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Screening of fruit pulp extracts of *Picralima nitida* against *in vitro* cultures of *Plasmodium falciparum* and acute oral toxicity in white albino mice

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ABSTRACT

The increasing resistance of *Plasmodium falciparum* to available antimalarial drugs currently justifies the quest for potential antiplasmodial agents from plants. *Picralima nitida* is used as a traditional remedy against malaria in Nigeria. In this study, phytochemical constituents of methanolic and aqueous extracts of *P. nitida* fruit pulp were screened, and tested *in vitro* against 20 clinical isolates of *P. falciparum* using the schizont growth inhibition assay. Acute oral toxicity of each extract was also evaluated in white albino mice. The results showed the presence of alkaloids, anthraquinones, flavonoids, saponins, and tannins in both extracts. Terpenes were only present in the methanolic extract. *In vitro* growth inhibition of *P. falciparum* by each extract was dose dependent. The methanolic extract exhibited moderate, but higher antimalarial activity with IC_{50} = 23.33 ± 0.76 µg/ml, compared to the aqueous extract with IC_{50} = 28.25 ± 0.95 µg/ml. The difference was significant (t = 16.72, df = 19, P < 0.001). The LD_{50} of each extract was 3807 mg/kg body weight. The moderate antiplasmodial, and toxicity of these extracts, may justify the use of *P. nitida*, fruit pulp for the treatment of malaria in Nigeria.

Keywords: phytochemical constituents, malaria, inhibitory concentration, lethal dose.

INTRODUCTION

Malaria is one of the most important infectious diseases in the tropical and sub-tropical regions of the world. Significant efforts to control, and perhaps eliminate malaria, beginning from the year 2000-2013 have yielded 30% global reduction in case incidence of malaria, currently estimated at 198 million cases. Also, a 47% reduction in global mortality, currently estimated at 584,000 deaths has been achieved (WHO, 2014). Despite these significant reductions, malaria continues to be one of the greatest health problems still facing Africa. The global scope of malaria, and the growth of drug resistant *Plasmodium falciparum* (Noedl et al., 2008; Dondorp et al., 2009; Miotto et al., 2013) have made the need for varied, effective and improved alternative therapies against the disease a necessity. According to WHO (2014), the burden of malaria is heaviest in WHO Africa region, where approximately 90% of global malaria deaths occur in children < 5 years old, who account for 78% of global malaria mortality.

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The endemic nature of malaria in some parts of the world could be attributed to the emergence and spread of drug resistant malaria to many disease endemic parts of the world (Packard, 2014). This unwanted phenomenon has led to treatment failures in many of the conventional antimalarial drugs, with the same threat extended to the most recent effective antimalarial drugs (Noedl et al., 2008; Dondorp et al., 2009; Miotto et al., 2013). Plants have always been considered as valuable sources of medicine against malaria, and other diseases (Bray, 1990; Balunas and Kinghorn, 2005). It is perhaps a common practice to investigate antimalarial activities from medicinal plants, suspected of having such activities against the malaria parasites. This is necessary in order to determine their potentials as sources of new antimalarial agents.

Picralima nitida, family; Apocynaceae (common name; Akuama plant, Igbo; Osi igwe), is a species occurring in Africa's forest regions. The Nigeria Natural Medicine Development Agency (NNMDA) (2008) has noted that the plant has a wide variety of applications, in the Nigerian herbal medicine. Previous reports have shown the activities of *P. nitida* as an antimicrobial (Fakeye et al., 2000), anti-inflammatory (Ezeamuzie et al., 1994), as well as an antiplasmodial agent (Bikii et al., 2007). The antimalarial effects of the bark, leaf, and seed of the plant have been well documented (Iwu and Klayman, 1992; François et al., 1996; Fakeye et al., 2000; Okokon et al., 2007; Adebayo and Krettli, 2011). However, the antiplasmodial potentials of the fruit pulp, the part of the plant usually discarded as not useful has not been well documented. Therefore, the main aim of this study was to determine whether the antimalarial activities associated with other parts of *P. nitida*, were equally present in the fruit pulp of the plant; a part of the plant that is often thrown away.

MATERIALS AND METHODS

Collection of plant materials

Fresh fruits of *P. nitida* plant were collected from Akabor in Oguta local government area of Imo State, Nigeria. The plant was identified by a plant taxonomist at the herbarium section of the International Centre for Ethnomedicine and Drug Development Nsukka and a herbarium specimen of the plant deposited at the centre.

