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Combinations of ascaridole, carvacrol, and caryophyllene oxide against *Leishmania*

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ABSTRACT

To date there are no vaccines against *Leishmania* and chemotherapy remains the mainstay for the control of leishmaniasis. The drugs currently used for leishmaniasis therapy are significantly toxic, expensive, and result in a growing frequency of refractory infections. In this study, we evaluated the effect of combinations of the main components of essential oil from *Chenopodium ambrosioides* (ascaridole, carvacrol, and caryophyllene oxide) against *Leishmania amazonensis*. Anti-leishmanial effects of combinations of pure compounds were evaluated *in vitro* and the fractional inhibitory concentration (FIC) indices were calculated. BALB/c mice infected with *L. amazonensis* were treated with different concentrations of ascaridole–carvacrol combinations by intralesional doses every 4 days. Disease progression and parasite burden in infected tissues were determined. *In vitro* experiments showed a synergistic effect of the combination of ascaridole–carvacrol against promastigotes of *Leishmania* with a FIC index of 0.171, while indifferent activities were observed for ascaridole–caryophyllene oxide (FIC index = 3.613) and carvacrol–caryophyllene oxide (FIC index = 2.356) combinations. The fixed ratio method showed that a 1:4 ascaridole–carvacrol ratio produced a better anti-protozoal activity on promastigotes, lower cytotoxicity, and synergistic activity on intracellular amastigotes (FIC index = 0.416). Significant differences ($p < 0.05$) in lesion size and parasite burden were demonstrated in BALB/c mice experimentally infected and treated with the ascaridole–carvacrol combinations compared with control animals. Carvacrol showed significant higher anti-radical activity in the DPPH assay compared with caryophyllene oxide. Electron spin resonance spectroscopy in combination with spin trapping suggested the presence of carbon-centered radicals after activation of ascaridole by Fe^{2+}. The intensity of the signals is preferably decreased upon addition of carvacrol. The ascaridole–carvacrol combination could represent a future alternative to monotherapeutic anti-leishmanial agents.

1. Introduction

Leishmaniasis is one of the serious health problems of the world. It is currently endemic in 98 countries worldwide. Overall annual prevalence of the disease is approximately 12 million people and the size of the population at risk is approximately 350 million. It is estimated that approximately 1.5 million new cases of cutaneous leishmaniasis and 500,000 cases of visceral leishmaniasis occur worldwide each year (Desjeux, 2004; WHO, 2010; den Boer et al., 2011). Furthermore, incidents of leishmaniasis are reported to be on the rise because of an increase in the number of vectors of the disease due to global warming (Peterson and Shaw, 2003). Among several drugs used in the treatment of leishmaniasis, pentavalent antimonials, considered a gold standard in the

Abbreviations: Asc, ascaridole; Car, carvacrol; Caryo, caryophyllene oxide; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; DMSO, dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EO, essential oil; ESR, electron spin resonance; FIC, fractional inhibitory concentration; GTM, glucantime; HFBS, heat-inactivated fetal bovine serum; IC_{50}, concentration at which inhibition of growth is 50%; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; p.i., post-infection; SI, selectivity index; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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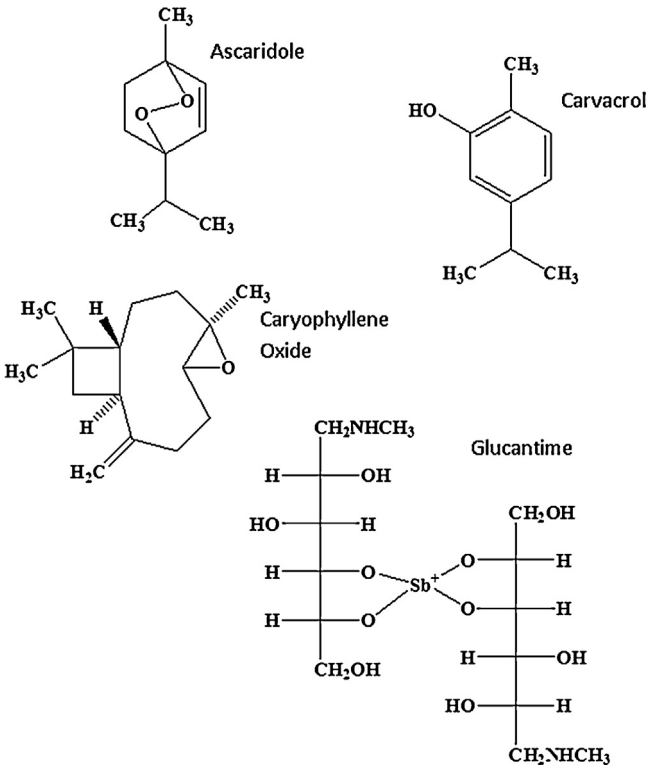

Fig. 1. Chemical structure of compounds used in this study.

treatment, are known to be toxic to humans. Resistance to anti-leishmanial drugs has also been observed in *Leishmania* parasites. Furthermore, other drugs used in treatment are expensive which limit their use in undeveloped and developing countries (Mishra et al., 2007; den Boer et al., 2011; Sundar and Chakravarty, 2012). Therefore, natural and traditional medicine can provide important treatment alternatives for leishmaniasis.

