In vivo effect of essential oil of *Mentha x villosa* and its active compound against *Schistosoma mansoni* (Sambon, 1907)

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Abstract

Schistosomiasis treatment is dependent on a single drug, praziquantel (PZQ). The development of resistance of PZQ has drawn the attention of many researchers to alternative drugs. One viable and promising treatment is the study of medicinal plants as a new approach to the experimental treatment for Schistosomiasis. The present work aimed to evaluate *in vivo* antischistosomal activity of effect of *Mentha x villosa* Oil Essential (Mv-EO) and rotundifolone (ROT) against *Schistosoma mansoni*. Thirty-day-old female *Swiss webster* mice (*Mus musculus*) weighing 50 grams were used. Mice were infected with 80 cercariae of *S. mansoni* (BH strain) and orally administered Mv-EO (50, 100 and 200 mg/Kg) and ROT (35.9, 70.9 and 141.9 mg/Kg) at 45-days post infection for 5 consecutive days. All mice were euthanized 60 days after infection. Praziquantel was the positive control in the experiment. Doses of 200 mg/kg (Mv-EO) and ROT (141.9 mg/Kg) resulted in a significant reduction in fluke burden (72.44% and 74.48%, respectively). There was also marked reduction in liver, intestinal and faecal and changed oogram pattern, compared to infected untreated mice. Considering the results obtained, further biological studies are required in order to elucidate the mechanism of schistosomicidal action on against adult *S. mansoni*.

Keywords: Mentha x villosa, óleo essencial, rotundifolona.

Efeito *in vivo* do óleo essencial de *Mentha x villosa* e seu composto ativo sobre *Schistosoma mansoni* (Sambon, 1907)

Resumo

O tratamento da esquistossomose é dependente de uma única droga, praziquantel (PZQ). O desenvolvimento da resistência de PZQ tem atraído atenção de muitos pesquisadores por medicamentos alternativos. Um tratamento viável e promissor é o estudo das plantas medicinais como uma nova abordagem para o tratamento experimental para esquistossomose. O presente trabalho objetivou avaliar a atividade esquistossomicida *in vivo* óleo essencial de *Mentha x villosa* (OE-Mv) e rotundifolona (ROT) contra *Schistosoma mansoni*. Foram utilizados camundongos *Swiss webster* (*Mus musculus*) fêmea de trinta dias de idade pesando 50 gramas. Os camundongos foram infectados com 80 cercárias de *S. mansoni* (cepa BH) e administrado por via oral OE-Mv (50, 100 e 200 mg/Kg) e ROT (35,9, 70,9 e 141,9 mg/Kg) após 45 dias de infecção durante 5 dias consecutivos. Todos os animais foram eutanasiados 60 dias após a infecção. Praziquantel foi o controle positivo no experimento. O tratamento dos camundongos infectados com doses de 200 mg/kg (OE-Mv) e rotundifolona (141,9 mg/Kg) resultaram em redução significativa dos vermes (72.44% e 74.48%, respectivamente). Foi observado também redução no fígado, intestino e fecal e alteração no padrão do oograma, em comparação aos camundongos infectados e não tratados. Considerando os resultados obtidos, mais estudos biológicos são necessários a fim de elucidar o mecanismo de ação esquistossomicida contra adultos de *S. mansoni*.

Palavras-chave: Mentha x villosa, essencial oil, rotundifolone.

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1. Introduction

Schistosomiasis is already a serious public health problem, caused by trematode flatworms of the genus Schistosoma, is one of the most significant, neglected tropical diseases in the world (Gryseels et al., 2006).

Some published studies have reported that widespread use of praziquantel (PZQ) has favored the emergence of isolates of *S. mansoni* refractory to treatment with this medicine drug (Jiwajinda et al., 2002). The considerable concern about the development of PZQ resistance has motivated the scientific community to develop novel and inexpensive drugs against schistosomiasis (Seif el-Din et al., 2014).