Extraction procedure

Seeds were extracted from the fruit, and the fruit pulp pulverized using an electrical crusher machine. Two thousand gram of the pulverized seed was subjected to extraction using the soxhlet extractor with 95% methanol and distilled water as solvents respectively. The different methanolic and aqueous extracts of the fruit pulp of *P. nitida* plant used were each concentrated to dryness using a water bath and weighed. The weighed extracts were then stored in a refrigerator at 4 ^{o}C for further experiments.

Phytochemical screening

Crude methanolic and aqueous extracts of the fruit pulp of *P. nitida* plant were screened for its phytochemical constituents using the method described by Harborne (1998). The plants phytochemical components tested for were alkaloids, anthraquinons, flavonoids, saponins, tannins, steroids and terpenes.

Blood sample collection for *in vitro* cultivation of *Plasmodium falciparum*

Blood samples were collected aseptically from patients at the clinic of National Institute for Pharmaceutical Research and Development (NIPRD) Abuja. The inclusion criteria were patients who had fever, with axillary temperature $\geq$ 37.5 ºC, and had not taken any anti-malarial drugs in the last two weeks. The absence of antimalarial drugs from their system was confirmed by a negative urine test of 4-aminoquinoloines and

sulfonamides by Dill-Glazko and Lignin test (WHO, 2001). Patients who satisfied these criteria, and gave oral informed consent were screened for *P. falciparum* mono infections. Thick blood films stained with 10% Giemsa stain for 10 minutes were examined under the microscope to confirm asexual parasitaemia and to estimate parasite density. The acceptable range of parasitaemia was 2000 – 80,000 asexual parasites/µl of blood. Methanol fixed thin smears were stained with 3% Giemsa stain for 30 minutes, and used to confirm mono infections of *P. falciparum*. In each confirmed case, 5 ml of blood were collected into EDTA bottles, the fresh blood plasma were removed and the blood pellets re-suspended and washed thrice in RPMI 1640 for *in vitro* cultivation of malaria parasites (Flyg et al., 1997).

Preparation of complete malaria culture medium

The complete malaria culture medium (CMCM) was prepared by dissolving 10.43 g of RPMI 1640 powder (Gibco), 6 g of Hepes and 2 g of $NaHCO_3$ in litre of distilled – demonized water. The solution was filtered using 0.22 µm membrane filters and 0.5 ml Gentamycin (from 50 mg/ml stock) was added. The mixture was then stored at 4 °C in aliquots of 45 ml. Before parasite cultivation, each aliquot was supplemented with 5% albumax II solution (Cranmer et al., 1997).

Preparation of stock solution of extracts' of *Picralima nitida*

Stock solutions of both methanolic and aqueous extracts were each prepared by dissolving 20 mg of the extract in 10 ml dimethylsulphoxide (DMSO) and sonicated for 30 minutes to yield a 20 mg/10 ml stock solution of the extracts. The stock solution was stored at 4 ºC until used, during *in vitro* drug susceptibility test of the extracts against *P. falciparum* parasites.

In vitro* cultivation of *P. falciparum* isolates and susceptibility test with extracts of *Picralima nitida

The assay was performed in duplicates, using a modification of WHO's schizont growth inhibition method (WHO, 2001). Briefly, 200 µl of a mixture of CMCM and *P. falciparum* positive blood from each individual patient, n = 20 was distributed in duplicates, on 96 well flat bottom micro titre plates. Then 200 µl of the stock solution was used to make a 7 well, two fold serial dilution of each sample, bringing the final plate concentration range of 3.13- 200 µg/ml. The 8^{th} well was maintained as control with a mixture of CMCM and *P. falciparum* positive blood without the extract. The plates were incubated at 37 °C in a candle jar under CO_2 atmosphere for 30 hours. After incubation, the plates were tilted at an angle, allowed to settle and 5 µl content drawn from the bottom of each well was removed. The cell content from each well was used to form a series of thick films on clean microscopy slides, and stained with 2.5% Giemsa stain for 45 minutes. The number of schizonts formed per 200 asexual parasites were counted and recorded from the control wells, as well as the wells containing the extracts (WHO, 2001).

