**Evaluation of antiparasitc activity of *Mentha* *crispa* essential oil, its major constituent rotundifolone and analogues against *Trypanosoma brucei***

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**Abstract**

Considering the pressing need for new drugs to treat sleeping sickness and nagana disease, *Mentha crispa* essential oil (MCOE), its principal constituent rotundifolone and four related p-menthane-type monoterpenes (two stereoisomers of limonene epoxide, perillyl alcohol and perillyl aldehyde) were investigated for their activity against bloodstream forms of *Trypanosoma brucei*. The general cytotoxicity of the compounds was determined with human myeloid HL-60 cells. The effect of the MCEO and the monoterpenes on the growth of parasite and human cells was evaluated in cell culture with the resazurin viability assay. Of all compounds tested, MCEO, rotundifolone and perillyl aldehyde showed the highest trypanocidal activities with 50% growth inhibition (GI50) and minimum inhibitory concentration (MIC) values of 0.3 μg/mL and 1 μg/mL, respectively. In contrast, HL-60 cells were considerably less sensitive to the compounds with MIC values of 100 μg/mL and GI50 values ranging between 3.4 to 13.8 μg/mL. As a consequence of this, GI50 and MIC ratios of cytotoxic to trypanocidal activity (selectivity index) of these three compounds were promising with values of 11-45 and 100, respectively. These results indicate that the p-menthane-type monoterpenes rotundifolone and perillyl aldehyde are interesting lead candidates for further rational anti-trypanosomal drug development.

**Key words** *Mentha crispa* essential oils; Monoterpenes; Rotundifolone; *Trypanosoma brucei*; HL-60 cells; Trypanocidal activity

**Introduction**

The genus *Mentha* (commonly known as mint) comprises approximately 19 species and 13 natural hybrids and is one of the most popular essential oil crops due to the remarkable chemical diversity and bioactivity of its secondary compounds (1-3). The plants are commonly known for their culinary use as spices and seasonings and as teas and infusions, while their essential oils and isolated compounds are used as flavourings in toothpaste, [antiseptic mouth rinses](https://en.wikipedia.org/wiki/Antiseptic_mouth_rinse), breath fresheners, [chewing gum](https://en.wikipedia.org/wiki/Chewing_gum), drinks, [desserts](https://en.wikipedia.org/wiki/Dessert), and [candies](https://en.wikipedia.org/wiki/Candy). However, mint is also used as medicinal herb in traditional medicine. In addition, extracts and essential oils of mint plants have been shown to display activity against parasites. For example, the essential oil of the hybrid species *Mentha crispa* (syn. *Mentha* x *villosa*) has been reported to exhibit larvidical activity against *Aedes aegypti* (4) and *Schistosoma mansoni* (5). A dry extract from leaves and stems of *M. crispa* is the active ingredient of the commercial formulation Giamebil® that displays amoebicidal and giardicidal activities (6). This remedy has also been shown to be effective in women with *Trichomonas vaginalis* infection (7).

One of the predominant compounds found in many *Mentha* species is the monoterpene rotundifolone (aka piperitenone oxide) (4,8-11). For instance, the essential oil of *M. crispa* contains generally around 70% of this secondary plant compound (4,10). Rotundifolone is a *p*-menthane-type epoxide and has been reported to exhibit strong trypanocidal activity against epimastigote and trypomastigote forms of *Trypanosoma cruzi*, the causative agent of Chagas disease in Latin America, with IC50 values of <10 μg/mL (12).

In search for new compounds with activity against African trypanosomes that cause sleeping sickness in humans and Nagana disease in cattle, we investigated the trypanocidal activity of *M. crispa* essential oil (MCEO) and its major constituent rotundifolone as well as some structurally related monoterpenes against bloodstream forms of *T. brucei*. In this respect it is noteworthy to mention that monoterpenes have previously been shown to display promising trypanocidal activities (13,14).