In previous studies, the anti-leishmanial activity of essential oil (EO) from *Chenopodium ambrosioides* was reported and major components were identified (Fig. 1): ascaridole (Asc), carvacrol (Car), and caryophyllene oxide (Caryo) (Monzote et al., 2006). Little or less specific activities were observed for the pure main components; while a potential anti-leishmanial effect was demonstrated by *Chenopodium* oil itself (Monzote et al., 2013, 2014). However, it is known that natural products exhibit also disadvantages, such as: the variation in chemical composition according to the parts of the plant used and the time or geographical area of collection (Gagnier et al., 2006). This clearly leads also to a variation of resulting biological effects. As a consequence, a long process of standardization would be required to obtain products in large-scales for pre-clinical/clinical studies or to develop a commercial pharmaceutical formulation (Kingston and Newman, 2005). For these reasons, a study with combinations of pure compounds found in *Chenopodium* oil was carried out with the aim to design a new therapeutic strategy against *Leishmania* based on active pure synthetic compounds.

2. Materials and methods

2.1. Parasite

The MHOM/77BR/LTB0016 strain of *Leishmania amazonensis* was kindly provided by the Department of Immunology, Oswaldo Cruz Foundation (FIOCRUZ), Brazil. Parasites were routinely isolated by aspiration with needle from mouse lesions and maintained as promastigotes at 26 °C in Schneider's medium (Sigma–Aldrich, St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (HFBS) (Sigma–Aldrich, St. Louis, MO, USA) and 100 μg of streptomycin/mL and 100 U of penicillin/mL. Cultures were passaged every 3 or 4 days. The parasites were not used after the tenth *in vitro* passage.

2.2. Compounds

Asc was synthesized as previously published (Monzote et al., 2009) and obtained with a purity >95%. Car and Caryo were obtained from Sigma–Aldrich (Vienna, Austria), with >98% and >95% of purity, respectively. The compounds were diluted in dimethyl sulfoxide (DMSO). Glucantime® (GTM, Fig. 1) obtained from Rhône-Poulenc (Rorer, Mexico) was diluted in sterile saline solution and used as reference drug.

2.3. Animals

Experiments with female BALB/c mice were carried out in accordance with the recommendations and guidelines for the care and use of laboratory animals of the Institutional Ethical Committee from the Institute of Tropical Medicine Pedro Kouri (Reference number: CEI-IPK 13–10). Mice were obtained from the National Center of Laboratory Animal Production (CENPALAB, Cuba) with a weight of 20–22 g and were maintained under standard conditions.

2.4. In vitro anti-promastigote activity of binary combinations of Asc, Car, and Caryo

Schneider's complete medium (100 μL) was plated in 96-well plates and additional 95 μL were added to the first column of wells. To obtain binary combinations of Asc–Car, Asc–Caryo, and Car–Caryo, 5 μL of each compound solution were added to first wells. Then, twofold serial dilutions were performed to give final concentrations of ranging from 1.25 to 125 μg/mL. Exponentially growing *L. amazonensis* promastigotes (100 μL, 2×10^5 promastigotes/mL) were then added to each well. Plates were incubated at 26 °C for 72 h and 20 μL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (SIGMA, St. Louis, MO, USA) solution at 5 mg/mL dissolved in saline solution was added to each well. After incubation for additional 4 h, the medium was removed and formazan crystals were dissolved by addition of 100 μL of DMSO. Absorbance was determined using an EMS Reader MF Version 2.4-0, at 560 and 630 nm as reference wavelengths. Median inhibitory concentration (IC_{50}) values were obtained from sigmoidal E_{max} model applied to dose–response curves (Sladowski et al., 1993; Dutta et al., 2005). Three replicates were performed and the average was calculated from the IC_{50} values obtained for each separate experiment.

Subsequently, the fractional inhibitory concentration (FIC) index was calculated according Johnson et al. (2004), by the following formulae: FIC index = [A]/IC_{50}A + [B]/IC_{50}B, where IC_{50}A and IC_{50}B are the IC_{50} values of each compound tested alone and [A] and [B] are the IC_{50} values of the compounds A or B when treatment was carried out in combination. A FIC index less than or equal to 0.5 indicates synergy, while an index greater than 4 indicates antagonism. A FIC index between 0.5 and 4 indicates indifference.

2.5. Isobologram construction using the fixed-ratio method from Asc to Car combination

The dynamic of the interaction between Asc and Car was studied by the ratio method (Seifert and Croft, 2006), adding the two compounds in fixed ratios (5:0, 4:1, 3:2, 2:3, 1:4, and 0:5; with final concentrations of 125:0, 100:25, 75:50, 50:75, 25:100, and