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Original Article

Investigation of Indian *Diospyros* Species for Antiplasmodial Properties

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Despite decades of intense research, malaria remains a deadly disease worldwide and new antimalarials are urgently needed due to increasing drug resistance of *Plasmodium falciparum* to existing drugs. This article reports the evaluation of four Indian *Diospyros* species viz., *Diospyros melanoxylon*, *D. peregrina*, *D. sylvatica*, *D. tomentosa* for antiplasmodial activities against chloroquine-sensitive (3D7) and chloroquine-resistant (K1) strains of *P. falciparum*. Six of eight methanolic extracts were found to have significant activity, ($IC_{50} = 16.5–92.9\,\mu g\,ml^{-1}$), against strain 3D7 and five of these showed similar activities against strain K1 ($IC_{50} = 20.5–121.6\,\mu g\,ml^{-1}$). *Diospyros sylvatica* was found to be the most active species ($IC_{50} = 16.5–29.4\,\mu g\,ml^{-1}$) and is worthy of further investigation.

Keywords: *Diospyros melanoxylon* – *Diospyros peregrina* – *Diospyros sylvatica* – *Diospyros tomentosa* – Ebenaceae – Malaria – *Plasmodium falciparum*

Introduction

Malaria is one of the most devastating diseases in the world responsible for more than 2.5 million deaths (1) and at least 300 million clinical cases per annum (2). Resistance of *Plasmodium falciparum* to chloroquine and some other antimalarial drugs continuous to increase. Efforts are now being directed towards the discovery and development of new chemically diverse antimalarial agents. *Diospyros* is one of the most important genera of the Ebenaceae and *Diospyros* species are well-known for their medicinal uses since ancient times in many traditional medicinal systems such as Ayurveda, Traditional Chinese Medicine and the African folklore (3–9). About 41 *Diospyros* species occur in India of more than 350 species identified (10,11). These are mostly trees and rarely shrubs. The literature revealed that these plants contain pentacyclic triterpenes and juglone-based 1,4-naphthoquinones and about 130 species have undergone some phytochemical and/or pharmacological investigations (8).

Two *in-vivo* studies by different groups revealed that the methanolic extracts of *Diospyros mespiliformis* (bark) and *D. v*ariegata (stem) possessed potent antipyretic properties supporting their folkloric use in relieving fevers (12,13) suggesting that species of *Diospyros* may be worth screening for antimalarial properties. *Diospyros melanoxylon*, *D. peregrina*, *D. sylvatica*, *D. tomentosa* have been used extensively in Indian traditional medicine to treat for a variety of diseases including diarrhea, cholera, dysentery, intermittent fevers, bleeding gums, bronchitis, carbuncles, cough, cramps, pneumonia, syphilis, tumors, etc. (8,14) and are widely available in the forests of Orissa (North East India). Therefore, these species have been collected and used for antiplasmodial screening.

Methods

Plant Collection

Plant materials were collected from the local forest, Bhubaneswar (20°15′ N, 85°50′ E), Orissa, India during June–August 2004. The identity of these plants was

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confirmed by taxonomist, Mr Murty K.S. (Ayurvedic Research Institute, Bhubaneswar, India) and the voucher specimens (040511–040518) are kept available in The School of Pharmacy, University of Bradford, UK.

Extraction

Method 1. Dried pulverized plant material (10 g) was extracted with methanol at room temperature; the methanol was decanted after 24 h and the extraction repeated three times. The pooled extracts were filtered and then concentrated under vacuum using a rotary evaporator at 40°C.

Method 2. Powdered stem bark (10 g) of *D. peregrina, D. sylvatica and D. tomentosa* were extracted (separately) in a Soxhlet apparatus successively with *n*-hexane, ethyl acetate and methanol and the extracts concentrated under vacuum.

Sample Preparation

Crude extracts and control drugs stock solutions (1 mg ml^{-1}) were prepared by weighing 1 mg accurately and dissolving in 50 µl of dimethyl sulphoxide (DMSO) or ethanol and made up to 1 ml with double distilled water. Later these solutions were diluted in complete medium.

Chemicals and Reagents

All the materials for the antiplasmodial assays were purchased from Sigma Chemical Co, Poole, UK, except for artemether which was a gift from Aventis Pharma.

Cultivation of *P. falciparum*

Chloroquine sensitive (3D7) and resistant (K1) strains were kindly supplied by Professor D. C. Warhurst (London School of Hygiene and Tropical Medicine). The strains were maintained in human A + erythrocytes suspended in RPMI 1640 medium supplemented with A + serum and D-glucose according methods of Trager and Jensen, 1976 (15) and Fairlamb *et al*. 1985 (16).

