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IN VITRO ANTIPLASMODIAL ACTIVITY AND CYTOTOXICITY OF LEAF EXTRACTS OF *JATROPHA TANJORENSIS* J.L. ELLIS AND SOROJA

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ABSTRACT
The in vitro antiplasmodial activity and cytotoxicity of extracts from Jatropha tanjorensis leaves was evaluated. The plant leaves were successively extracted into three (3) extract forms (aqueous extract, ethanolic extract and hydro-ethanolic (50:50 v/v)) using standard procedures. For quality control, high performance thin layer chromatography (HPTLC) was performed on extracts. Antiplasmodial activity was assessed in vitro by using a 3D7 chloroquine sensitive clone of NF-54 isolate of Plasmodium falciparum. Cytotoxicity of extracts against non-cancerous vero cell lines was determined with doxorubicin serving as the standard cytotoxic drug. The spectra from the chemical finger-print of the extracts revealed that the ethanolic extract contained eleven peaks in contrast to the hydro-ethanolic (eight peaks) and aqueous (six peaks) extracts. The ethanolic extract had the highest antiplasmodial activity (IC_{50} 10.86 ± 1.52 ug/ml), low cytotoxicity (IC_{50} 86.8 ± 4.8 ug/ml) and selectivity index (SI) of 8.0 when compared with the hydro-ethanolic ($IC_{50plasmodial}$ 48.0 ± 1.34 ug/ml, $IC_{50vero\ cells}$ 547 ± 9.4 ug/ml, and SI of 11.4) and aqueous ($IC_{50plasmodial}$ 44.0 ± 2.4 ug/ml; $IC_{50vero\ cells}$ > 1000 ug/ml) extracts. The antiplasmodial activity of the crude ethanolic extract was however moderate when compared with the standard antimalaria drug chloroquine (IC_{50} 0.087 ± 0.0003 ug/ml). The results therefore suggest moderate antiplasmodial activity and low cytotoxicity of ethanolic extract of Jatropha tanjorensis leaves against chloroquine sensitive strain of Plasmodium falciparum. The antiplasmodial activity of the plant leaves supports local claims on its efficacy in the treatment of malaria infection.
Keywords: Malaria, cytotoxicity, Jatropha tanjorensis, Plasmodium falciparum, chemical finger-print.

INTRODUCTION

Malaria is one of the most serious pathogenic diseases in endemic areas of the world, particularly in Africa, Asia, and Latin America (Ravikumar *et al*., 2012; Sha'a *et al*., 2011). In Africa alone, it is estimated that more than 300 million people are infected annually by the parasite, *Plasmodium falciparum*, and over one million deaths have been recorded in children under five years (Ramazani *et al*., 2010; Zofou *et al*., 2011). The problem is further compounded by the upsurge in the resistant strains of the parasite against conventional antimalaria drugs such as chloroquine, quinine, and recently, artemisinin derivatives (Sha'a *et al*., 2011; Zofou *et al*., 2011). Thus, the continuous search for novel and more effective antimalarial compounds especially from medicinal plants extracts is of utmost importance in combating malaria infection (Asase *et al*., 2005; Ramazani *et al*., 2010; Ravikumar *et al*., 2012).

In Africa, the use of indigenous plants plays an important role in the traditional methods of malaria treatment by providing good sources for the detection of novel antiplasmodial compounds (Hilou *et al*., 2006; Ouattara *et al*., 2006; Chukwujekwe et al., 2009). The therapeutic properties ascribed to most of these plants are linked to the phytochemical compounds contained in them. Phytochemicals such as alkaloids, glycosides, phenols, saponins, triterpenoids, flavonoids, etc have been suggested to possess antimalarial property (Madureira *et al*., 2002; Kraft *et al*., 2003; Tona *et al*., 2004; Mbatchi *et al*., 2006; Omoregie *et al*., 2011; Zofou *et al*., 2011; Ravikumar *et al*., 2012).

Jatropha tanjorensis (Euphorbiaceae family), is a perennial herb, a hybrid species which shows intermediacy in phenotypic characters between *Jatropha curcas* and *Jatropha gossypifolia* (Prabakaran and Sujatha, 1999; Omoregie and Osagie, 2007). The common names include: catholic vegetables, *Jatropha,* 'Hospital too far', *Iyana ipaja (*Yoruba) (Omoregie and Osagie, 2011; Oyewole *et al*., 2012). The plant leaves were initially and popularly consumed in Nigeria as soups and as a tonic with the claim that it increases blood volume. The leaves are also employed traditionally in the treatment of anaemia (as a haematinic agent), diabetes and cardiovascular diseases (Iwalewa *et al*., 2005; Orhue *et al*., 2008; Omoregie and Osagie, 2011; Oyewole *et al*., 2012). However, the plant's popularity has been doused by unproven claims that the whitish latex emanating from the leaf stem and stalk may be toxic to man (Omoregie and Osagie, 2011).

