In Vitro Evaluation of Schistosomicidal Activity of Essential Oil of *Mentha x villosa* and Some of Its Chemical Constituents in Adult Worms of *Schistosoma mansoni*

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- Lamiaceae
- *Mentha x villosa*
- *Schistosoma mansoni*
- schistosomicidal activity
- natural products

Abstract

This study aimed to determine the composition of the essential oil of *Mentha x villosa* and to evaluate its biological effects *in vitro* on adult worms of *S. mansoni*. Rotundifolone (70.96%), limonene (8.75%), trans-caryophyllene (1.46%), and *β*-pinene (0.81%) were shown to be the major constituents of this oil. Adult worms of *S. mansoni* were incubated with different concentrations of the essential oil (1, 10, 100, 250, 500, and 1000 µg/mL) and of its constituents rotundifolone (0.7, 3.54, 7.09, 70.96, 177.4, 354.8, and 700.96 µg/mL), limonene (43.75 µg/mL), trans-caryophyllene (7.3 µg/mL), and *β*-pinene (4.03 µg/mL). No schistosomicidal activity was identified at the trans-caryophyllene and *β*-pinene concentrations studied. However, use of the essential oil (10 µg/mL), rotundifolone (7.09 µg/mL), and limonene (43.75 µg/mL) resulted in decreased worm motility continuing until 96 hours of observation. At higher concentrations (100 and 70.96 µg/mL, respectively), both the essential oil and rotundifolone caused mortality among adult worms of *S. mansoni*. The positive control praziquantel caused the death of all parasites after 24 h of evaluation. The results from this study suggest that the essential oil of *Mentha x villosa* presents schistosomicidal efficacy.

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Introduction

For treating schistosomiasis, WHO recommends praziquantel (PZQ) because it has low toxicity and can be administered in a single dose orally. However, indiscriminate use has resulted in appearance of strains of *Schistosoma mansoni* (Sambon, 1907) refractory to this drug [1]. Besides praziquantel, oxamniquine is also recommended by WHO as an alternative available for treating schistosomiasis, but because of adverse effects, its use has been restricted in Brazil [2].

The search for new effective drugs for treating schistosomiasis has directed studies towards extracts and compounds isolated from plants that can provide schistosomicidal activity [3–6].

M. x villosa Hudson, which is usually reported in the literature as *M. crispa*, belongs to the family Lamiaceae and is known popularly as the mint-leaf-girl, creeping mint, and mint-in-pot. After identification by Dr. R. Harley, of the Royal Botanic Gardens, Kew (UK), it was shown that *M. x villosa* is a hybrid originating from spontaneous crossing of *M. spicata* and *M. suaveolens* [7]. As for bioavailability, there are no studies in the literature that show bioavailability in humans or animals.

Some biological studies have been conducted to evaluate the therapeutic activity of the essential oil of *M. x villosa* (EOMv), and these have also added information about this plant and its main compounds.

Rotundifolone ($C_{10}H_{14}O_2$; molecular weight 166) is a naturally occurring monoterpenic ketone of plant origin and an important chemical constituent of the essential oils of many *Mentha* species (*M. rotundifolia, M. suaveolens, M. spicata, M. longifolia*, and *M. x villosa*) [8]. This most abundant constituent has been evaluated in relation to the following different biological activities: cardiovascular [8], hypotensive, bradycardic [9,10], antimicrobial, antifungal [11], and antinociceptive [12] properties.

This study is given greater impact to rotundifolone because it represents more than 50% (can reach 65%) of the total oil of *M. x villosa*. It is the substance (monoterpene) present in greater amounts in the leaves of *M. x villosa* [13–17].

The following forms of activity have been reported in the literature with regard to rotundifo-

Substance	(%)	*KI from experiment	KI in literature*
Sabinene	0.23	972	976
β-Pinene	0.81	976	980
Myrcene	3.10	990	991
Limonene	8.75	1029	1031
Cis-β-ocimene	0.85	1035	1040
Rotundifolone	70.96	1363	1363
β-Bourbonene	0.09	1386	1384
β-Elemene	0.24	1393	1391
Trans-caryophyllene	1.46	1420	1418
α-Humulene	0.21	1454	1454
Trans-β-farnesene	0.39	1457	1458
Germacrene D	3.81	1481	1480
Cis-calamenene	0.22	1521	1521
ç-Elemene	0.31	1523	1524
α-Muurolol	0.49	1653	1645

Table 1 Chemical constituents of EOMv, as shown using GC-MS.

* Retention Index = Kovats Index (KI). Components are identified based on GC-MS and listed according to elution order in the column

lone: as analgesic [13], relaxative [14], hypotensive, bradycardic [15], antinociceptive [16], antimicrobial, antifungal [11], and spasmolytic [17]. Studies demonstrated a possible mechanism of action involved in the relaxative effect exhibited by rotundifolone, which they correlated with the influx of calcium channels [18].

