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In vitro schistosomicidal effects of the essential oil of *Tagetes erecta*

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Abstract: The *in vitro* schistosomicidal effects of the essential oil obtained from *Tagetes erecta* L. Asteraceae, leaves (TE-EO) collected in Brazil against *Schistosoma mansoni* worms are reported in this paper. The oil caused a significant decrease in the motor activity at 50 µg/mL as minimal concentration after 24 h. This oil also caused death of all the parasites and the separation of coupled pairs into individual male and female at 100 µg/mL after 24 h. The viability of adult worm groups treated with the TE-EO at 100 µg/mL was similar to that of groups treated with praziquantel (positive control). In addition, the oil promoted the inhibition of eggs development at all the tested concentrations. These data indicate that the TE-EO could be considered as a promising source for the development of new schistosomicidal agents.

Introduction

Schistosomiasis, or bilharzia, is a parasitic disease caused by trematode flatworms of the genus *Schistosoma* (El Shenawy et al., 2008). It is one of the most prevalent parasitosis in the world, second behind malaria. The World Health Organization (WHO) estimates that approximately 200 million people are currently contaminated, and that 800 million are at risk of contracting this disease (Magalhães et al., 2010; Steinmann et al., 2006). Today, praziquantel (PZQ) is the most widely employed drug for the treatment of Schistosomiasis, and it plays a key role in population-based disease-control programs (Melo et al., 2011). However, PZQ does not prevent re-infections, it is inactive against juvenile schistosomes, has limited effect on parasite liver forms (Utzinger et al., 2003). Moreover, the reliance on one single antischistosomal drug has culminated in the development of resistant schistosome strains at an alarming rate (Wang et al., 2010; Engels et al., 2002; Ismail et al., 1999), thus the above mentioned observation motivated us to investigate *Tagetes erecta* L. aiming at the design of novel and inexpensive drugs against Schistosomiasis.

The search for antiparasitic compounds from natural sources has increased over the last decade, and plants continue to be a major source of biologically active compounds that may provide lead structures for the development of new drugs (Magalhães et al., 2010; Pontin et al., 2008). In this scenario, some essential oils have been recently pointed out as a promising alternative against *Schistosoma mansoni* (Melo et al., 2011; Caixeta et al., 2011; Parreira et al., 2010).

Tagetes erecta L., Asteraceae, commonly known as "marigold" in many countries and as "cravo-de-defunto" in Brazil, is an annual aromatic and branched herb native to Mexico. It is frequently used as an ornamental plant (Sowbhagya et al., 2004) and is often used in folk medicine against rheumatism, headache, dysmenorrheal, and bronchitis. Moreover, the essential oil from its leaves is utilized as antihelminthic in the Amazonia region (Stasi & Hiruma-Lima, 2002). Despite its popular use as antihelminthic, the anti-parasite effects of its essential oil have not yet been

investigated. To the best of our knowledge, only its antioxidant, larvicidal, and fungicidal potentials have been reported (Lopes & Ritter, 2009; Martinez et al., 2009; Gutierrez et al., 2006). Thus, as a part of our ongoing project on the prospection of biologically active natural products (Keles et al., 2011; Melo et al., 2011; Peixoto et al., 2011; Ferreira et al., 2010), this paper will report the extraction of the essential oil from *T. erecta* leaves as well as on the investigation of its in vitro schistosomicidal activity against *Schistosoma mansoni* worms.

Material and Methods

Tagetes erecta L., Asteraceae, was collected at "Sítio 13 de maio" (20°26'S 47°27'W 977 m) near the city of Franca, State of São Paulo, Brazil. The sample collection was held on May 8th, 2009 at 8 am. A voucher specimen (SPFR10014) was deposited at the Herbarium of Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, São Paulo, Brazil (Herbarium SPFR).

Fresh leaves of *T. erecta* were submitted to hydrodistillation in a Clevenger-type apparatus, for 3 h. After manual collection of the essential oil, remaining traces of water were removed by freezing the sample below 0 °C, followed by transfer of the unfrozen essential oil to a new vial. The yield was calculated from the weight of the fresh leaves.