Antiplasmodial Assay

Cultures containing predominantly early ring stages of *P. falciparum* were used for testing. After adding sample solutions to 96-well microtiter plates in duplicate, 2-fold serial dilutions were made with RPMI 1640 medium and infected erythrocytes were added to give a final volume of 100 µl with 2.5% hematocrit and 1% parasitemia. Chloroquine diphosphate and artemether were used as positive controls, and uninfected and infected erythrocytes without sample solutions were incubated in each test. Plates were placed into a modular incubator gassed with 93% nitrogen, 3% oxygen and 4% carbon dioxide and incubated at 37°C for 48 h. Parasite growth was assessed by measuring parasite lactate dehydrogenase activity (pLDH) as described by Makler *et al*. 1993 (17). The reagent used contained the following in each microlitre acetylpyridine adenine dinucleotide (APAD), 0.74 mg; lithium lactate, 19.2 mg; diaphorase, 0.1 mg; Triton X-100, 2 µl; nitroblue tetrazolium, 1 mg; and phenazine ethosulfate, 0.5 mg. Fifty microliters of this reagent was added to each well and mixed, and plates were read at 550 nm using a Dynatech Laboratories MRX microplate reader, and per cent inhibition of growth was calculated by comparison with control values. Optical densities were read at 550 nm using a Dynatech Laboratories MRX microplate reader and percentage inhibition of growth was calculated by comparison with control values. IC_{50} values were determined using linear regression analysis (Microsoft Excel). A minimum of three separate determinations was carried out for each sample.

Results

The antiplasmodial activities of the extracts and control drugs are shown in Tables 1 and 2. Data are expressed as the mean ± standard deviation of the IC_{50} of three independent experiments on different days for each strain. The IC_{50} values ranged from 16.5 to >500 µg ml^{-1}. The effect of the methanolic extract of root, *D. sylvatica* on growth of *P. falciparum* strain K1 is shown in Fig. 1. No significant difference was found in IC_{50} values against strains 3D7 and K1 in the case of artemether (0.008 and 0.014 µg ml^{-1}, respectively), but ~20-fold difference was found in CQ between the strains 3D7 and K1 (0.02 and 0.38 µg ml^{-1}, respectively) showing that, as expected strain K1 is resistant to CQ, but not to artemether. The effect of CQ on growth of *P. falciparum* strain K1 is shown in Fig. 2.

Discussion

Six of the eight methanolic extracts from the four *Diospyros* species were found to have significant activities against strain 3D7 ($IC_{50} \leq 93$ µg ml^{-1}; see Table 1) and five extracts were active in case of strain K1 ($IC_{50} \leq 122$ µg ml^{-1}; see Table 1) suggesting that the latter do not exhibit cross-resistance with chloroquine. Interestingly, the extracts of *D. sylvatica* possessed very good activity ($IC_{50} = 16.5$–29.4 µg ml^{-1}; see Table 1) against both strains 3D7 and K1 and were the most active of these *Diospyros* extracts. *Diospyros melanoxylon*, stem heartwood and root possessed significant activities against strain 3D7 ($IC_{50} = 55.6$ and 78.4 µg ml^{-1}, respectively), but the heartwood extract was 2-fold less active against strain K1 compared with strain 3D7

Table 1. *In vitro* activities of MeOH extracts and antimalarial drugs against *P. falciparum*

S. No.	Species and control	Ext. Part	IC_{50} μg ml^{-1} against *Pf* [Mean ± SD]	
			Strain 3D7	Strain K1
1.	*D. melanoxylon*	Root	78.4 ± 1.2	121.6 ± 0.9
2.		St. heartwood	55.6 ± 1.7	201.7 ± 2.1
3.		St. bark	>500	>500
4.	*D. peregina*	St. bark	92.9 ± 1.8	120.2 ± 2.3
5.	*D. sylvatica*	Root	27.3 ± 0.6	29.4 ± 1.3
6.		St. heartwood	16.6 ± 0.4	20.5 ± 0.7
7.		St. bark	16.5 ± 0.2	21.9 ± 0.3
8.	*D. tomentosa*	St. bark	>500	>500
9.	Positive controls	Chloroquine	0.02 ± 0.001	0.38 ± 0.03
10.		Artemether	0.008 ± 0.002	0.014 ± 0.01

Note. Pf: *P. falciparum*; SD: Standard deviation; St.: Stem; Ext.: Extract; $n = 3$.

Table 2. *In vitro* activities of successive extracts against *P. falciparum*

S. No.	Species and control	Ext. of St. bark	IC_{50} μg ml^{-1} against *Pf* [Mean ± SD]	
			Strain 3D7	Strain K1
1.	*D. peregina*	Hexane	>500	>500
2.		Ethyl acetate	>500	>500
3.		Methanol	44.7 ± 0.7	66.7 ± 1.6
4.	*D. sylvatica*	Hexane	>500	>500
5.		Ethyl acetate	19.4 ± 1.1	29.3 ± 0.8
6.		Methanol	51.5 ± 1.6	81.5 ± 1.3
7.	*D. tomentosa*	Hexane	>500	>500
8.		Ethyl acetate	>500	>500
9.		Methanol	>500	>500

Note. Pf: *P. falciparum*; SD: Standard deviation; St.: Stem; Ext.: Extract; $n = 3$.

