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Antimalarial alkaloids isolated from *Annona squamosa*

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Abstract

Aim of the investigation was to isolated antimalarial compounds from *Annona squamosa,* which is traditionally used in diseases including infections associated with malarial parasites. N-Nitrosoxylopine (**1**), Roemerolidine (**2**) and Duguevalline (**3**) were isolated from the extract of bark. All compounds showed moderate activity against chloroquine-sensitive strain (D10) and a chloroquine resistant strain (Dd2) of *Plasmodium falciparum* with IC_{50} values ranging between 7.8 and 34.2 μM/mL. N-Nitrosoxylopine also showed cytotoxicity in MTT assay while no cytotoxicity was observed for other two compounds.

Keywords: *Plasmodium falciparum*, *Annona squamosa*, alkaloids, MTT

Introduction

Malaria is a major disease throughout the tropical and subtropical regions and continues to be one of the greatest causes of serious illness and death. According to the WHO Malaria Report 2008, there was an estimated 247 million malaria cases among 3.3 billion people at risk in 2006, causing nearly a million of deaths, mostly of children under 5 years. 109 countries were endemic for malaria in 2008, including 45 within the WHO African region. Malaria treatment relies on a handful of accepted, affordable and effective drugs, many of which are chemically similar. However, an increasing incidence of drug resistant strains of Plasmodium spp. highlights the need for novel antimalarial compounds. Plants represent an important source of novel Antimalarial compounds, as most famously evidenced

by the Antimalarial agents quinine and artemisinin initially isolated from Cinchona spp. (Smith, 1976) and Artemisia annua (Graziose et al., 2010), respectively. New compounds with antimalarial activity continue to be isolated from plant sources (Bero et al., 2009; Kaur et al., 2009) often representing the culmination of ethnobotanical investigations of plants used to treat malaria in endemic regions (Bourdy et al., 2008). However, malaria was once much more widespread than it is today (Hay et al., 2004), and although often overlooked, many plants native to the United States were important sources of antimalarial treatments before the disease was eradicated from the country.

Members of the *Annonaceae* family (about 128 genera) are generally well-known as insecticides and parasiticides and mostly distributed in America and Asia, are now cultivated for its edible fruits in Europe. *Annona squamosa* (Annonaceae) is a tree occurs wild and is also cultivated. Its leaves are used as insecticidal and antispasmodic agents and are used in the treatment of rheumatism and painful spleen. The plant is reported to possess analgesic, anti-inflammatory (Dash et al. 2001), antipyretic, antiulcer, and antiseptic and abortifacient activities (Akolkar et al. 1992). Its use as an insecticidal agent has been investigated by several workers (Cheema et al. 1985) and various phytochemical, pharmacological, antibacterial and antiovulatory studies have already been carried out with the seed extracts (Vohra et al. 1975). In the study we have reported isolation of compounds with anti-malarial properties

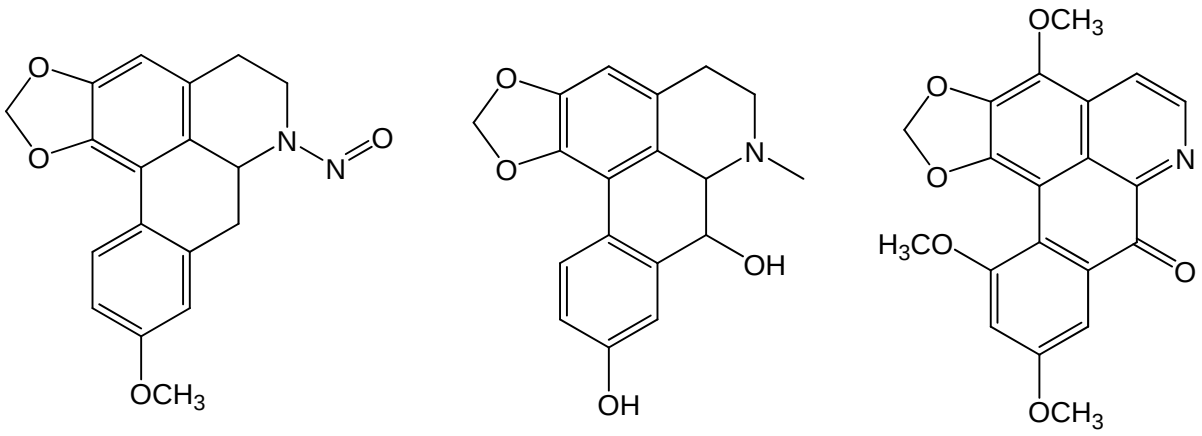
Materials and Methods

Plant Material

Bark of *Annona squamosa* was collected from Penang Island, west coast of West Malaysia. After identification, plant material was dried under shade for several weeks. After drying, bark was powdered to facilitate extraction procedure.