The trend of using natural plant extracts as new and safe is promising and constitutes the basis for the development of lead chemicals for therapeutics (Manneck et al., 2009).

The literature showing that artemisinin and its derivatives presented anti-schistosomal potential and were approved as schistosomiasis prevention drugs by the Chinese Ministry of Health. They are active against *S. japonicum*, *S. mansoni* and *S. haematobium*, mainly targeting the immature, pre-adult stage, the schistosomulum (Abdel-Hameed et al., 2008; Botros et al., 2004).

Mentha x villosa (Hudson) such as *M. crispa* usually reported in the literature belongs to the family Lamiaceae and is known popularly as hortelã-da-folha-miúda, hortelã-rasteira e hortelã-de-panela (Lorenzi and Matos, 2002).

Some studies have been conducted to evaluate therapeutic activities of to the Essential Oil of *M. x villosa* (Mv-EO) thus adding information about this plant and its major compounds. In the present study, both Mv-EO as and rotundifolone (ROT), its major constituents, is being evaluated for different biological activities such as cardiovascular (Lahlou et al., 2002), hypotensive and bradycardic (Guedes et al., 2004a, b), antimicrobial (Arruda et al., 2006) and antinociceptive (Sousa et al., 2009) for Mv-EO.

Regarding ROT some effects reported in the literature, analgesic (Almeida et al., 1996), relaxant (Sousa et al., 2007), hypotensive and bradycardic (Guedes et al., 2002), antinociceptive (Sousa et al., 2007, 2009), antimicrobial (Arruda et al., 2006) and spasmolytic (Sousa et al., 2008). Studies have also demonstrated a possible mechanism of action involved in the relaxing effect exhibited by ROT (Silva et al., 2011).

Considerable efforts are ongoing in the development of novel drugs for the prevention and treatment of schistosomiasis. Recent studies by our research group have demonstrated *in vitro* schistosomicidal activity of Mv-EO and ROT, its major constituent (Matos-Rocha et al., 2013). These results led us to continue the study of the in vivo activity of Mv-EO and ROT on schistosomes. The present study aimed to evaluate *in vivo* effect of Mv-EO and its active compound, ROT, against *S. mansoni* infection in mice.

2. Material and Methods

2.1. Ethical standards

All experiments involving the use of experimental animals were performed in accordance to the ethical standards of Fundação Oswaldo Cruz (FIOCRUZ) and were approved by the in Animal ethics committee (CEUA-FIOCRUZ/PE, No. 06/2010).

2.2. Medicinal plant

Fresh leaves of the species *M. x villosa* were used. They were gathered from the Medicinal Plants Garden of the Research Institute of Drugs and Medicines (IPeFarM), Federal University of Paraiba (UFPB) between April and June 2011, where they were identified and authenticated *in locum* by Dr. F. J. Abreu Matos (Laboratory of Natural Products, Federal University of Ceará) and by Dr. Raymond Harley of the Royal Botanic Gardens, Kew, England. A voucher specimen was deposited in the Prisco Bezerra Herbarium of the Federal University of Ceará (No. 14996).

2.3. Essential oil of M. x villosa

To extract Mv-EO, 10 kg of the leaves were steam-distilled for 8h. The oil obtained (0.1%) was dried over anhydrous sodium sulfate in the usual manner and stored at 4ºC. We used a gas chromatograph coupled to a mass spectrometer (GC-MS) (Shimadzu QP-500) under the following analytical conditions: capillary colunn, OV-5 (30 m x 0.25 mm x 0.25m); injector (Ohio Valley Specialty Chemical, Inc.) 240º; detector, 230º; electron impact, 70 eV; gas drag, He; flow, 1.0 mL/min; split, 1/20; program temperature, 60º C-240º C at 3º C/min; and solution injection volume, 1 μL (1 μL of Mv-EO per 1mL of ethyl acetate). The compounds were identified by comparing their mass spectra using the GC-MS database system (Nist 62 lib.) and the Kovats retention index (Matos-Rocha et al., 2013).