Determination of IC_{50} of *Picralima nitida* extracts

The number of schizonts counted from the extract free control wells, and the wells with extracts from each cultured isolate were fed into a specialized malaria *in vitro* inhibitory concentration determination software, HN-NonLin. The concentrations required to inhibit schizont maturation by 50%, 90% and 95% were then determined. HN-Non Lin is available free of charge at http://malaria.farch.net.

Experimental animals

The animals used for acute toxicity test were adult male white Albino mice weighing 22-30 g. The mice were obtained from the animal house of the University of Nigeria Nsukka. They were housed in standard cages,

and were handled according to local rules and regulation of Experimental Animals at the University of Nigeria, Nsukka, Nigeria.

Acute toxicity test

The acute toxicity of *P. nitida* extracts in mice was carried out as reported by Lorke (1983) with a slight modification. A total of 24 male white albino mice were divided into 2 sets of 6 groups of 2 mice each. The mice were treated orally with single doses of 10, 100, 1000, 1600, 2900, 5000, mg/kg body weight. Both the methanolic and aqueous extracts of the fruit pulp were separately administered after the mice were starved for 24 hours. Two additional mice were only given distilled water as control animals. The animals in each group were routinely inspected for appearance or signs of toxicity such as, weakness, falling off of hair, coma or even death for a period of 24 hours.

Determination of LD_{50} of *Picralima nitida* extracts

Lethal dose LD_{50} of each extract was estimated by calculating the geometric mean of the maximum dose with 0% mortality and the minimum dose with 100% mortality (Lorke, 1983).

Statistical analysis

Inhibitory concentrations (ICs) of the two extracts were compared by paired sample t-test, using SPSS software version 20, IBM Inc. 2011. The level of significance (α) was = 0.05.

RESULTS

The phytochemical screening of the aqueous and methanolic extracts from the fruit pulp of *P. nitida* revealed the presence of alkaloids, anthraquinones, flavonoids, saponins, tanins, and terpenes (Table 1). Steroids were absent in both extracts, while terpenes were present in the methanolic extract but absent in the aqueous extract.

Each of the extract produced a dose dependent inhibitory activity against clinical parasite isolates of *P. falciparum* at the different concentrations used. Of the two, the methanolic extract showed a comparably higher antimalarial activity, with mean IC_{50} of 23.33 ± 0.76 µg/ml, compared to the aqueous extract with IC_{50} of 28.25 ± 0.95 µg/ml. The mean IC_{50} values of the two extracts were significantly different ($t = 16.72$, $df = 19$, $P < 0.001$). The difference was consistently significant at higher inhibitory concentrations - IC_{90} and IC_{95} (Table 2).

The acute oral toxicity of both the methanolic and aqueous extracts of the fruit pulp of *P. nitida* demonstrated that a dose of 5000 mg/kg produced physical signs of toxicity in white albino mice 10 hours after administration of the extracts, leading to death. These signs included writhing, decreased motor activity, gasping and subsequently death. All the animals treated with 5000 mg/kg body weight of the extracts died. The LD_{50} of each extract was therefore estimated to be = 3705.15 (Table 3).

Table 1: Phytochemicals present in the fruit pulp of *Picralima nitida* extracts.

Phytochemicals	Methanolic extract	aqueous extract
Alkaloids	+	+
Anthraquinones	+	+
Flavonoids	+	+
Saponin	+	+
Tannins	+	+
Terpens	+	-

+ = present, - = absent

Table 2: Mean inhibitory concentrations (ICs) of extracts of *P. nidita* in µg/ml ± SD.

ICs	Methanolic extract	Aqueous extract	t - test	Df	P - value
IC_{50}	23.33 ± 0.76	28.25 ± 0.95	16.72	19	< 0.001
IC_{90}	92.53 ± 1.01	110.42 ± 1.29	51.11	19	< 0.001
IC_{95}	116.57 ± 1.17	138.60 ± 14.32	6.79	19	< 0.001

Table 3: Lethal Dose $(LD)_{50}$ of fruit pulp extracts of *P. nitida*.

