600nm and optical density to 0.1 on UV/visible spectrophotometer (SHIMADZU) (Tereschuck *et al.,* 1997). On the MHA plate, 6mm holes of the diameter (8mm thick) were made and filled with 150 µL of the oil sample as well as std. drug(s) dissolved in DMSO as 10 mg/mL. For screening, the strains were maintained at 4°C on agar slant and were activated at 37°C for 24hrs on Sabouraud glucose agar (SGA) or nutrient agar (NA) for bacteria and for fungi, respectively. The diameter of the growth was measured to determine the zone of inhibition in the given antimicrobial activity. The mean diameter was measured and the assay was repeated triple time.

For the comparison, the essential with the standard drugs/ antibiotics, Imipenum, Miconazole and Amphotericin B were used in the assay.

Table 1. Composition of Essential oil from the *Tagetis minuta*.

Scan (RI)	TIC	Compound (NIST)	%	R.T	m/z Base
9	351648	2,3,5-trimethylfuran	0.87	3.10	43
23	94160	Acetic acid, pentyl ester	0.23	3.28	43
91	70096	à-chloropropionaldehyde diethyl acetal	0.17	4.56	47
121	1517888	à-phellandrene	3.75	5.35	93
193	3401776	D-limonene	8.41	61.7	68
224	1373728	Z-ocimene	3.40	7.49	93
235	4727360	Unknown	11.69	8.03	57
258	1480000	2,7,7-trimethyl-3-oxatricyclo[4.1.1.02]octane	3.66	8.33	67
271	426912	5-methyl-2-(1-methylethyl)-2-cyclohexene-1-one	1.06	8.50	110
324	2220016	3,4-dimethyl-2,4,6-octatriene	5.49	9.58	121
328	610864	3,6-dimethyl-2,3,3a,4,5,7a-hexahydrobenzofuran	1.51	10.04	137
361	3557920	4-(5-methyl-2-furanyl)-2-butanone	8.80	10.27	95
424	2835120	3-methyl-6-(1-methylethylidene)-2-cyclohexen-1-one	7.01	12.08	150
493	10462624	Verbenone	25.88	13.37	107
513	1074432	3-methyl-6-(1-methylethenyl)-,(S)-2-cyclohexen-1-one	2.66	14.03	82
592	196400	Aciphyllene	0.49	15.46	95
682	1172048	Caryophyllene	2.90	17.42	41
721	440208	1,1,4,8-tetramethyl-,cis,cis,cis-4,7,10-cycloundecatriene	1.09	18.33	93
753	358736	à-cubebene	0.89	19.14	161
772	2335360	Germacrene B	5.78	19.39	121
1321	767536	4-(4,8-dimethyl-3,7-nonadienyl)-,(E)-2(5H)-furanone	1.90	31.30	150
1446	955520	Chichipegenin	2.36	34.00	83

RT; Retention time, RI; Retention indices

The cytotoxicity activity was assessed on lethality bioassay of Brine shrimp (Khalil *et al.,* 2017), while *Lemna minor* was used for the phytotoxic activity. The insecticidal activity was carried out by direct contact method (Atta-ur-Rahman *et al.,* 2001). The test insect was applied to *Callosbruchus analysis, Sitophilus oryzae, Tribolium castaneum, Rhyzopertha dominica* and *Trogoderma granarium*."

Results and Discussion: Keeping in view that various conditions have a significant effect on yield and chemical constituents of the essential oil of the *Tagetes minuta* L., the plant was chosen for investigation from the high altitudes of Sheringal in Pakistan. The oil of flowering shoot biomass of *T. minuta* L. yielded 4.9731g of oil/200g of biomass (2.48655%). The oil was characterized by GC/MS (JEOL MSRoute) and it was found that the oil composition of the *T. minuta* L. was found quite different from previously reported results from various other locations of the world (Omidbaigi *et al.,* 2008; Meshkatalsadat *et al.,* 2010; Craveiro *et al.,* 1988; Rao *et al.,* 2006; Moghaddam *et al.,* 2007; Chamorro *et al.,* 2008). In our current investigation, we identified about 22 compounds in which verbenone (25%), Unknown(11.6%) were the major constituents followed by 4-(5-methyl-2-furanyl)-2-butanone (8.8%). The GC data showed the peaks on RT (25.8, 26.5, 34.8 and 42.01 minutes, which were used to find the concerned concentration of the constituents. The GCMS analysis data of the essential oil of *T.minuta* L. revealed the presence of verbenone (25%), Unknown(11.6%), 4-(5-methyl-2-furanyl)-2-butanone (8.8%) and D-limonene (8.4%), 3-methyl-6-(1-methylethylidene)-2-cyclohexane-1-one (7.0%) and 3,4-dimethyl-2,4,6-octatriene (5.4%) etc. (Table 1).

The oil showed antibacterial activities against *S. flexenari* and *S. aureus* with a zone of inhibition 22mm and 20mm respectively (Table 2), while the other species were not inhibited. The oil exhibited the most interesting antifungal activities against *A. flavus* with 60% inhibition. The *Candida albicans* was inhibited as 30%, while all the other strains showed resistance (Table 3). Our results showed that the of *T. minuta* essential oil has a good phytotoxic activity of *L. minor* at a given concentration of 10,100 and 1000 µg/mL and caused inhibition of 25, 35 and 60% respectively (Table 4). The oil possessed insecticidal activities and caused 50% mortality against *Sitophilus oryzae* (Table 5*)*. The essential oil was also screened for Brine shrimp lethality bioassay techniques, but it displayed no significant activity (Table 6).

Table 2. Antibacterial activity of the essential of *Tagetes minuta* L.

Bacteria	Zone of inhibition of sample (mm)	Standard drugs	
		Name	Zone of Inhibition (mm)
Escherichia coli	--	Imipenum	35
Bacillus subtilis	--	Imipenum	35
Shigell flexenari	22	Imipenum	40
Staphylococcus aureus	20	Imipenum	50
Pseudomonas aeruginosa	--	Imipenum	20
Salmonella typhi	--	Imipenum	31

Table 3. Antifungal activity of the essential of *Tagetes minuta* L.

Fungi	Linear growth (mm)		% Inhibition	Standard drugs	
	Control	Sample		Name	MIC (µg/ml)
Candida albicans	100	80	30	Miconazole	110.8
Aspergillus flavus	100	40	60	Miconazole	20.0
Microsporum canis	100	100	0	Amphotericin B	98.4
Fusarium solani	100	100	0	Miconazole	73.25
Candida glabrata	100	100	0	Miconazole	110.8

Table 4. *In vitro* phytotoxicity of the essential of *Tagetes minuta* L.

Plant	Concentration of sample (µg/mL)	No. of fronds		% Growth regulation	Standard drug (paraquat) (µg/mL)
		Sample	Control		
Lemna minor	10	15	20	25	0.015
	100	13		35	
	1000	8		60	

Table 5. Insecticidal activity of the essential of *Tagetes minuta* L.

Insects	% Mortality		Sample 1019.10µg/cm^3
	+iv control	-iv control	
Tribolium castaneum	100	0	0
Sitophilus oryzae	100	0	50
Rhyzopertha dominica	100	0	0
Callosbruchus analis	100	0	0
Trogoderma granarium	100	--	--

Table 6. Brine shrimp (*Artemia salina*) lethality bioassay of the essential of *Tagetes minuta* L.

Dose (µg/mL)	No. of shrimps	No. of survivors	LD50 (µg/mL)	Standard drug	LD50 (µg/mL)
1000	30	30			
100	30	30	--	Etopside	7.4625
10	30	30			

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