2.4. Obtaining of rotundifolone

The essential oil of *Mentha x villosa* was subjected to thin-layer chromatography (Si-gel PF254, 40 x 20 cm plates; Merck, Darmstadt, Germany). The plates were developed three times with n-hexane as solvent. Two well-separated bands were visible under a UV lamp. The bands were cut and extracted in the usual way using CH_2Cl_2 and ROT was obtained from the slower moving band with 99.9% of purity determined by high-performance liquid chromatograph.

2.5. Obtaining of praziquantel

Praziquantel tablets were commercially available through Sigma-Aldrich (Sigma chemical, St Louis, MO, USA) with purity of 99.9%. The batch of Mv-EO, rot and PZQ that was been tested and used previously in our laboratory, was used in the present study (Matos-Rocha et al., 2013).

2.6. Experimental animals

Female *Swiss webster* mice (30-day old, weight ~50 grams) were obtained from CPqAM-FIOCRUZ/PE. Animals were maintained under environmentally controlled conditions at 23±2 ºC with 12/12h light/dark cycle, and fed on standard diet and normal drinking water *ad libitum* during the duration of the experiment. Infection of 72 mice with cercarie of *S. mansoni* was carried out using a tail

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immersion technique (80±cercarie/mice). After 45 days post exposure to cercarie, the faecal samples of mice were examined for the presence of *S. mansoni* eggs. The infected mice were then separated and used in the experiments.

2.7. Maintenance of parasite life-cycle

Eggs of *S. mansoni* were collected from excrements originating from individuals native of the city of Belo Horizonte, Minas Gerais, Brazil, after reading and signing the terms of agreement. Parasitological analyses were done through the method of Kato-Katz. The *Biomphalaria glabrata* snails were placed individually in wells of culture plates containing 3 mL of distilled water, where were added eight to ten miracidia of *S. mansoni* per well. A total of 68 snails were infected, for a period of 2 h, under heat and intense light.

After 30 days of infection, the snails were displayed to the heat and the intense light for 2 h, for elimination of the cercariae that were used for the female mice (*Mus musculus*) infection. The evolutive cycle of the parasite was kept in the schistosomiasis Laboratory of the Department of Parasitology, Oswaldo Cruz Institute, Pernambuco (FIOCRUZ-PE).

Mice with 30-day old, weighing ~50 g were housed in cages (30×20×13 cm) containing sterile wood shaving bed. Standard diet (Labina®, Ralston Purina Ltda, São Paulo, Brazil) and water were available *ad libitum*. Room temperature was kept at 22±2 °C and 12:12 h light/dark cycle (Pereira et al., 2013).

2.8. Protocol of treatment

Mice were randomized into eight groups with nine mice each: Group I, received 50 mg/Kg, Group II, received 100 mg/Kg and Group III, received 200 mg/Kg of Mv-EO; Group IV, received 35.9 mg/Kg, Group V, received 70.9 mg/Kg and Group VI, received 141.9 mg/Kg of ROT; Group VII, received 200 mg/Kg of PZQ and Group VIII, received suspension in 7% tween-80 and 3% ethanol. For all treatments, Mv-EO and ROT were administered daily for five consecutive days orally (gavage) using appropriate tube containing a volume of 400 µl per mice. Sixty days after treatment with the compounds, the animals were anesthetized with an intraperitoneal injection of ketamine hydrochloride (115 mg/Kg) associated with xylazine hydrochloride (10 mg/Kg) (Pereira et al., 2013). The recovery of *S. mansoni* worms from the hepatic portal system and mesenteric veins of sacrificed mice was done by the perfusion technique described by Smithers and Terry (Duvall and Dewitt, 1967).