**Results and Discussion**

The present work investigated the *in vitro* anti-trypanosomal and cytotoxic activities of MCEO, its major constituent rotundifolone and four analogous monoterpenes ((+)-limonene epoxide, (-)-limonene epoxide, (-)-perillyl alcohol and (-)-perillyl aldehyde) (**Fig. 1**) using the resazurin (Alamar blue) assay described previously (15). The trypanocidal activity of the compounds was determined with *T. brucei* bloodstream forms 427-221a (16) while the general cytotoxicity was evaluated with human myeloid leukaemia HL-60 cells (17). MCEO and the other compounds all showed a dose-dependent effect on the growth of trypanosomes with MIC (minimum inhibitory concentration, i.e., that concentration of the compounds at which all cells were killed) values varying between 1 and 100 μg/mL and GI50 (50% growth inhibition, i.e., that concentration of a compound necessary to reduce the growth rate of cells by 50% to that of controls) values ranging from 0.3 to 13.3 μg/mL (**Table 1**). MCEO, rotundifolone and (-)-perillyl aldehyde were the most trypanocidal agents with identical MIC and GI50 values. The anti-trypanosomal activity of MCEO is most likely due to its major constituent, rotundifolone (the MCEO used in this study contained 58.11% rotundifolone, **Table 2**), as the essential oil and the monoterpene have similar trypanocidal activities. When compared with the MIC value and the GI50 value of suramin (reference control), one of the drugs used in the treatment of sleeping sickness, the three compounds were 10 and 6 times less trypanocidal, respectively (**Table 1**). The cytotoxicity of the compounds towards human HL-60 cells was generally lower with MIC values of 100 or >100 μg/mL and GI50 values ranging between 3.4 to >100 μg/mL (**Table 1**). As a result, the MIC and GI50 ratios of cytotoxic to trypanocidal activities (selectivity indices) were found to be in a modest range for most compounds (**Table 1**). Only MCOE, rotundifolone and (-)-perillyl aldehyde had a substantial selectivity index for the MIC ratio of 100. For comparison, the reference drug suramin had a MIC ratio and a GI50 ratio of >1000 and >2000, respectively (**Table 1**).

Structure-activity relationship analysis revealed some structural characteristics that can be correlated with the anti-trypanosomal effect of the compounds tested. It appears that the trypanocidal activity increases with the presence on an α,β-unsaturated carbonyl, since rotundifolone and perillyl aldehyde containing this functional group were the most potent compounds. This suggestion is supported by previous findings that the presence of a carbonyl group (aldehyde or ketone) conjugated to a C-C double bond seems to be important for the biological activity of many compounds (4,18-20). In line with this is the finding that perillyl alcohol, which contains a hydroxyl group, was 40 to 100 times less trypanocidal than its analogue perillyl aldehyde. In addition, the increased hydrophilicity of perillyl alcohol due to the presence of a hydroxyl group may also contribute to its lower anti-trypanosomal activity. It is well established that lipophilicity is a critical parameter for membrane and cell permeation of drugs (21). This is corroborated by the finding that the limonene epoxide stereoisomeres, which do not contain a carbonyl or a hydroxyl group, exhibited trypanocidal activities that ranged between those of perillyl alcohol and perillyl aldehyde. Another interesting observation is that both enantiomers of limonene epoxide display the same anti-trypanosomal activity. Likewise, no difference in larvicidal activity against *A. aegypti* has been recently reported for the enantiomers (+)-limonene epoxide and (-)-limonene epoxide (4). However, enantioselectivity was observed for the cytotoxic activity of (+)-limonene epoxide and (-)-limonene epoxide against HL-60 cells. This example shows that determination of the activity of stereoisomers can be worthwhile as one enantiomer may be active while the other one inactive. In this context it would have been interesting to see whether (+)-perillyl aldehyde would have a reduced cytotoxicity but the same trypanocidal activity as its stereoisomer (-)-perillyl aldehyde. Unfortunately, the required parent compound (+)-perillyl alcohol for synthesis of (+)-perillyl aldehyde is not commercially available at an affordable price.