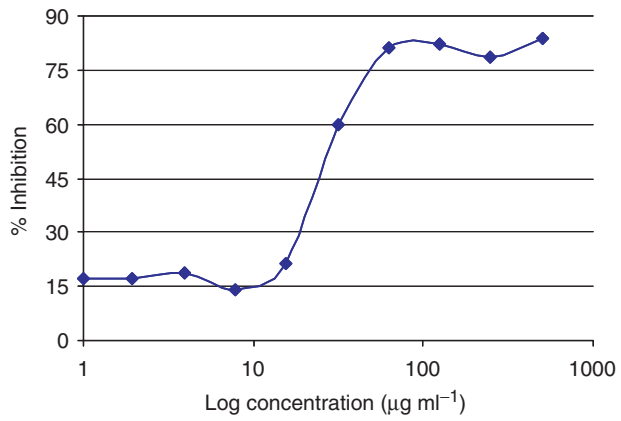

Figure 1. Effect of extract of *D. sylvatica* on growth of *P. falciparum* strain K1.

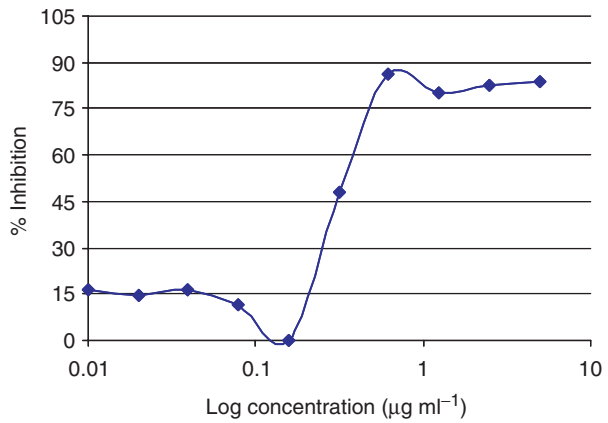

Figure 2. Effect of chloroquine diphosphate on growth of *P. falciparum* strain K1.

($IC_{50} = 201.7$ and 121.6 μg ml^{-1}, respectively). *Diospyros peregrina* stem bark possessed activity against both strains 3D7 & K1 ($IC_{50} = 92.9$ and 120.2 μg ml^{-1}, respectively). Methanolic extracts of the stem barks of *D. melanoxylon* and *D. tomentosa* were inactive ($IC_{50} > 500$ μg ml^{-1}). Stem barks of *D. peregrina*, *D. sylvatica* and *D. tomentosa* were fractionated successively with *n*-hexane, ethyl acetate and methanol

and screened against both parasite strains (Table 2). Only the methanolic fraction from *D. peregrina* was active while in the case of *D. sylvatica* the ethyl acetate fraction was the most active, but some activity remained in the methanol fraction. No activity was seen in the fractions from *D. tomentosa*, consistent with the lack of activity found in the cold methanolic extract.

A literature study of *Diospyros* species revealed that only the leaves of *D. melanoxylon* have previously been tested against *P. falciparum* (3D7) and the ethanolic extract was found to be active ($IC_{50} = 97\,\mu g\,ml^{-1}$) (18). *Diospyros peregrina*, *D. sylvatica*, *D. tomentosa* and the root, heartwood and stem bark of *D. melanoxylon* have been screened for the first time in this study.

A large number of compounds have been reported from *D. melanoxylon* and *D. peregrina* including lupeol, betulin, betulinic acid, ursolic acid, oleanolic acid, β-sitosterol, etc. (8). A few studies have been carried out with *D. sylvatica*. Lupeol, betulin and betulinic acid have been isolated from the bark (19) and 2-methylanthraquinone, plumbagin, diosindigo, diospyrin, iso-diospyrin and microphyllone reported from roots by Ganapaty *et al.* 2004 (20). The antiplasmodial activities of some of these compounds have been reported (21–27); plumbagin (25) and diospyrin (26) have been shown to have potent activities against *P. falciparum in vitro* ($IC_{50} = 0.27$ and $0.21\,\mu g\,ml^{-1}$, respectively) while betulinic acid and lupeol, ursolic acid and oleanolic acid were only weakly active. The presence of these compounds, especially plumbagin and diospyrin may explain the antiplasmodial activities of the extracts tested in this study but further studies will be needed in order to confirm this.

Conclusion

Extracts of three of the four species of Indian *Diospyros* tested in this study showed antiplasmodial activities, with the best activity shown by *D. sylvatica*. This species especially is worthy of further investigation to determine which of its constituents are responsible for the activity.

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