Phytomedicine 24 (2017) 111–118

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.com/locate/phymed

Original article

In vitro and *in vivo* antimalarial potential of oleoresin obtained from *Copaifera reticulata* Ducke (Fabaceae) in the Brazilian Amazon rainforest

Giovana A.G. de Souza[a], Nazaré C. da Silva[a], Juarez de Souza[b], Karen R.M. de Oliveira[c], Amanda L. da Fonseca[d], Leopoldo C. Baratto[e,*], Elaine C.P. de Oliveira[f], Fernando de Pilla Varotti[d], Waldiney P. Moraes[a]

[a] *Laboratório de Farmacologia Experimental, Universidade Federal do Oeste do Pará – UFOPA, Brazil*
[b] *Laboratório de Farmacologia, Instituto Esperança de Ensino Superior – IESPES, Brazil*
[c] *Laboratório de Neurofarmacologia Experimental, Universidade Federal do Pará – UFPA, Brazil*
[d] *Laboratório de Bioquímica Medicinal, Universidade Federal de São João del-Rei – UFSJ, Brazil*
[e] *Faculdade de Farmácia, Universidade Federal do Rio de Janeiro – UFRJ, Brazil*
[f] *Laboratório de Biotecnologia Vegetal, Universidade Federal do Oeste do Pará – UFOPA, Brazil*

ARTICLE INFO

Article history:
Received 22 August 2016
Revised 22 November 2016
Accepted 30 November 2016

Keywords:
Plasmodium berghei
Plasmodium falciparum
Copaifera reticulata
Oleoresin
Malaria
Antimalarial activity

ABSTRACT

Background: In view of the wide variety of the flora of the Amazon region, many plants have been studied in the search for new antimalarial agents. *Copaifera reticulata* is a tree distributed throughout the Amazon region which contains an oleoresin rich in sesquiterpenes and diterpenes with β-caryophyllene as the major compound. The oleoresin has demonstrated antiparasitic activity against *Leishmania amazonensis*. Because of this previously reported activity, this oleoresin would be expected to also have antimalarial activity.
Purpose: In this study we evaluated the *in vitro* and *in vivo* antimalarial potential of *C. reticulata* oleoresin.
Methods: *In vitro* assays were done using *P. falciparum* W2 and 3D7 strains and the human fibroblast cell line 26VA Wi-4. For *in vivo* analysis, BALB/c mice were infected with approximately 10^6 erythrocytes parasitized by *P. berghei* and their parasitemia levels were observed over 7 days of treatment with *C. reticulata*; hematological and biochemical parameters were analyzed at the end of experiment.
Results: The oleoresin of *C. reticulata* containing the sesquiterpenes β-caryophyllene (41.7%) and β-bisabolene (18.6%) was active against the *P. falciparum* W2 and 3D7 strains ($IC_{50} = 1.66$ and 2.54 µg/ml, respectively) and showed low cytotoxicity against the 26VA Wi-4 cell line ($IC_{50} > 100$ µg/ml). The *C. reticulata* oleoresin reduced the parasitemia levels of infected animals and doses of 200 and 100 mg/kg/day reached a rate of parasitemia elimination resembling that obtained with artemisinin 100 mg/kg/day. In addition, treatment with oleoresin improved the hypoglycemic, hematologic, hepatic and renal parameters of the infected animals.
Conclusion: The oleoresin of *C. reticulata* has antimalarial properties and future investigations are necessary to elucidate its mechanism of action.

Abbreviations: W2, chloroquine-resistant *Plasmodium falciparum* strain; 3D7, chloroquine-sensitive *Plasmodium falciparum* strain; 26VAWi-4, human fibroblast cell line; RPMI 1640, "Roswell Park Memorial Institute" 1640 culture medium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BALB/c, albino laboratory-bred mouse strain; IC_{50}, half maximal inhibitory concentration; ATCC, American Type Culture Collection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; SI, Selectivity Index; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; PbA, *Plasmodium berghei* ANKA strain; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ANOVA, analysis of variance; G1, Group 1 non-infected mice (control); G2, Group 2 non-treated *P. berghei*-infected mice; G3, Group 3 *P. berghei*-infected mice treated orally with artemisinin 100 mg/kg/day; G4, Group 4 *P. berghei*-infected mice treated orally with *C. reticulata* oleoresin at 200 mg/kg/day; G5, Group 5 *P. berghei*-infected mice treated orally with *C. reticulata* oleoresin at 100 mg/kg/day; G6, Group 6 *P. berghei*-infected mice treated orally with *C. reticulata* oleoresin at 10 mg/kg/day.