Over the past years, essential oils and their constituents have been proven to be profitable sources for compounds with anti-parasitic activity. This study has shown that rotundifolone, the major constituent of the essential oil of *M. crispa*, and the related monoterpene perillyl aldehyde display promising trypanocidal activity towards bloodstream forms of *T. brucei*. Both compounds almost fulfil the activity criteria for drug candidates for African trypanosomiasis (GI50 <0.2 μg/mL; selectivity >100) (22). However, one should bear in mind that in the present study a cancer cell line was used for determining the selectivity. Therefore, compared with non-malignant cells, it is likely that the cytotoxicity of the compounds are overestimated. Further optimisation of the compounds through structural modification may lead to molecule with improved trypanocidal activity and reduced cytotoxicity.

**Materials and Methods**

Reagents

The essential oil of *M. crispa* (MCEO) was obtained from Hebron® today (Brazil). (-)-Perillyl alcohol (purity 96%, (GC)), (+)-limonene (purity 97%, (GC)), (-)-limonene (purity 96%, (GC)) and suramin sodium salt (purity ≥99%, (TLC)) were purchased from Sigma-Aldrich (USA or UK).

GC/MS analysis of MCEO

MCEO was analysed by gas chromatography-mass spectrometry (GC/MS) using a Hewlett Packard system (gas chromatograph model 5890 equipped with a mass spectrometer model 5988A) and an OV-5 capillary column (30 m × 0.25 mm, bonded 0.25 μm). The following analytical condition were used: electron impact, 70 eV; carrier gas, helium; flow rate, 1 mL/min; temperature, programmed from 60-240 °C at 3 °C/min; injection temperature, 240 °C; detection temperature, 230 °C; split ratio, 1/20. The injected volume was 1 μL of a solution containing approximately 0.1 μL of MCEO in 1 mL ethyl acetate. The identification of each component was determined by comparing their mass spectra with a GC/MS database (Nist 62 library) and Kovats retention indices (23). The results of the GC/MS analysis of MCEO are shown in **Table 2**.

Isolation of rotundifolone

The terpenic ketone rotundifolone was isolated from MCEO as previously described (24). MCEO was subjected to preparative silica gel thin layer chromatography using hexane as mobile phase. The plates were exposed to UV light (254 nm) and rotundifolone was identified as the major component of MCEO. Rotundifolone was removed from the plates and recovered by extraction with CH2Cl2 followed by filtration and evaporation under reduced pressure to obtain a yellowish oil. The oil was confirmed as rotundifolone by 1H and 13C Nuclear Magnetic Resonance (NMR) analysis, by infrared (IR) spectroscopy and by comparison with published data (25).

*Rotundifolone*: 1H NMR (CDCl3, 200 MHz): δH 1.41 (s, 3H), 1.74 (s, 3H), 1.92-1.78 (m, 2H), 2.04 (s, 3H), 2.40-2.24 (m, 2H), 3.17 (s, 1H); 13C NMR DEPT (CDCl3, 50 MHz): δC 21.7, 23.0, 23.0, 24.0, 27.7, 63.2, 63.4, 127.5, 149.2, 198.4; IR (cm-1): ν 3050, 2990, 1700, 1640 and 880; purity: >93% (1H NMR and TLC).

Synthesis of (+)-limonene epoxide, (-)-limonene epoxide and (-)-perillyl aldehyde

(+)-Limonene epoxide and (-)-limonene epoxide were synthesized from (+)-limonene and (-)-limonene, respectively, by endocyclic epoxidation of the double bond using *meta*-chloroperbenzoic acid (*m*-CPBA) as oxidising agent and CH2Cl2 as solvent system as previously described (26). In brief, to a solution of (-)-limonene or (+)-limonene (7.35 mmol) in 40 mL of dry CH2Cl2, a 70% solution of *m*-CPBA (7.35 mmol) in CH2CL2 was added dropwise. The mixture was stirred at 0°C (ice bath) for 4 h, followed by washing 4 times with 50 mL of an aqueous solution of 10% NaHSO3. The aqueous layer was extracted with CH2Cl2 (2 × 50 mL) and the organic layers washed with an aqueous solution of 5% NaHCO3 (2 × 50 mL). The combined organic solutions were dried over anhydrous Na2SO4. After evaporation of the solvent under reduced pressure, the product was purified by column chromatography on silica gel.