The essential oil of *T. erecta* was analyzed by GC-MS on a Shimadzu QP2010 Plus (Shimadzu Corporation, Kyoto, Japan) system equipped with an AOC-20i autosampler under the following conditions: Restek Rtx-5MS fused silica capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) composed of 5%-phenyl-95%-methylpolysiloxane operating in the electron ionization mode at 70 eV; carrier gas Helium (99.999%) at a constant flow of 1.0 mL/min; sample injection volume 0.1 µL (split ratio of 1:10); injector temperature 240 °C; and ion-source temperature 280 °C. The oven temperature was programmed to increase from 60 to 240 °C at 3 °C/min. The mass spectra were recorded with a scan interval of 0.5 s within the mass range 40-600 Da. Quantification of each constituent was estimated by internal normalization (%). The identification of the TE-EO components was based on their retention indices, relative to a homologous series of *n*-alkanes (C_8-C_{20}) measured on an Rtx-5MS capillary column under the same operating conditions. Computer matching was accomplished with the aid of the Wiley 7, NIST 08, and FFNSC 1.2 spectra libraries. The mass spectra of the constituents were also compared to those reported in the literature (Adams, 1995).

The LE strain of *S. mansoni* was maintained in *Biomphalaria glabrata* snails and Balb/c mice. After eight weeks, *S. mansoni* adult worms (males and females) were recovered under aseptic conditions from mice previously infected with 200 cercariae by perfusion of the livers and mesenteric veins (Smithers & Terry, 1965). The worms were washed in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen), kept at pH 7.5 with HEPES 20 mM, and supplemented with penicillin (100 UI/mL), streptomycin (100 µg/mL), and 10% bovine fetal serum (Gibco). All experiments were authorized by the Ethical Committee for Animal Care of the University of São Paulo and University of Franca, and were in accordance with the nationally and internationally accepted principles for laboratory animal handling and care.

For the *in vitro* test with *S. mansoni*, the essential oil of *T. erecta* (TE-EO) was dissolved in 1% DMSO and used at concentrations of 10, 50, and 100 µg/mL. The essential oil in the desired concentration was added to the medium containing one adult worm pair after a period of 24 h of adaptation to the culture medium. The parasites were kept for 120 h and monitored every 24 h, for evaluation of their general condition, as judged from motor activity, alterations in the tegument, and mortality rate (Xiao et al., 2007). Also, changes in pairing, egg production, and egg development were examined by using an inverted microscope (Leitz) (Magalhães et al., 2009; Michaels & Prata, 1968). RPMI 1640 medium and RPMI 1640 with 1% DMSO were employed as negative control groups; praziquantel (PZQ) was used as positive control group at a concentration of 10 µg/mL. The experiments were carried out in quadruplicate and repeated at least 3 times.

Pairs of adult worms were incubated with TE-EO (10, 50, or 100 µg/mL) for 120 h, and the viability assay was performed by means of the MTT assay (Comley et al., 1989). After incubation, each pair of adult worms was placed individually into wells (96-well plates) containing 100 µL phosphate-buffered saline with 5 mg MTT per milliliter for 30 min, at 37 °C. The solution was carefully removed and replaced with 200 µL DMSO, and the worms were allowed to stand in DMSO at room temperature for 1 h. The absorbance was read at 550 nm using a microplate reader (Sunrise, TECAN). Parasites in RPMI 1640 medium and RPMI 1640 with 1% DMSO were used as negative control groups, and heat-killed worms at 56 °C and PZQ (10 µg/mL) were used as positive control groups. Four replicates of each experiment were accomplished. Results are expressed as mean±SE. Data were statistically analyzed by one-way analysis of variance, followed by Tukey's multiple comparison test.

Results and Discussion

Tagetes erecta L., Asteraceae, leaves furnished a greenish essential oil (TE-EO) in 0.36%

yield (w/w). The GC-MS analysis revealed that monoterpenes (97.3%) are the main constituents of TE-EO, being α-terpinolene (17.9%), (*E*)-ocimenone (12.9%), dihydrotagetone (11.8%), piperitone (8.75%), verbenone (9.67%), and limonene (10.4%) its major constituents (Table 1). These chemical constituents had also been identified in *T. erecta* essential oils obtained from specimens collected in other regions of the world (Krishna et al., 2004; Sefidkon et al., 2004; Singh et al., 2003; Baslas & Singh, 1981).

Table 1. Chemical constituents of the essential oil of *Tagetes erecta*.