M. x villosa has been used in traditional medicine due to its antiparasitic property. It is known popularly as hortelã-rasteira (creeping mentha), hortelã comum (regular mentha), or hortelã-da-folha-miúda (small-leaved mentha) [19]. Giamebil® is a commercial formulation that has as its active compound the dry extract from the leaves and stem of *M. x villosa*, with amebicidal (*Entamoeba histolytica*) and giardicidal (*Giardia lamblia*) activities [20].

Recent studies have also demonstrated efficacy of *M. x villosa* in *Trichomonas vaginalis* [21]. However, there are no published studies that show the activity of the essential oil from the leaves of *M. x villosa* against adult worms of *S. mansoni*.

New molecules have been harvested from natural sources to fulfill a need for new chemical designs with distinctive pharmacological activities. The present study aimed to evaluate the *in vitro* effect of the essential oil of schistosomicidal *M. x villosa* and each of its main compounds (rotundifolone, limonene, trans-caryophyllene, and β-pinene) in adult worms of *S. mansoni*.

Results and Discussion

Several studies have shown that some natural products or compounds isolated from them present schistosomicidal activity [22–28]. Studies have also reported that some medicinal plants have been used clinically against schistosomiasis, such as myrrh (Mirazid®), a resinous oil obtained from *Commiphora molmol* (*Commiphora myrrha*), which is used in Egypt [29].

However, few studies *in vitro* have demonstrated any schistosomicidal activity of essential oils of medicinal plants against *S. mansoni* [30–33]. No work has been reported demonstrating the antiparasitic activity of EOMv and compounds isolated from it until now.

The EOMv yield was 0.1%, based on the dry weight of the plant, and its composition, analyzed using GC-MS, showed the presence of nineteen substances (**Table 1**). These were identified through comparison of their spectral masses, using the GC-MS database system (Nist 62 lib.) and Kovats retention index [34]. The main compounds were: rotundifolone (70.96%), limonene (8.75%), trans-caryophyllene (1.46%), and β-pinene (0.81 µg/mL).

Antiparasitic activity of EOMv and its separate compounds against the adult worms of *S. mansoni* was observed during the biological assays *in vitro*. At the concentration of 100 µg/mL, worm mortality was observed from 72 h onwards; at the concentration of 250 µg/mL from 48 h; at the concentration of 500 µg/mL from 24 h, and at the concentration of 1000 µg/mL within 24 h of exposure (**Table 2**).

Using EOMv at concentrations of 1, 5, and 10 µg/mL, worm mortality was not observed at any of the times examined. However, in terms of relative motility at a concentration of 10 µg/mL, minor loss of the movements of the tail, suckers, and gynaecophoric canal membrane was observed after 96 h of exposure.

Biological assays evaluating the essential oil of *Ageratum conyzoides L.* (EOAc) demonstrated that it had schistosomicidal activity on adult worms of *S. mansoni* at concentrations of 10 to 100 µg/mL. At the concentration of 100 µg/mL, worm mortality was observed when evaluated for up to 120 h [31]. Similarly to the results from the present study, the concentration of 10 µg/mL of EOMv caused a reduction in the motor activity of adult worms and at the concentration of 100 µg/mL, worm mortality was observed at times up to 72 h. These results were also reported from a study on the schistosomicidal activity of the essential oil of *Plectranthus neochilus* (EOPn) [32].

Differently from the findings of another study in which the *in vitro* schistosomicidal activity of the essential oil of *Piper cubeba* (EOPc) was evaluated at concentrations of 12.5 and 25 µg/mL at 120 h of exposure with regard to motility, a viability similar to that of the negative control group of adult worms of *S. mansoni* was observed [33]. At a concentration of 100 µg/mL, reduction in the movements of adult worms of *S. mansoni* was observed up to 72 h. These results were superior to those observed in other studies evaluating EOAc, in which 75% mortality of the worms after this same time was reported [31].

Differently from the above results, biological assays testing the essential oil of *Baccharis dracunculifolia* found 100% mortality of the worms after 24 h at the concentration of 10 µg/mL [30]. Also in this same study, 100% mortality of adult worms was demonstrated for concentrations of 250 µg/mL at 48 h, and of 100 µg/

Groups	Incubation period (h)	Number of dead worms (%)	Changes in motor activity
Control	120	–	+++
DMSO 1%	120	–	+++
PZQ	24	100	–
1	120	–	+++
5	120	–	+++
10	96	–	++
100	72	100	–
250	48	100	–
500	24	100	–
1000	24	100	–

Negative control (RPMI 1640; RPMI 1640 + DMSO 0.1%); positive control with 0.5 µg/mL. EOMv: +++ normal activity; ++ slight loss of movement, with activity of the tail, suckers, and gynaecophoric canal membrane; + movement of the tail and suckers alone; – no movement

Table 2 *In vitro* effect of different concentrations of EOMv on mortality of adult worms of *S. mansoni*.