(-)-Perillyl aldehyde was prepared from (-)-perillyl alcohol by selective oxidation using pyridinium chlorochromate (PCC) as oxidising agent and dry CH2Cl2 as solvent according to the method described in (27). In brief, to a solution of (-)-perillyl alcohol (59.21 mmol) in 462 mL of dry CH2Cl2, a solution of PCC (177.63 mmol) in CH2Cl2 was slowly added. The mixture was stirred at room temperature for 4 h and the filtered through a Büchner funnel containing silica gel. The filtrate was concentrated on a rotavapor and the product purified by silica gel column chromatography.

The identity of all three monoterpenes were confirmed by 1H and 13C NMR analysis, by IR spectroscopy and by comparison with published data (27,28).

(*+*)*-Limonene epoxide*: 1H NMR (CDCl3, 200 MHz): δH 1.30-0.90 (m, 2H), 1.22 (s, 3H), 1.72-1.63 (m, 2H), 1.68 (s, 3H), 2.05-1.74 (m, 2H), 2.18-2.10 (m, 1H), 2.97 (t, *J*=4.2 Hz, 1H), 4.62 (d, *J*=0.8 Hz, 1H), 4.66 (d, *J*=0.8 Hz, 1H); 13C NMR DEPT (CDCl3, 50 MHz): δC 20.1, 23.0, 24.2, 29.7, 30.6, 40.6, 57.1, 59.2, 109.2, 148.9; IR (cm-1): ν 3040, 3000, 1670, 1250 and 860; purity: >93% (1H NMR and TLC).

(*-*)*-Limonene epoxide*: 1H NMR (CDCl3, 200 MHz): δH 1.40-1.00 (m, 2H), 1.23 (s, 3H), 1.72-1.63 (m, 2H), 1.68 (s, 3H), 2.05-1.74 (m, 2H), 2.18-2.10 (m, 1H), 2.96 (m, 1H), 4.62 (d, *J*=1.0 Hz, 1H), 4.68 (d, *J*=1.0 Hz, 1H); 13C NMR DEPT (CDCl3, 50 MHz): δC 21.0, 24.2, 28.4, 29.7, 30.4, 36.2, 57.5, 60.4, 109.5, 149.1; IR (cm-1): ν 3050, 3000, 1690, 1260 and 890; purity: >93% (1H NMR and TLC).

(*-*)*-Perillaldehyde*: 1H NMR (CDCl3, 200 MHz): δH 1.46-1.36 (m, 2H), 1.72 (s, 3H), 1.89-1.84 (m, 2H), 2.24-2.18 (m, 2H), 2.48-2.42 (m, 1H), 4.71 (s, 2H), 6.01-5.77 (m, 1H), 9.40 (s, 1H); 13C NMR DEPT (CDCl3, 50 MHz): δC 20.5, 21.4, 26.2, 31.6, 40.6, 109.4, 141.1, 148.2, 150.5, 193.8; IR (cm-1): ν 3080, 2980, 1700, 1680 and 880; purity: >93% (1H NMR and TLC).