2.9. Evaluation of the efficacy of treatment

The evaluation of the efficacy of the Mv-EO and ROT was determined by observing reduction in the percentage of parasitic load in each group treated using the following equation:

reduction of fluke $(\%)$ =nº of fluke in the control group - nº of fluke in the treatment group× 100 / nº of fluke in the control group.

2.10. Percentage egg developmental stages

Three fragments of the distal portion of the intestine were washed in normal saline solution and slightly dried on absorbent paper. Subsequently, each intestinal fragment

was squeezed between the slide and the cover slip and analyzed in a microscope to quantify the eggs. For each fragment 100 eggs were counted and classified according to their developmental stage. The eggs were classified as immature, viable eggs (from the 1st to the 4th stage); mature, viable eggs; and non-viable eggs (calcified, with retracted miracidium, semitransparent) (Fallon et al., 1995).

2.11. Counting the eggs in the liver and intestine

Three fragments of the liver and the intestine of each mouse subjected to euthanasia were taken after perfusion and digested in 4% potassium hydroxide (KOH) (Botros et al., 2004). The recovered eggs found were quantified with the aid of a cell-counting "Sedgewick Rafter" camera (Graticules Limited: model S50, Tonbridge-England) (Pellegrino and Faria, 1965).

2.12. Counting the eggs in the faecal

The mice were tested for the elimination of eggs in the faecal through HelmTest Kit which is based on an adaptation of the Kato-Katz (Fashuyi, 1981), available at Biomanguinhos Laboratory Fiocruz/PE. The faecal were subjected to sieving filter provided by the kit and rode up a blade, with certain amount of stool through the fill hole with known diameter (provided by the kit). The preparations were covered with cover slip impregnted with malachite green cellophane paper, aiming at the conservation of faecal and whitening *S. mansoni* eggs. We carried out the reading of the blades and counting of eggs and calculated the number of eggs per gram of faecal using the following formula: Sample number of eggs / gram of feces = number of eggs found on the blade 24 x factor (standardized by kit HelmTest - BioManguinhos).

2.13. Statistical analysis

Data were expressed as the mean±SD. Statistical analysis was performed using the Tukey test. Differences were considered to be significant when *p<0.001* for all cases. The software used was GraphPad Prism 5.0.

3. Results

3.1. Worm burden

The Table 1, the mean numbers of *S. mansoni* recovered after treatment with Mv-EO and ROT are presented in Table 1. Reduction rate was 74.48% and 72.44% in infected

Table 1. Fluke recovery of *S. webster* mice infected with *S. mansoni*, treated or nontreated with Mv-EO and ROT.

Group	Efficacy (%)
I	8.16
II	53.06
III	72.44
V	13.26
IV	59.18
VI	74.48
VII	95.91
VIII	-

Group I: Mv-EO/50 mg/Kg; group II: Mv-EO/100 mg/Kg; group III: Mv-EO/200 mg/Kg; group IV: ROT/35.9 mg/Kg; group V: ROT/70.9 mg/Kg; group VI: ROT/141.9 mg/Kg; group VII: PZQ/200 mg/Kg and group VIII: suspension in 7% tween-80 and 3% ethanol.

mice treated with 141.9mg/Kg and 200 mg/Kg of ROT and Mv-EO, respectively. PZQ treatment seemed to affect the fluke burden with a reduction of 95.91% as compared to the infected group.

3.2. Intestinal, hepatic and faecal eggs count

Table 2 presents the mean number of *S. mansoni* eggs in the intestinal, hepatic and faecal samples of infected mice treated with Mv-EO and ROT at a dose of 141.9 mg/Kg (Group III) and 200 mg/Kg (Group VI) (*p<0.001*). ROT affected the viability of both mature and immature eggs as indicated by the increase in the percentage of dead eggs and the decrease in the percentage of live ones.

This reduction of faecal egg count could be related to a possible lethal effect of the PZQ on eggs (*p<0.001*) (Fashuyi, 1981; El-Shenawy et al., 2000; Nahed et al., 2009). Treatment of infected mice with the drugs tested, not caused a significant reduction in the number of intestinal eggs, hepatic eggs, faecal eggs excreted per gram of faecal. In this study, three microscopic slides were analysed and all analyses were performed by double-blind observers.