Groups	Incubation period (h)	Number of dead worms (%)	Changes in motor activity
Control	120	–	+++
DMSO 1%	120	–	+++
PZQ	24	100	–
0.7	120	–	+++
3.54	120	–	+++
7.09	96	–	++
70.96	72	100	–
177.4	48	100	–
354.8	24	100	–
700.96	24	100	–

Negative control (RPMI 1640; DMSO + RPMI 1640); positive control with 0.5 µg/mL. Rotundifolone: +++ normal activity; ++ slight loss of movement, with activity of the tail, suckers, and gynaecophoric canal membrane; + movement of the tail and suckers alone; – no movement

Table 3 *In vitro* effect of different concentrations of rotundifolone on mortality of adult worms of *S. mansoni*.

Groups	Incubation period (h)	Number of dead worms (%)	Changes in motor activity
Control	120	–	+++
DMSO 1%	120	–	+++
PZQ	24	100	–
(1) 43.75 µg/mL	120	–	++
(2) 7.3 µg/mL	120	–	+++
(3) 4.03 µg/mL	120	–	+++

Negative control (RPMI 1640; RPMI 1640 + DMSO 0.1%); positive control with 0.5 µg/mL. +++ Normal activity; ++ slight loss of movement, with activity of the tail, suckers, and gynaecophoric canal membrane; + movement of the tail and suckers alone; – no movement

Table 4 *In vitro* effect of different concentrations of limonene (1), trans-caryophyllene (2), and *β*-pinene (3) on mortality of adult worms of *S. mansoni*.

mL at 72 h of observation, and similarly to our results, a dose-dependent effect was observed at the concentrations evaluated.

At the concentration of 70.96 µg/mL, mortality and absence of movement of the worms were observed from 72 h onwards, at the concentration of 177.4 µg/mL from 48 h, at the concentration of 354.8 µg/mL from 24 h, and at the concentration of 700.96 µg/mL within 24 h of exposure (**Table 3**).

Using rotundifolone at concentrations of 0.7, 3.54, and 7.09 µg/mL, no worm mortality was observed at any of the times examined. Regarding motility at a concentration of 7.09 µg/mL, minor loss of movement of the tail, suckers, and gynaecophoric canal membrane was observed after 96 h of exposure.

Using the limonene concentration of 43.75 µg/mL, no worm mortality was observed at any of the times examined. In relation to motility at this concentration, decreased motor activity was observed after 96 h of exposure. The compounds *β*-pinene at 4.03 µg/mL and trans-caryophyllene at 7.3 µg/mL did not cause mortality of adult worms of *S. mansoni* at any time analyzed (**Table 4**). Furthermore, the worms in the negative control groups (RPMI 1640 and RPMI 1640 + DMSO 1.6%) remained viable throughout the analysis period. The positive control PZQ caused the death of all parasites after 24 h of evaluation, as seen in **Tables 2**, **3**, and **4**.

After studying the schistosomicidal effect of EOAc in that same study, the authors also evaluated the two major compounds of the essential oils precocene (I) and (E)-caryophyllene, in which schistosomicidal effects similar to that of EOAc were observed [31].

In that study, the major compound of EOMv (i.e., rotundifolone) demonstrated a schistosomicidal effect similar to EOMv [31].

Evaluation of the effect of other compounds (limonene, trans-caryophyllene, and β-pinene) that form part of EOMv showed that only limonene at the concentration of 43.75 µg/mL produced decreased motility of adult worms of *S. mansoni*. This suggests that rotundifolone is responsible for the schistosomicidal effect. On the other hand, another study showed results differing from the above in an evaluation of the biological effect of essential oil of *Baccharis dracuncufolia* (EOBd) on adult worms of *S. mansoni*. It was observed that nerolidol, the major constituent of EOBd, did not show any schistosomicidal effect on the adult worms at any of the concentrations tested [30].

The results suggest that EOMv and rotundifolone caused mortality of adult worms of S. mansoni when evaluated *in vitro* at concentrations greater than 10 µg/mL for EOMv and 7.09 µg/mL for rotundifolone.

In this context, the schistosomicidal activity is probably related to rotundifolone because when analyzed alone, it showed activity similar to EOMv at the respective concentration in the composition of total EOMv. The results indicate that rotundifolone can be considered to be a promising source for development of novel antischistosomal agents.