* Corresponding author at: Universidade Federal do Rio de Janeiro-UFRJ, Faculdade de Farmácia, Departamento de Produtos Naturais e Alimentos-DPNA, Centro de Ciências da Saúde - Bloco A, 2° andar - Sala 30, Ilha do Fundão, CEP: 21.941-590, Rio de Janeiro-RJ, Brazil.
E-mail address: leopoldo@pharma.ufrj.br (L.C. Baratto).

http://dx.doi.org/10.1016/j.phymed.2016.11.021
0944-7113/

Introduction

Malaria is one of the most important human parasitic disease, considered to be a global issue, although mainly occurring in tropical and subtropical regions (Saei and Ahmadian 2009). In recent years, the number of malaria cases has decreased, althought 3.2 billion of people live in areas of high risk. A total of 214 million cases of malaria were estimated to have occurred in 2015, with 438,000 deaths (WHO, 2015). In Brazil, a 75% reduction in the number of cases was recorded from 2002 to 2013, with the lowest number, about 143,000, recorded in 2014 (MS, 2015).

The etiological agent of malaria is a parasite belonging to genus *Plasmodium* and the species that affect humans are *P. falciparum, P. vivax, P. malariae* and *P. ovale* (Gomes et al., 2011). Humans can be occasionally infected by simian species such as *P. knowlesi* that has arisen as an important cause of human malaria in South-East Asia (Rossati et al., 2016; Shearer et al., 2016). Natural transmission occurs during a blood meal of infected female *Anopheles* mosquitoes. In Brazil, the highest incidence of malaria occurs in the Amazon region and *A. darlingi* is the most important Brazilian malaria vector (Rosa-Freitas et al., 1998; Brasil 2010), followed by *A. marajoara* (Laporta et al., 2015).

In the absence of an effective malaria vaccine, malaria control relies on a limited chemotherapeutic arsenal (Barik, 2015). Currently, WHO recommends artemisinin-based combination therapies (ACT) as first-line treatment for *P. falciparum* malaria (WHO, 2015), although one of the most important issues concerning malaria is the appearance of artemisinin drug-resistant *P. falciparum* strains. Artemisinin resistance in *P. falciparum* is now widespread in Southeast Asia, hampering malaria control measures (Woodrow and White, 2016).

In this context, new and safe antimalarial drugs are urgently needed and natural products can represent a vast source of leading molecules (Oliveira et al., 2009). Many plants are used therapeutically in alternative and traditional medicine to treat infectious diseases. The largest part of the Amazonian Rainforest is located in the Brazilian territory, offering a huge diversity of plants that could represent new sources of antimalarial agents (Maciel et al., 2002). One of these plants is *Copaifera reticulata* Ducke (Fabaceae), known as “copaíba”, spread throughout Latin America and Western Africa (Sachetti et al., 2009). Its oleoresin is a mixture of sesquiterpenes and diterpenes and the major compound is β-caryophyllene which has anti-inflammatory, antibacterial and antifungal properties, as well as antileishmanial activity against *L. amazonensis* (Veiga Jr. and Pinto 2002; Santos et al., 2008a, b) Other components of the oleoresin are β-bisabolene (with anti-inflammatory and analgesic activities), α-humulene, α- and β-selinene, α-bisabolol, β-elemene, γ-cadinene, α-cadinol etc. (Veiga Jr. and Pinto 2002). *C. reticulata* oleoresin showed $IC_{50} = 5.0$, 15.0 and 20.0 μg/ml against promastigotes, axenic amastigotes and intracellular amastigotes of *Leishmania amazonensis*, respectivelly, and low cytotoxicity to J774G8 macrophages (Santos et al., 2008b). This oleoresin was also found to be active against promastigotes and amastigotes of *L. chagasi* with $IC_{50} = 7.88$ and 0.52 μg/ml, respectively, with no toxicity to murine monocytes RAW 264.7 (Rondon et al., 2012).