Compound	Retention (min)	RI_{lit}	RI_{exp}	RA (%)	Identification
α-pinene	5.187	939	931	0.82	RL MS
camphene	5.620	953	947	0.21	RL MS
sabinene	6.204	976	971	0.85	RL MS
α-phellandrene	7.196	1005	1007	0.49	RL MS
limonene	7.937	1004	1027	10.4	RL MS
(*Z*)-β-ocimene	8.134	1040	1033	4.16	RL MS
(*E*)-β-ocimene	8.504	1050	1044	0.48	RL MS
dihydrotagetone	8.692	1054	1049	11.8	RL MS
α-terpinolene	9.930	1088	1084	17.9	RL MS
linalool	10.496	1098	1100	0.39	RL MS Co
1,3,8-*p*-mentatriene	10.998	1111	1112	0.78	RL MS
(*Z*)-ocymene oxide	11.653	1128[c]	1128	0.56	RL MS
(*E*)-tagetone	12.576	1146	1151	6.96	RL MS
linalool propyonate	14.376	1174	1194	0.56	RL MS
verbenone	15.895	1218[a]	1230	9.67	RL MS
(*E*)-ocimenone	16.224	1239	1238	12.9	RL MS
piperitone	16.838	1282	1252	8.75	RL MS
piperitenone	20.404	1342[b]	1335	9.65	RL MS
trans-caryophyllene	23.768	1418	1414	1.24	RL MS
precocene I	25.598	1467[a]	1458	1.43	RL MS
Monoterpene hydrocarbons: 45.8%; oxygenated monoterpenes: 51.5%, Sesquiterpene hydrocarbons: 1.24%; others: 1.43%					

RT: retention time in an Rtx-5MS column; RI: retention indices relative to *n*-alkanes C_8-C_{20} on Rtx-5MS capillary column; RA: relative area (peak area relative to the total peak area); RL: comparison of the retention index with literature (Adams, 1995).

The *in vitro* effects of TE-EO against adult *S. mansoni* worms are summarized in Table 2. The positive control (PZQ, 10 μg/mL) resulted in total decreased motor activity, death of parasites, and partial or extensive tegument alterations within 24 h, without separation of worms, whereas the negative controls (RPMI medium and RPMI medium plus 1% DMSO) did not exert any effects on mortality, motor activity, couple separation, or tegument. TE-EO at 50 μg/mL caused the death of 25% of *S. mansoni* male and female adult worms after 24 h of incubation. However, the incubation with TE-EO at 100 μg/mL culminated in death of all *S. mansoni* adult forms after 24 h. The 24 h and 120 h LC50 of TE-EO on adult worms in vitro was calculated to be 81.47 and 52.23 μg/mL, respectively. It has previously been reported that male and female *S. mansoni* worms can exhibit different susceptibilities to treatment with natural products, such as the *Zinger officinalis* extract (Sanderson et al., 2002) and the essential oil of *Ageratum conyzoides* (Melo et al., 2011). In this sense, these results are remarkable not only because they demonstrated the in vitro schistosomicidal activity of TE-EO, but also because they indicate that male and female *S. mansoni* worms have the same susceptibility to this oil.

The viability of the adult worms was also evaluated during their *in vitro* incubation with TE-EO at 10, 50, and 100 μg/mL (Figure 1). In the case of the groups treated with TE-EO at 10 μg/mL, the viability of the adult worms was similar to that obtained for the negative control groups at 120 h of incubation. However, the incubation of the group of adult worms with TE-EO at 50 and 100 μg/mL for 120 h led to significantly diminished viability when compared to the negative control group. Moreover, this viability was similar to that observed for the positive control (PZQ). These results revealed an interesting non-linear dose-response effect for TE-EO at the tested concentrations and corroborated the microscopic analysis results.

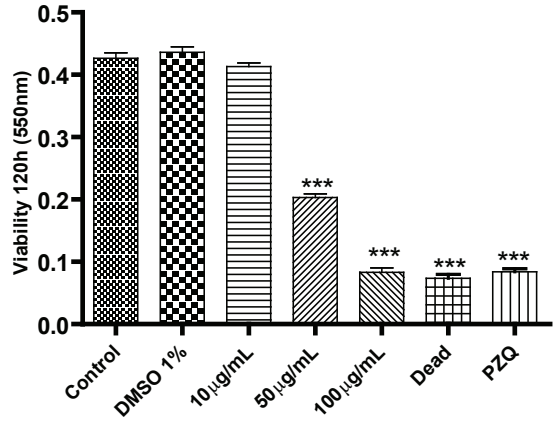

Figure 1. *In vitro* effect of Te-EO on the viability of the *S. mansoni* adult worms. Pairs of adult worms were treated with different Bs-EO concentrations for 120 h, and the viability was measured by MTT assay at 550 nm. RPMI 1640 medium and 1% DMSO in RPMI 1640 medium were used as negative control groups. Praziquantel (PQZ, 10g/mL) and heat-killed worms at 56 °C were used as positive control group. Data are presented as the mean from four experiments (***p<0.001).

The *S. mansoni* worms incubated with TE-EO at 50 and 100 μg/mL displayed significantly reduced motor activities after 24 h of incubation (Table 2), but

Table 2. *In vitro* effects of TE-EO against adult *S. mansoni* worms.