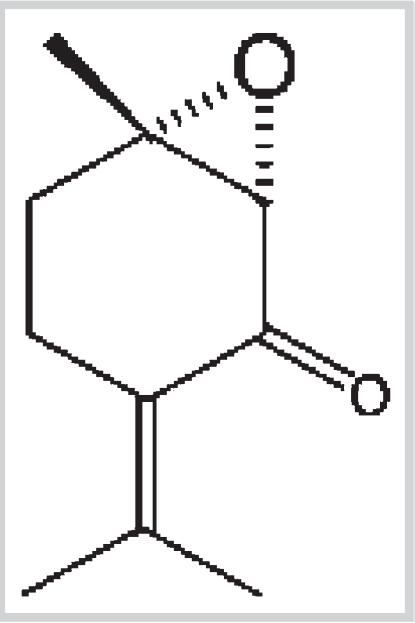

Fig. 1 Chemical structure of rotundifolone.

Materials and Methods

Medicinal plant

Fresh leaves of the species *M. x villosa* were used. They were gathered from the Medicinal Plants Garden of the Pharmaceutical Technology Laboratory, Federal University of Paraíba (LTF-UFPB) between April and June 2011, where they were identified and authenticated 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).

Extraction of the essential oil of *M. x villosa* and analysis using GC-MS

To extract EOMv, 10 kg of the leaves were steam-distilled for 8 h. The oil obtained (0.1%) was dried over anhydrous sodium sulfate in the usual manner and stored at 4 °C [13]. We used a gas chromatograph coupled to a mass spectrometer (Shimadzu QP-5000) under the following analytical conditions: capillary column, OV-5 (30 m × 0.25 mm×0.25 µm); injector (Ohio Valley Specialty Chemical, Inc.), 240 °C; detector, 230 °C; electron impact, 70 eV; gas drag, He; flow, 1.0 mL/min; split, 1/20; program temperature, 60 °C – 240 °C at 3 °C/min; and solution injection volume, 1 µL (1 µL of essential oil per 1 mL 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 [34].

Isolation and identification of rotundifolone

The oil was subjected to preparative layer chromatography (Merck silica gel PF254 plates, 40 × 20 cm). The plates were eluted three times using hexane as solvent. Pure rotundifolone (**Fig. 1**) was obtained from the green leaves of *M. x villosa* accordingly. The chemical purity of rotundifolone (more than 99.9%) was determined by high-performance liquid chromatography [34].

Limonene, β-pinene, trans-caryophyllene, and praziquantel

Limonene, β-pinene, trans-caryophyllene, and praziquantel, obtained from the lower band in 99.9% purity, were purchased commercially from the company Sigma-Aldrich.

Animals and parasite

S. mansoni strain BH (Belo Horizonte) was maintained in the Schistosomiasis Laboratory of the Department of Parasitology, Oswaldo Cruz Institute, Pernambuco (FIOCRUZ-PE), using mice of Swiss Webster lineage as definitive hosts and the snail species *Biomphalaria glabrata* as intermediate host. Each mouse was exposed to around 120 cercariae.

Eight weeks after infection of the mice, adult worms of *S. mansoni* were recovered from the hepatic portal system through infusion. They were washed in medium RPMI 1640, buffered with 20 µM of HEPES (pH 7.5), supplemented with penicillin (100 UI/mL), streptomycin (100 µg/mL), and 10% fetal bovine serum (Gibco) and placed into sterile Petri dishes containing 2 mL of culture medium [35].

In vitro antischistosomal assay

For *in vitro* assay with *S. mansoni,* the compounds were dissolved in 100% dimethyl sulfoxide (DMSO). Each well received five worms, which were then incubated at 37 °C in an atmosphere containing 5% CO_2. After two hours (the time allowed for the worms to adapt to the culture medium), EOMv isolates and compounds were added at a range of concentrations: a) EOMv (1.10, 100, 250, 500, and 1000 µg/mL); b) rotundifolone (0.7, 3.54, 09.07, 70.96, 177.4, 354.8, and 700.96 µg/mL); c) limonene (43.75 µg/mL), β-pinene (4.03 µg/mL), and trans-caryophyllene (7.3 µg/mL). As a negative control, adult worms were incubated in RPMI 1640 and RPMI 1640 + 1.6% DMSO. As a positive control, worms were incubated in 0.5 µg/mL of PZQ.

The parasites were incubated and monitored every 24 h until 120 h to evaluate changes in motor activity and consequently the mortality rate [36, 37]. The evaluation of the viability of adult worms was based on standard procedures for screening schistosomicidal compounds used within the WHO Special Program for

Research and Training in Tropical Diseases, in which: (+ + +) indicates normal activity; (+ +) slight loss of movement, with active tail, suckers, and gynaecophoric canal membrane; (+) movement of tails and suckers alone; and (−) no movement. The worms were considered to be dead when no movement was identified after 3 min of observation using an inverted microscope [38].

Ethical standards

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

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Conflict of Interest

The authors declare that there is no conflict of interest.

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