*In vitro* toxicity assays

Cytotoxicity assays were performed as previously described (15). In brief, cells were seeded in 96-well plates in a final volume of 0.2 mL of appropriate culture medium (*T. brucei*: Baltz medium (29); HL-60 cells: RPMI 1640 medium (30)) supplemented with 16.7% heat-inactivated foetal calf serum and containing 10-fold serial dilutions of test compounds (10-1 to 10-6 mg/mL) dissolved in 100% DMSO. Controls contained DMSO alone. In all experiments, the final DMSO concentration was 1%. The seeding densities were 104 trypanosomes/mL and 105/HL-60 cells/mL. After 24 h incubation at 37°C in a humidified atmosphere containing 5% CO2, 20 µL of a 0.44 mM resazurin solution prepared in PBS was added and the cells were incubated for a further 48 h so that the total incubation time was 72 h. It should be also noted that the addition of 20 μl resazusin resulted in a 9% dilution of the test compounds which, however, does not have a significant effect on the determination of MIC and GI50 values. This was previously shown when the resazurin assay was compared with direct cell counting (15). Thereafter, the absorbance was read on a microplate reader using a test wavelength of 570 nm and a reference wavelength of 630 nm. GI50 values were determined by linear interpolation according to the method described in (31). MIC values were determined microscopically.

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**Conflict of Interest**

The authors have declared that there is no conflict of interest.

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**Figure 1**

   

 rotundifolone (+)-limonene epoxide (-)-limonene epoxide

  

 (-)-perillyl alcohol (-)-perilly aldehyde

**Fig 1**  Chemical structures of p-menthane-type monoterpenes tested for trypanocidal acivity.

**Table 1** Bioactivity of MCEO and *p*-menthane-type monoterpenes against *T. brucei* bloodstream forms and HL-60 cells.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Compound | *T. brucei* |  | HL-60 |  | Selectivity |  |
|  | **MIC [µg/mL]a** | **GI50 [µg/mL]b** | **MIC [µg/mL]a** | **GI50 ([g/mL]b** | **MIC ratioc** | **GI50 ratiod** |
| MCEO | 1 | 0.33±0.03 | 100 | 8.31±2.10 | 100 | 25 |
| Rotundifolone | 1(6.0)e | 0.32±0.05(1.93) | 100(602) | 3.40±1.21(20.5) | 100 | 10.6 |
| (+)-Limonen epoxide | 10-100(66-655) | 3.03±0.47(19.9) | >100(>655) | >100(>655) | >1-10 | >33 |
| (-)-Limonene epoxide | 10(66) | 2.94±0.15(19.3) | 100(655) | 34.6±0.7(227) | 10 | 11.7 |
| (-)-Perillyl alcohol | 100(655) | 13.3±1.6(87) | 100(655) | 34.6±0.6(227) | 1 | 2.6 |
| (-)-Perillyl aldehyde | 1(6.7) | 0.31±0.02(2.06) | 100(666) | 13.8±4.1(92) | 100 | 45 |
| Suraminf | 0.1(0.07) | 0.050±0.003(0.035) | >100(>70) | >100(>70) | >1000 | >2000 |

a Data shown are mean values of three independent experiments; b data shown are mean values ± SD of three independent experiments; c defined as MIC(HL-60)/MIC(*T. brucei*); d defined as GI50(HL-60)/GI50(*T. brucei*); e values in brackets are concentrations in μM; f reference control

**Table 2** Composition of the essential oil from leaves of *Mentha crispa* determined by GC/MS.

|  |  |  |
| --- | --- | --- |
| RIa | Constituent | %b |
| 934 | α-Pinene | 2.00 |
| 972 | Sabinene | 1.08 |
| 976 | β-Pinene | 4.43 |
| 990 | Myrcene | 7.79 |
| 1029 | Limonene | 10.58 |
| 1035 | *cis*-β-Ocimene | 5.01 |
| 1363 | Rotundifolone | 58.11 |
| 1386 | β-Bourbonene | 0.17 |
| 1420 | *trans*-Caryophyllene | 2.00 |
| 1454 | α-Humulene | 0.22 |
| 1457 | trans-β-Farnesene | 0.37 |
| 1481 | Germacrene | 6.55 |
| Total |  | **98.31** |

a Retention index determined on an OV-5 column; b constituent’s percentage