Although the activity *in vivo* have demonstrated a significant change in the number of fluke in the treated groups, changes in oogram related to other stages of the eggs of *S. mansoni* was observed (Table 3).

4. Discussion

Plants of the genus *Mentha* contain substances that have been shown to exhibit therapeutic activity against several schistosomiasis (Matos-Rocha et al., 2013). The promising antischistosomal properties of Mv-EO and rot reported in this study could be added to its known potency in traditional folk medicine (Lima et al., 2011). Swiss albino mice were used to evaluate the *in vivo* action of Mv-EO and ROT on survival of *S. mansoni* fluke.

A study conducted by Oliveira et al. (2017) demonstrated in vivo schistosomicidal activity evaluation of crude hexanic (HE) and ethanolic (EE) extracts obtained from *Phyllanthus amarus* in mice infected with Schistosoma mansoni (BH strain). Mice were treated orally with a single dose of 100 or 250 mg/kg, on two different infection periods, 30 and 45 days post-infection (dpi). Parameters such as worm recovery, faecal egg count, intestinal tissue egg count and liver histopathology were evaluated. Treatment against young adult (30 dpi) and adult (45 dpi) worms were more effective compared to the control group treated with PZQ. At a concentration of 250 mg/kg (30 dpi) EE showed a 54.4% female reduction and a 61.2% total worm reduction whilst at a concentration of 100 mg/kg (45 dpi) HE showed a 40.6% female worm reduction and a 45.3% total worm reduction.

Table 2. Mean number of *S. mansoni* eggs in the intestinal, hepatic and faecal of mice treated with Mv-EO and ROT.

Mice	Intestinal egg count	Hepatic egg count	Faecal egg count
Group I	6023±135.5	6634±247.1	409.6±13.2
Group II	5415±159.6	5725±103.2	361±12.9
Group III	4846±125.7	5000±111.3	314.3±8.4
Group IV	5921±101.0	6300±207.2	405.4±17.2
Group V	5119±149.6	5714±110.2	366.4±10.6
Group VI	4931±133.1	5014±93.6	320±6.1
Group VII	1401±65.23*	1857±84.1*	48.29±8.4*
Group VIII	6357±105.1	6900±87.2	499.6±14.1

Values are expressed as means±SD; *Significant different at *p<0.001* indicate the percentage of reduction compared with infected control group (group VIII); Group I: Mv-EO/50 mg/Kg; group II: Mv-EO/100 mg/Kg; group III: Mv-EO/200 mg/Kg; group IV: ROT/35.9 mg/Kg; group V: ROT/70.9 mg/Kg; group VI: ROT/141.9 mg/Kg; group VII: PZQ/200 mg/Kg and group VIII: suspension in 7% tween-80 and 3% ethanol.

Table 3. Efficacy of Mv-EO and ROT on different stages of development of eggs of *S. mansoni*.

Mice	% egg developmental stages		
	Immature	Mature	Dead
Group I	64.54 ± 9.80	57.34 ± 8.83	0.52 ± 2.38
Group II	47.29 ± 19.76	48.74 ± 7.65	6.85 ± 2.86
Group III	37.83 ± 16.32	35.08 ± 8.65	14.52 ± 6.69
Group IV	63.32 ± 9.58	59.34 ± 8.89	0.57 ± 2.72
Group V	48.22 ± 19.33	45.24 ± 7.47	6.49 ± 2.54
Group VI	38.73 ± 11.56	37.62 ± 8.14	15.48 ± 6.16
Group VII	0.72 ± 2.33*	14.8 ± 1.86*	88.21 ± 11.09*
Group VIII	66.11 ± 9.23	41.24 ± 8.47	0.49 ± 2.27

Values are expressed as means±SD; *Significant different at *p<0.001* indicate the percentage of reduction compared with infected control group (group VIII); Group I: Mv-EO/50 mg/Kg; group II: Mv-EO/100 mg/Kg; group III: Mv-EO/200 mg/Kg; group IV: ROT/35.9 mg/Kg; group V: ROT/70.9 mg/Kg; group VI: ROT/141.9 mg/Kg; group VII: PZQ/200 mg/Kg and group VIII: suspension in 7% tween-80 and 3% ethanol.

