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Effects of linalool and eugenol on the survival of *Leishmania* (*L.*) *infantum chagasi* within macrophages

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ABSTRACT

The most commonly used drugs against visceral leishmaniasis are based on pentavalent antimonial compounds, which have played a fundamental role in therapy for over 70 years. However, the treatment is painful and has severe toxic side effects that can be fatal. Antimonial resistance is spreading and reaching alarming proportions. Linalool and eugenol have been shown to kill *Leishmania* (*L.*) *amazonensis* and *Trypanosoma cruzi* at low doses. In the present study, we demonstrate the effects of linalool and eugenol, components of essential oils, on *Leishmania* (*L.*) *infantum chagasi*, one of the causative agents of visceral leishmaniasis. We compared the effects of those compounds to the effects of glucantime, a positive control. In *L. infantum chagasi* killing assays, the LD_{50} for eugenol was 220 μg/ml, and that for linalool was 550 μg/ml. *L. infantum chagasi* was added to cultures of peritoneal mouse macrophages for four hours prior to drug treatment. Eugenol and linalool significantly decreased the number of parasites within the macrophages. Eugenol and linalool enhanced the activities of the *L. infantum chagasi* protein kinases PKA and PKC. Linalool also decreased *L. infantum chagasi* oxygen consumption. In conclusion, both linalool and eugenol promoted a decrease in the proliferation and viability of *L. infantum chagasi*. These effects were more pronounced during the interaction between the parasites and peritoneal mouse macrophages.

1. Introduction

Leishmania parasites are transmitted by phlebotomine insects to several animals, including humans; these parasites have tropism for mononuclear phagocytic cells. Depending on the species, the parasites may cause lesions at the site of the bite (skin and/or mucosa) or may infect organs, especially the spleen, liver, bone marrow and lymphoid tissues. Leishmaniasis refers to a group of diseases that vary from self-healing skin ulcers to visceral leishmaniasis, which is usually fatal without treatment. Severe visceral leishmaniasis infection leads to over 50,000 deaths per year, with an incidence of 200–400 thousands new cases (Vannier-Santos et al., 2002; Singh and Sundar, 2014). *Leishmania* (*L.*) *infantum chagasi* is considered the singular causative agent of American visceral leishmaniasis (AVL) in the New World, including Brazil. *L. donovani* and *L. infantum* are the causative agents of visceral leishmaniasis in the Old World.

Pentavalent antimonials are first-line drugs against leishmaniasis, though they cause severe side effects, induce drug resistance (Van Griensven and Boelaert, 2011) and require hospitalization for intravenous administration (Mitropoulos et al., 2010). The macrolide antibiotic, amphotericin B, is the first line treatment in India, because of the high rates of resistance to pentavalent antimonials in that country. Amphotericin B is also extremely toxic and its administration requires prolonged hospitalization. A new liposomal formulation of this drug is highly effective and presents low toxicity; however, its downside is the high cost and the need

Abreviations: AVL, American visceral leishmaniasis; BALBc, an albino, laboratory-inbred strain of the house mouse; cAMP, 3′,5′-cyclic adenosine monophosphate; DMSO, dimethylsulfoxide; EGTA, ethylene glycol tetraacetic acid; FCS, fetal calf serum; HepG2, a perpetual cell line derived from hepatocellular carcinoma; LD_{50}, median lethal dose. It is the amount of the substance required to kill 50% of the test population; MAPK, mitogen-activated protein kinases; MIC, minimum inhibitory concentration; MPK13, mitogen-activated protein kinase 13; MPK3, mitogen-activated protein kinase 3; MPK9, mitogen-activated protein kinase 9; Na/KATPase, sodium-potassium adenosine triphosphatase; NF-KB, nuclear factor kappa-light-chain-enhancer of activated B cells; PBS, phosphate-buffered saline; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; ROS, reactive oxygen species; RPMI, medium used for the culture of many types of cultured cells.

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of intravenous administration (Katsuno et al., 2015). Pentamidine, only efficient against some cutaneous leishmaniasis in South America, is very toxic and requires intramuscular administration (De Menezes et al., 2015). Paromycin is effective, well tolerated and relatively cheap, but its efficacy varies between *Leishmania* species; for instance, it is not efficient in East Africa (De Menezes et al., 2015). Miltefosine is an orally drug, well tolerated and usually safe, even though it is contraindicated in pregnancy, as there are suggestions of teratogenicity. Miltefosine is very effective against visceral leishmaniasis in India, but not in Africa (Kapoor et al., 1999; Papagiannaros et al., 2005; Peyron et al., 2005; Katsuno et al., 2015). Other form to treat the disease is the rational combination of drugs, reducing individual doses, treatment duration, adverse effects and the incidence of resistance (Mesquita et al., 2014; De Menezes et al., 2015). Even with all these efforts, the increasing number of patients infected with that are drug-resistant strains is certainly a major issue (Mitropoulos et al., 2010). Taken together, these facts make it imperative to search for new antileishmanial lead drug candidates (Singh and Sundar, 2014).