Group	Incubation period (h)	Number of separated worms (%)	Number of dead worms (%)		Decrease in motor activity				Number of worms with tegumental alteration			
					Slight (%)		Significant (%)		Partial (%)		Extensive (%)	
			M	F	M	F	M	F	M	F	M	F
Control[a]	24	0	0	0	0	0	0	0	0	0	0	0
	120	0	0	0	0	0	0	0	0	0	0	0
DMSO 1%[b]	24	0	0	0	0	0	0	0	0	0	0	0
	120	0	0	0	0	0	0	0	0	0	0	0
PZQ[c]	24	0	100	100	0	0	100	100	25	25	50	50
	120	0	100	100	0	0	100	100	25	25	50	50
TE-EO	24	0	0	0	75	75	25	25	0	0	0	0
(10 μg/mL)	120	0	0	0	75	75	25	25	0	0	0	0
TE-EO	24	75	0	0	0	0	100	100	0	0	0	0
(50 μg/mL)	120	75	25	25	0	0	100	100	0	0	0	0
TE-EO	24	100	100	100	0	0	100	100	0	0	0	0
(100 μg/mL)	120	100	100	100	0	0	100	100	0	0	0	0

[a]RPMI 1640; [b]DMSO+RPMI medium; [c]Tested at concentration of 10 μg/mL. M: males; F: females.

no tegument changes were detected, even at higher concentrations after 120 h. PZQ (10 μg/mL) caused significant decrease in the motor activity of all the parasites within 24 h of incubation, as well as tegument alterations in 75% of the worms treated during this same period, in accordance with previous studies on PZQ (Shushua et al., 2000)

It is known that couples of schistosomes are permanently paired throughout their lifespan in the blood system of their vertebrate host. This fact causes an intense oviposition rate, which is responsible for the resulting immuno-pathological lesions, characterized by inflammation and fibrosis in the target organs (Knobloch et al., 2006). Thus, in order to investigate the effects of TE-EO on these important features associated with the schistosomicidal activity, additional experiments to assess the impact of this oil on pairing (Table 2) and changes in egg production and egg development (Figure 2) were undertaken. As shown in Table 2, TE-EO promoted separation of 75% and 100% of the coupled pairs of worms after 24 h at concentrations of 50 and 100 μg/mL, respectively. On the other hand, parasites incubated with TE-EO at 10 μg/mL and those belonging to the negative control groups (RPMI 1640 medium and DMSO 1% plus RPMI 1640 medium) remained coupled, even after 120 h. TE-EO at 50 μg/mL was observed to cause a slight decrease in the number of eggs compared to the negative control (data not reported here).

In spite of the mild effect of TE-EO on egg production, this oil significantly reduced the percentage of developed eggs in a dose-dependent manner after 120 h of incubation (Figure 2). This is a noteworthy result, once ceasing embryogenesis and blocking propagation and development of this disease is also a fundamental step in the control of schistosomiasis (Freitas et al., 2007). PZQ, the most widely used in the treatment of schistosomiasis, was not tested, because it has been reported to be inactive against developing schistosomes (Doenhoff et al., 2008).

In summary, herein we have reported an investigation of the *in vitro* schistosomicidal potential of the essential oil of *Tagetes erecta* (TE-EO). We have concluded that such oil exhibits *in vitro* schistosomicidal activity against adult *S. mansoni* worms and demonstrated that TE-EO prompted an interesting reduction in the number of developed eggs in a dose-dependent manner. This significant activity offers the way for a new schistosomicidal drug, since PZQ, the most widely employed drug for the treatment of this disease, is known to be only active against adult forms of the parasite. In this context, the essential oil of *T. erecta* could be considered a promising source for the development of new schistosomicidal agents.

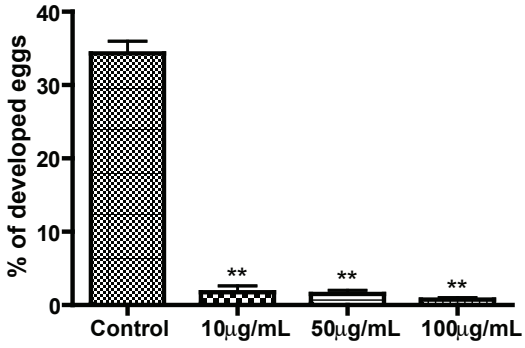

Figure 2. *In vitro* effects of the TE-EO on eggs development. Quantitative analysis of phenotype development. After treatment, the eggs were microscopically examined and scored as developed or undeveloped on the basis of the presence or absence of the miracidium. Data are presented as mean of developed eggs from three separate experiments (**p<0.01).

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