The effectiveness on total load of the fluke of *S. mansoni* is considered a modest result, since according to literature date, a compound has a low efficiency when a reduction in the number of fluke is less than 30% (Botros et al., 2004). All these findings may suggest a possible anti-*S. mansoni* activity of the present compounds.

A study conducted by Guimarães et al. (2018) demonstrated the efficacy of epiisopilosine (EPIIS) in a murine model of schistosomiasis. A single dose of EPIIS (100 or 400 mg/kg) administered orally to mice infected with adult *S. mansoni* resulted in reduced worm burden and egg production. The treatment with the lower dose of EPIIS (100 mg/kg) significantly reduced total worm burden by 60.61% (P <0.001), as well as decreasing hepatosplenomegaly and egg excretion. Scanning electron microscopy revealed morphological changes in the worm tegument after treatment. Despite good activity of EPIIS in adult S. mansoni, oral treatment with single dose of EPIIS 100 mg/kg had only moderate effects in mice infected with juvenile *S. mansoni*.

Jatsa et al. (2009), El-Ansary et al. (2007), Melo et al. (2011) and Seif el-Din et al. (2014) also obtained considerable reductions of egg in the faecal treating *S. mansoni*-infected mice with *Clerodendrum umbellatum* extract, *Curcuma longa* oil extract, cramoll 1.4 lectin see extract and *Zingiber offinale* extract. The reduction of eggs count is probably the consequence of the noticeable decrease of fluke burden. This is possibly due to a positive linear relationship between the egg output and the fluke burden, where the reduction of the number of worms is correlated with the reduction in the eggs count (Nahed et al., 2009).

Unlike the results obtained in our study, El-Shenawy et al. (2000) observed in their study that the extract of *Cleome droserifolia* affected oogram with a high percentage of dead eggs pattern. In treated mice, high percentage of dead eggs immature and mature on live compared with infected mice may be related to a possible lethal action of *C. droserifolia* extract eggs. In the study, the authors did not evaluate whether the eggs were viable or not.

A study conducted by Oliveira et al. (2014) demonstrated the in vivo efficacy of *Baccharis trimera* against schistosomula, juvenile and adult worms of Schistosoma mansoni BH strain. In the experiment, mice were treated with DE, AF and PZQ (40 and 200mg/kg) over the period of larval development (3 and 30 post-infection; pi), and adult worms (60days post-infection; pi). The in vitro results show that the DE and AF effects are dose-dependents, being the 130μg/mL the most effective one in a shorter period of incubation. The exposure of the in vitro samples over adult parasites were able to inhibit 100% of the oviposition infemales. Likewise caused the mortality of the parasites with morphological alterations on the tegument, on the suckers, oral and acetabulum, in both males and females after 6-72h of exposure. Additionally, the in vivo treatments against juvenile and adult infection were more effective compared to the control group untreated. Administrations of AF and DE in day 30pi (juvenile worms) show female worm total burden reductions of 75% and 68% respectively.

At the same period of infection reductions of respectively 98% and 97% egg/g in the faeces were seen.

In this case, mice may be treated orally using single or multiple oral doses at different life-cycle stages (eggs, schistosomula and juvenile worms). In addition, toxicological studies (e.g., acute oral LD_{50}) should be examined.

5. Conclusion

Considering the results obtained, further biological studies are required in order to elucidate the mechanism of schistosomicidal action on against adult *S. mansoni*.

Acknowledgements

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