Mankind has used plants to treat disease for thousands of years. Medicinal plants currently play a key role in disease treatment, primarily as a source of compounds that would otherwise be difficult to synthesize. Additionally, flora provides basic compounds that can be modified to become either less toxic or more effective (Turolla and Nascimento, 2006). Recent studies have shown the importance of phytotherapy in parasitic diseases such as leishmaniasis (Carvalho and Ferreira, 2001; Derda and Hadaś, 2014). Essential oils are among the most variable compounds extracted from plants. A multiplicity of compounds is found in essentials oils, including terpene hydrocarbons, alcohols, aldehydes, ketones, esters, phenols and organics acids. Two such compounds, linalool and eugenol, are present in many plant extracts, such as *Croton cajucara*, *Achillea millefolium*, *Ocimum gratissimum* and *Syzygium aromaticum* (Rosa et al., 2003; Ueda-Nakamura et al., 2006; Santoro et al., 2007). However, the exact mechanisms of actions of isolated compounds of various traditionally used plant extracts still remain to be elucidated in many cases (Ramalingum and Mahomoodally, 2014).

Eugenol (4-allyl-2-methoxyphenol) (Fig. 1) is a significant chemical constituent of the essential oils derived from aromatic plants such as *Eugenia caryophyllus, Dicipelium cariophyllatum* and *Pimenta dioica.* The essential oil of *Ocimum gratissimum* and the eugenol derived from it demonstrate antileishmanial activities against *Leishmania amazonensis* (Ueda-Nakamura et al., 2006).

Linalool (3,7-dimethyl-octa-1,6-diene-3-ol) (Fig. 1) is a monoterpene alcohol produced by many plants by an enzyme called linalool synthase, which ionizes and rearranges the structure of geranyl diphosphate (Sugiura et al., 2011). The compound has a stereocenter carbon, allowing the existence of the enantiomers (−)-linalool (coriandrol) and (+)-linalool (licaerol) (Orav et al., 2006). Essential oils containing linalool were shown to have antimicrobial, anti-inflammatory and anesthetic activities. Rosa and co-workers showed that a linalool-rich essential oil extracted from *Croton cajucara* kills *L. amazonensis* in vitro (Rosa et al., 2003).

Considering that low doses of linalool and eugenol-rich essential oils are effective inhibitors of growth and are fatal in several species of *Leishmania* (Rosa et al., 2003; Ueda-Nakamura et al., 2006; Rodrigues et al., 2013; Islamuddin et al., 2014), our goal in the present study was to explore these effects in *L. infantum chagasi* promastigotes and axenic amastigotes at baseline and during the infection of peritoneal mouse macrophages. Instead of using the essential oils, we decided to analyze the effects of purified linalool and eugenol, which enabled us to delineate some of the basic mechanisms involved in these phenomena in the most correct manner (Ramalingum and Mahomoodally, 2014). Our results show that linalool and eugenol have promising antileishmanial potential and could be considered as new lead compounds in the search for novel antileishmanial drugs.

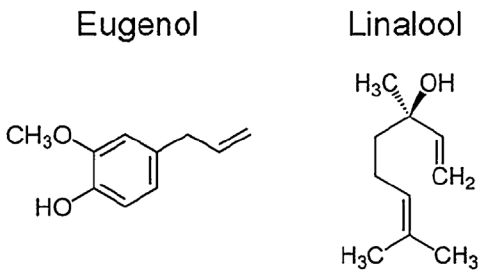

Fig. 1. Representation of chemical structures of eugenol and linalool. Eugenol (4-allyl-2-methoxyphenol); linalool (3,7-dimethyl-octa-1,6-diene-ol).

2. Materials and methods

2.1. Materials

Fetal bovine serum was purchased from Cultilab (São Paulo, Brazil). Schneider's *Drosophila* medium, linalool ((±)-3,7-dimethyl-1,6-octadiene-3-ol – purity 97%), eugenol (2-methoxy-4-(2-propenyl)phenol, 4-allyl-2-methoxyphenol – purity 99,8%), DMSO and resazurin sodium salt were purchase from Sigma-Aldrich (St. Louis, MO, USA). Kemptide (PKA peptide substrate) and neurogranin (PKC peptide substrate) were purchased from Promega Corporation (Alexandria, NSW, Australia). All other compounds used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA). The fluorimeter (Gemini XPS microplate reader) was purchased from Molecular Devices (Sunnyvale, CA, USA), the Kinase-Glo® kit Luminescent Kinase Assay Platform was obtained from Promega Corporation, a Sirius Single Tube Luminometer was obtained from Berthold Detection Systems (Pforzheim, Germany) and a high-resolution respirometer (Oroboros Oxygraph) was obtained from Oroboros Instruments (Innsbruck, Austria).

2.2. Linalool and eugenol solutions

Both eugenol and linalool were diluted in DMSO and then in distilled water for the final solution. The highest concentration of DMSO used in the experiments was 0.06%. Cells (parasites, macrophages or both) were treated with 0.06% DMSO or different concentrations of eugenol or linalool in DMSO and then subjected to the assays as described.

2.3. Microorganisms

Promastigotes of *Leishmania infantum chagasi* L579 (MHOM/BR/1974/PP 75) were maintained by weekly transfers in Schneider's *Drosophila* medium, pH 7.2, supplemented with 20% fetal bovine serum at 28 °C. The parasites were harvested after 5 days in culture and then used to obtain axenic amastigotes and for the assays below. Axenic amastigotes were obtained from promastigotes after incubation of promastigotes in Schneider's insect medium at 35 °C for 13 days and then used in the assays below (Teixeira et al., 2002).

2.4. Effects of eugenol or linalool on the viability of L. infantum chagasi

The parasites were incubated in 96-well plates (2×106 cells/well) for 24 h with linalool (50–750 ug/ml) or eugenol (50–650 ug/ml) and then for 12 h with 6 mM resazurin sodium salt. The assays with promastigotes were performed at 28 °C and