The plant leaves were selected for this study as a result of claims by traditional healers, of their efficacy in the treatment of anaemia and malaria fever in the southern region of Nigeria (Orhue *et al*., 2008; Omoregie and Osagie, 2011; Oyewole *et al*., 2012). This study therefore reports the *in vitro* cytotoxicity of extracts from *Jatropha tanjorensis* leaves in non-cancerous vero cell lines. Also, the *in vitro* antiplasmodial activity of extracts of the plant was tested in chloroquine sensitive *Plasmodium falciparum* 3D7 (NF-54) clone.

MATERIALS AND METHODS

Collection of Plant Materials

Jatropha tanjorensis leaves were collected during the rainy season between April and June, 2010 from private farms at different locations in Benin City, Nigeria. The leaves were authenticated by a Botanist, and voucher specimens of the plant leaves (with voucher number: JT/104/07) was deposited in the herbarium of the University of Benin, Benin City, Nigeria.

Chemicals

All chemicals used were of analytical grade (from E-Merck India Ltd). Chloroquine standard (Cat. No – C6628) and doxorubicin were purchased from Sigma Chemicals Ltd. (Bangalore, Karnataka India).

Preparation and Chemical Finger-Print of the Extracts

The plant extracts (alcoholic, aqueous, and hydro-ethanolic (50:50 v/v)) were prepared from air-dried leaves powder of the plant according to previously reported standard procedures (Ouattara *et al*., 2006). The extracts were subjected to HPTLC Chemical Finger-Printing for quality assurance. The HPTLC system consisted of TLC plates 10 × 10 cm silica gel $60F_{254}$ (Merck). One hundred milligram of plant extract was dissolved in 1.0ml of methanol by means of ultra-sonication for 30 minutes, and centrifuged at 8000rpm for 10 minutes. The supernatant obtained was used for the HPTLC chemical profile with chloroform: methanol (9:1, v/v) serving as the mobile phase. Sample application plates were developed for one hour in a Camag 10 × 10 twin trough glass solvent developing chamber initially pre-saturated with the mobile phase. The plates were allowed to air-dry, and then scanned using a Camag TLC Scanner model 3 (with slit size 10 × 0.40mm and wavelength of 254nm) equiped with Wincats software and absorption-reflection scan mode. The calibration curve of peak area vs. concentration was prepared.

In vitro Antimalarial Activity of Plant Extracts

Plasmodium falciparum 3D7 (clone NF-54), obtained from Central Drug Research Institute (CDRI) laboratory, Lucknow, India, was maintained in our laboratory at 5% hematocrit (human type O-positive red blood cells) in complete RPMI 1640 medium (RPMI 1640 medium supplemented with 25 mM HEPES, 370 µM hypoxanthine, 40 μgml^{1} gentamycin, 0.25 $\mu g\ ml^{-1}$ Fungizone and 0.5% [wt/vol] AlbuMax II) in 60mm petri dish by modified candle jar method (Trager and Jensen, 1976; Srivastava *et al*., 2007). The culture was routinely monitored through Geimsa staining of the thin smears.

Standard drug (chloroquine) and extracts (at different concentrations of 1, 5, 10, 50, 100, 500, and 1000ug/ml) were prepared in distilled water (chloroquine; Sigma) and DMSO (test extracts) and then diluted to achieve the required concentrations. The synchronized culture with parasitaemia of 1.5% and 3% haematocrit were incubated in 96-well microtitre plate predisposed with multiple concentrations of compounds/extracts for 48 hrs at 37°C in candle jar. Blood smears from each well were fixed in methanol, stained with Giemsa's stain and the numbers of infected RBCs per 5000 cells were counted. The antimalarial activity of the test extract was expressed as 50% inhibitory concentration (IC_{50}) determined from dose-response curve by non-linear regression analysis (curve-fit) using Graph Pad Prism (version 4) software. All experiments were performed in triplicates and the results were expressed as percentage of growth inhibition. Crude extracts with IC_{50} values > 50 µg/ml were considered to be inactive (Kraft *et al*., 2003).