Type of extract	LD_{50}
Methanolic extract	3705.15
Aqueous extract	3705.15

DISCUSSION

Phytochemical compounds found in plants are reported to act in synergy with nutrients and dietary fibre to protect against diseases. Compounds such as terpenoids are commonly implicated in the antiprotozoal and antiplasmodial activities of many plants that have being studied (Phillipson and Wright, 1991; Asase et al., 2004). A notable example of a terpenoid from plant is artemisinin, the main active ingredient in the traditional Chinese antimalarial qinghaosu (Tan et al., 1998). Currently, artemisinin derivatives in combination with other antimalarial compounds are the most effective antimalarial drugs recommended for the treatment of malaria (WHO, 2010). In one study, flavonoids were reported to show significant anti-parasitic activities against different strains of malaria, trypanosomes and leishmania (Kim et al., 2004). The presence of these phytochemical compounds in the fruit pulp extracts of *P. nitida* suggest that perhaps, these compounds may be acting in synergy with one or more compounds to exert the observed antimalarial activity, albeit moderate, as seen from the fruit pulp extracts of *P. nitida.* The absence of terpenoids in the aqueous extract despite its presence in the methanolic extract could be the in ability of water to extract the compound due to its non-polar nature (Bhat et al., 2005). Perhaps its presence in the methanolic extract could be a reason why the extract exhibited moderately higher antimalarial activity *in vitro* compared to the aqueous extract.

Both *in vivo* and *in vitro* antiplasmodial activities of ethanolic extracts of *P. nitida* plant have been reported (Iwu and Klayman, 1992; Okokon et al., 2007). One of the studies linked the antiplasmodial activities of the various parts of the plant to indole alkaloids present in the plant (Iwu and Klayman, 1992). Alkaloids were also found in both the aqueous and methanolic extracts of the fruit pulp of *P. nitida* in the present study. The latter extract with an IC_{50} of 23.33 ± 0.76 µg/ml exhibited moderate, but significantly higher anti-malarial activity on clinical isolates of *Plasmodium falciparum* compared to the former's IC_{50} of 28.25 ± 0.95 which was also moderate. The threshold classification of the present *in vitro* antimalarial activities of extracts from the pulp of *P. nitida* seeds was based on the classification according to Gessler et al. (1994), which classified extracts with IC_{50} less than 10 µg/ml as very good; 10-50 µg/ml as moderate and over 50 µg/ml as having low activity. In one study the Cameroon, the antimalarial activity of the methanolic extract of *P. nitida* against chloroquine resistant W2 strain of *P.*

falciparum was over 2 fold higher (IC_{50} = 10.90 ± 1.10) than the values recorded in this study (Bikii et al., 2007). However, the part of the plant used was not clearly stated. Our knowledge of the various inhibitory activities of *P. nitida* extracts from different parts of the plant on *P. falciparum* indicated a death of data on extracts from the fruit pulp. This study has shown moderate activity using methanol as the extracting solvent. The antimalarial activity of the aqueous extract in the present results which also showed a moderate activity was in agreement with the results obtained by Iwu and Klayman (1992), as well as François et al. (1996) with various parts of the plant.

The acute toxicity potential of both the methanolic and aqueous extracts of the fruit pulp of *P. nitida* in white albino mice showed moderate toxicity (Homburger, 1989) with LD_{50} = 3807 mg/kg body weight. A recent study by Solomon et al. (2014) observed that ethanolic extracts of *P. nitida* seeds induced toxic effects on the testis of male adult Wistar rats, which led to a reduction in the seminal fluid parameters of the rats. Our study did not investigate a similar histopathological effect on mice, but it reminds one of the toxic effects the plants' extracts might bring to bear on body organs. Such a distortion is capable of compromising the fertility potentials of the rats. However, it appears that reduction in the seminal fluid of rats by some herbal extract might be a common attribute of herbs which exhibit antimalarial properties (Raji et al., 2003; Oze et al., 2006). A similar property has been observed in some standard antimalarial drugs, like chloroquine and halofantrine (Adeeko and Dada 1993; Orisakwe et al., 2003). These drugs were used extensively in the treatment of malaria without necessarily compromising human fertility.

The fruit pulp of *P. nitida* is not always considered useful by those who frequently utilize the plant. Consequently, it is frequently thrown away. Our finding has demonstrated the medicinal value of this part of the plant with some antimalarial activity, albeit moderate in nature. However the moderate toxicity also recorded in the fruit pulp may justify the future use of this plant's part as a complementary remedy for the treatment of malaria.

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