Isolation

Dried and powdered bark of *A. squamosa* (750 g) was defatted with hexane (8 L) at room temperature for 24 h, dried, and extracted with 95% ethanol (8 L, 2×) at room temperature for 48 h. The ethanolic extract was separated by filtration and concentrated under vacuum to yield 49.8 g of crude extract that showed an antiplasmodial activity of 12.3 μg/mL. This crude extract was acidified with 3% HCl solution and extracted with chloroform (450 mL, 3×) to remove phenolic compounds, and the remaining aqueous extract was basified with 4N NH_4OH solution and extracted with chloroform (45000 mL, 3×), which was concentrated under vacuum to yield 0.58 g of extract. This basic extract was purified by reverse phase HPLC (Waters RP-8 300 mm × 19.0 mm, 7 μm, 30–95% methanol in water which contains 0.01% TFA, over 60 min, flow rate 10 mL/min; UV detector, 254 nm) to collect three fractions, Fr-A (74 mg), Fr-B (57 mg), and Fr-C (109 mg), respectively. Fr-A was further purified by reverse phase HPLC (Phenomenex Synergy Hydro-RP 80A 250 mm × 21.20 mm, 4 μm, 30–95% methanol in water with 0.01% TFA, over 60 min, flow rate 10 mL/min; UV detector, 254 nm) to give two subfractions, Fr-A-1 (51 mg) and Fr-A-2 (23 mg), respectively. Fr-A-2 further purified by reverse phase HPLC (Phenomenex Synergy



N-Nitrosoxylopine **(1)** Roemerolidine (**2**) Duguevalline (**3**)

Figure 1. Compounds isolated from bark of *Annona squamosa.*

Hydro-RP 80 Å 250 mm × 4.60 mm, 4 μm, 30–95% methanol in water with 0.01% TFA, over 60 min, flow rate 1 mL/min; UV detector, 254 nm) to give compound **1** (43 mg). Similarly, Fr-C gave two sub fractions, Fr-C-1 (89 mg), and Fr-C-2 (21 mg). Compound **2** (52 mg) was isolated from Fr-C-1 and compounds **3** (26 mg) were isolated from Fr-C-1 and -2, respectively.

Antimalarial activity

A chloroquine-sensitive strain (D10) and a chloroquine resistant strain (Dd2) of Plasmodium falciparum were continuously cultured according to a modified method (Trager and Jensen, 1976). The in-vitro antiplasmodial assays were performed using the parasite lactate dehydrogenase (pLDH) activity assay to measure parasite viability. Chloroquine diphosphate (Sigma) was used as a positive control. Crude extracts and fractions were tested against the D10 strain at three concentrations: 20, 10, and 5μg/mL. Pure compounds were tested in triplicate against both the D10 and Dd2 strains. IC_{50} values were calculated from a 10-point dose response curve (the samples were serially diluted 2× from 100 to 0.2μg/mL, CQ was serial diluted 2× from 1000 to 2 ng/mL) using a non-linear dose-response curve fitting analysis via Graph Pad Prism v. 4.0 software.

Cytotoxicity

Cytotoxicity was assessed against a Chinese Hamster Ovarian (CHO) cell line using the 3-(4,5-dimethylthiazol-2yl)-2,5- diphenyltetrazoliumbromide (MTT) assay (Mosmann, 1983). Emetine hydrochloride (Sigma) was used as a positive control. Crude extracts and fractions were assayed in parallel with the antiplasmodial assay at three concentrations: 20, 10, and 5μg/mL. IC_{50} values of pure compounds were tested in triplicate and were calculated from a 10-point dose response curve (samples were serially diluted 2× from 100 to 0.2μg/mL, emetine hydrochloride was serial diluted 2× from 100 to 0.001μg/mL) using a nonlinear dose-response curve fitting analysis via GraphPad Prism v.4.0 software.

Results and Discussion

Present investigation was focused on isolation and characterization of the antiplasmodial compounds from *A. squamosa*. Three known aporphine alkaloids were isolated from bark. Structures of compounds were identified as N-Nitrosoxylopine (**1**), Roemerolidine (**2**) and Duguevalline (**3**) via comparison with spectral data with literature (Carollo et al., 2006, Pérez et al., 2004, Rasamizafy et al., 1987). The aporphine alkaloids isolated by antiplasmodial activity-guided fractionation of *A. squamosa* bark display *in vitro* antiplasmodial activates with IC_{50} values ranging between 7.8 and 34.2 μM/mL (Table 1). The antiplasmodial activity of compounds 1, 4 and 5 showed considerable antiplasmodial activity against both the CQ sensitive D10 strain. Compound 2 and 3 showed moderate antiplasmodial activities with no observable cytotoxicity. However compound 1 was found to active against both of the plasmodium strains but also exhibited cytotoxicity as well which might be attributed to nitroso moiety of compound **1.** Our results suggest that the antiplasmodial activity of compounds isolated from *A. squamosa* has enough potential to further considered for detailed mechanistic studies for getting insights to mechanism of action and resistance to develop new therapeutic agents

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