In vitro Cytotoxicity Test

Cytotoxicity assay was performed in 96-well microplates using neural red uptake method as described by Borenfreund and Puerner (1985) and Repetto *et al.* (2008). The cytotoxicity of the plant extracts was assessed against Vero cell line (kidney cells from the African green monkey) cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum; 0.2% NaHCO3 at 37°C in an atmosphere of 95% humidity, 5% CO2. Concentration ranges tested were between 0.3 – 1000 mg/ml for plant extracts and 0.3 – 100 mg/ml for doxorubicin. All cultures were performed in triplicates (three assays). Doxorubicin hydrochloride was used as the standard cytotoxic drug. IC_{50} was calculated from dose-response curve as earlier described.

Statistical Analysis

All values were analyzed using Graph Pad Prism (version 4) software and the results were expressed as means ± standard deviation (SD). One-way analysis of variance (ANOVA) was performed to test for differences between the groups mean of IC_{50s}. Significant differences (P values < 0.05) between the means were compared non-parametrically by the Duncan's multiple range test (Sokal and Rohlf, 1995).

RESULTS AND DISCUSSION

The chemical profile of extracts of *Jatropha tanjorensis* leaves showed several peaks in the spectra suggesting the presence of some phytochemical compounds (figures 1 to 3). Eleven peaks were observed in the ethanolic extract (between 22.8 - 427.8 AU), followed by eight peaks in the hydro-ethanolic extract (between 33.1 – 234.4 AU) and six peaks shown by the aqueous extract (between 19.5 – 211.2 AU).

In vitro antiplasmodial study revealed that the ethanolic extract of *Jatropha tanjorensis* was most active, when compared with the other extracts against the chloroquine sensitive 3D7 strain of *Plasmodium falciparum,* with 50% inhibitory concentration of parasite growth ($IC_{50plasmodium}$) observed at 10.86 ± 1.52 µg/ml when compared with that of the aqueous (($IC_{50plasmodium}$ = 44.0 ± 2.40 µg/ml) and hydro-ethanolic (($IC_{50plasmodium}$ = 48.0 ± 1.34 µg/ml) extracts (Figures 4 to 6 and Table 1).

From literature, an extract is regarded as highly active if IC_{50} < 10 µg/ml, moderately active if IC_{50} is between 10µg/ml and 50µg/ml and inactive if IC_{50} > 50µg/ml (Ramazani *et al*., 2010). Based on this classification, the three extracts forms were found to be moderately active against *Plasmodium falciparum* when compared with the standard antimalarial drug chloroquine (($IC_{50plasmodium}$ = 0.087 ± 0.0003 ug/ml). All the extracts, that is, ethanolic extract (IC_{50vero} = 86.8 ± 4.8 ug/ml), hydro-ethanolic (IC_{50vero} = 547 ± 9.4 ug/ml) and aqueous (IC_{50vero} > 1000 ug/ml), showed little or no cytotoxic activity against non-cancerous vero cells relative to the standard cytotoxic drug doxorubicin (IC_{50vero} = 1.8 ± 0.42 ug/ml). The selectivity index (SI) was however lower for ethanolic extract (8) when compared with the hydro-ethanolic extract (11.4) (Table 1).

The antiplasmodial property of the plant extracts may be attributed to presence of some phytochemicals which might have conferred some protective / antioxidative effect against oxidative stress induced in the host parasitized red blood cells (RBCs) by the malaria parasite (Becker *et al*., 2004; Nethengwe *et al*., 2012). In malarial infection, oxidant damage to the erythrocyte membrane has been reported with evidences revealing a causal relationship between haemichrome production and band 3 aggregations in oxidatively stressed RBCs. This relationship could account for the deposition of band 3-specific autologous IgG and consequent deposition of fragments of complement C3c. Thus, leading to alterations of the surface of the infected RBCs and subsequent phagocytosis by macrophages (Becker *et al*., 2004).