Material and methods

Collection and characterization of oleoresin from Copaifera reticulata

Oleoresin of *C. reticulata* was collected in Floresta Nacional do Tapajós - FLONA, kilometer 67, located in Belterra, Pará State, Brazil. The species was identified by the taxonomist Regina Célia Viana Martins da Silva and a voucher specimen was deposited in the Herbarium of Embrapa Oriental under registration NID: 69/2011. Oil extraction for scientific purposes was authorized and approved by SISBIO (*Sistema de Autorização e Informação em Biodiversidade*) (protocol number: 44,380–1). Oleoresin was extracted during the Amazon summer (dry period) by the method of Oliveira et al., (2006), *i.e.*, by random mechanical perforation of the trees with a traditional auger (2 cm in diameter and 45 cm in length) producing two holes at 1 m and 1.50 m from the soil, respectively. Oleoresin samples were stored in plastic containers protected from light and then transferred to glass vials (10 ml) for later analysis. After the full flow of oleoresin, the tree holes were sealed with a PVC-type pipe (¾ diameter and 10 cm length) covered with a plastic cap in order to facilitate the next collections and to avoid wood waste.

The oleoresin used in the present experiment was the same as that chemically characterized by Baldissera et al., (2014). Samples were analyzed in duplicate by gas chromatography (GC, injection volume: 1 μl) according to the following conditions: Agilent Technologies 6890 N GC-FID system, DB-5 capillary column (30 m × 0.25 mm; film thickness 0.25 mm), flame ionization detector (FID), injector and detector temperatures 280 °C, carrier gas: helium, flow rate: 1.3 ml/min, thermal programmer: 50–300 °C (rate of 5 °C/min. Component relative concentrations were calculated based on GC peak areas without using correction factors. GC–MS analyses (injection volume: 1 μl) were performed as follows: Agilent Technologies AutoSystem XL GC–MS system, EI mode (70 eV), split/splitless injector (250 °C), transfer line temperature: 280 °C, carrier gas: helium (1.5 ml/min), capillary columns: HP 5MS (30 m × 0.25 mm; film thickness 0.25 mm) and HP Innowax (30 m × 0.32 mm i.d., film thickness 0.50 mm). The temperature program was the same as that used for the GC analyses. The constituents of oleoresin were identified on the basis of retention index (RI) and by comparison with the mass spectra library search (NIST and Wiley) and with the mass spectra literature data. The relative amounts of individual components were calculated based on the CG peak area (FID response).

GC–MS analysis demonstrated that the major compounds were sesquiterpenes identified as β-caryophyllene and β-bisabolene, 41.7% and 18.6%, respectivelly (complete characterization of the chemical composition of *C. reticulata* oleoresin can be ckecked in Supplementary material). In the present study, β-caryophyllene and β-bisabolene were considered to be analytical markers since they were the major components of the test sample. Similarly, β-caryophyllene was considered to be an active marker since the literature has confirmed its antimalarial activity. On the other hand, due to the complexity of the sample, it is difficult to state that this molecule is responsible for the antimalarial potential of *C. reticulata* oleoresin, with a synergistic effect probably playing an important role in the activity.

The oleoresin tested in the present antimalarial assays was endotoxin free, as determined according to the instructions of the Lonza Kinetic-QCL Chromogenic Limulus Lysate (LAL) Endotoxin Assay Kit (Walkersville, MD), with a sensitivity range of 0.005–50.0 EU/ml.

Antimalarial assays

In vitro *tests*

Culture of the intraerythrocytic phases of Plasmodium falciparum. The parasite lines W2 (chloroquine-resistant) and 3D7 (chloroquine-sensitive) were cultured *in vitro* in human erythrocytes under the conditions established by Trager and Jensen (1976), with modifications (Andrade-Neto et al., 2004; Carvalho et al., 1991). Parasites with 5% hematocrit were cultured in RPMI-1640 medium supplemented with 25 mM Hepes, 21 mM sodium bicarbonate, 300 μM hypoxanthine, 11 mM glucose, 40 μg/ml gentamicin, and 10% (*v/v*) inactivated human serum. Parasites were kept at 37 °C with a controlled oxygen level and parasitemia was