In our present study, the plant extracts may function as antioxidant by reversing these changes due to their bioactive principles. Besides, previous studies on *Jatropha tanjorensis* leaves showed that it contained some phytochemical principles including alkaloids, saponins, anthraquinones, tannins, and flavonoids (Olayiwola *et al*., 2004; Omoregie and Osagie, 2007), chemical classes with demonstrated effective antioxidant and/or antimalarial activities (Hilou *et al*., 2006; Chukwujekwe et al., 2009; Valdes *et al*., 2010; Sha'a *et al*., 2011; Zofou *et al*., 2011; Omoregie and Osagie, 2011; Omoregie *et al*., 2011). The HPTLC chemical finger-print profile of the plant's crude extracts further attested to the presence of these phytochemical compounds evidenced by the several yet to be identified absorption peaks in this study. A similar reversal has been observed by a known reducing and oxidant scavenging agent (β-mercaptoethanol), indicative of the oxidative character of the parasite–induced modifications (Becker *et al*., 2004).

The plant extracts may also prevent the detoxification of free oxidized haem (FP – Fe III), one of the by-products of haemoglobin degradation, by intercalating with the iron-carboxylate bond which links the haem units of malaria pigment (hemozoin) thereby inhibiting their polymerization (Becker *et al*., 2004; Ravikumar *et al*., 2012). Some bioactive agents from plants sources have been implicated as metal chelators containing orthodiphenol and carboxyl functions that can chelate the parasite indispensable inner cations (including Ca^{2+}, Fe^{2+} and Mg^{2+}). These cations serve as cofactors of the *Plasmodium* enzyme ribonucleotide reductase (RNR). The plant extracts may contain constituents that can inhibit *Plasmodium* growth by blocking the parasite choline intracellular transport necessary for the biosynthesis of the phosphatidylcholines which are essential molecules for the *Plasmodium* (Hilou *et al*., 2006). Therefore, phytochemical principles present in the plant extracts may have a proportional link with antiplasmodial activity (Ahmed *et al*., 2010; Ravikumar *et al*., 2012).

CONCLUSION

The present study provides evidence that the ethanolic extract of *Jatropha tanjorensis* leaves exhibited the highest antiplasmodial activity relative to the other two extracts against chloroquine sensitive strain of *Plasmodium falciparum*. All the plant extracts however possess moderate antimalarial activity when compared with standard antimalaria drug chloroquine. The plant extracts showed little or no cytotoxicity against vero cells line

The results therefore justify the traditional use of the plant leaves in the treatment of malaria. Nevertheless, further work is necessary to ascertain the *in vivo* toxicity of the plant, identify its active principles and optimum dosage in order to provide effective and low cost intervention during malaria infection.

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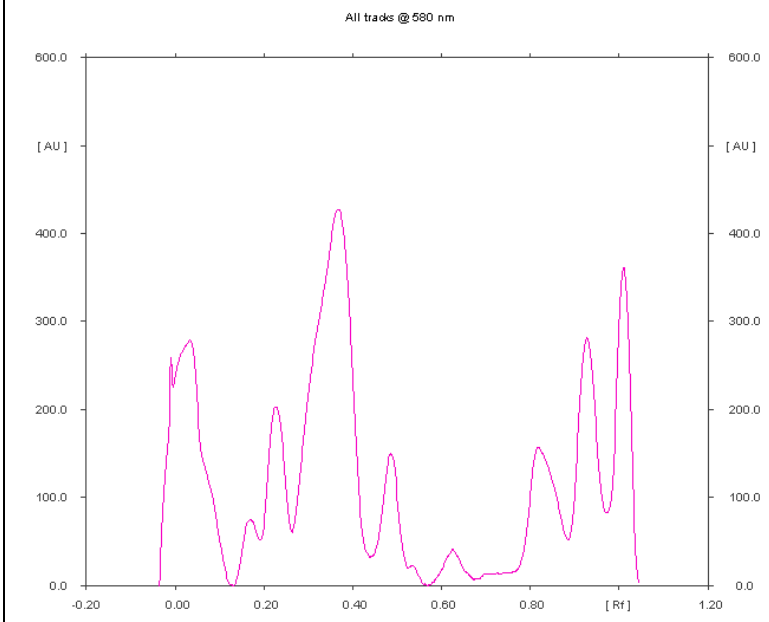

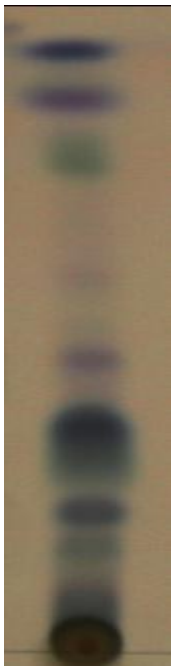

TLC Plate at 580 nm after derivatization

Figure 1: Chemical Fingerprint of Ethanolic Extract of *Jatropha tanjorensis* Leaves

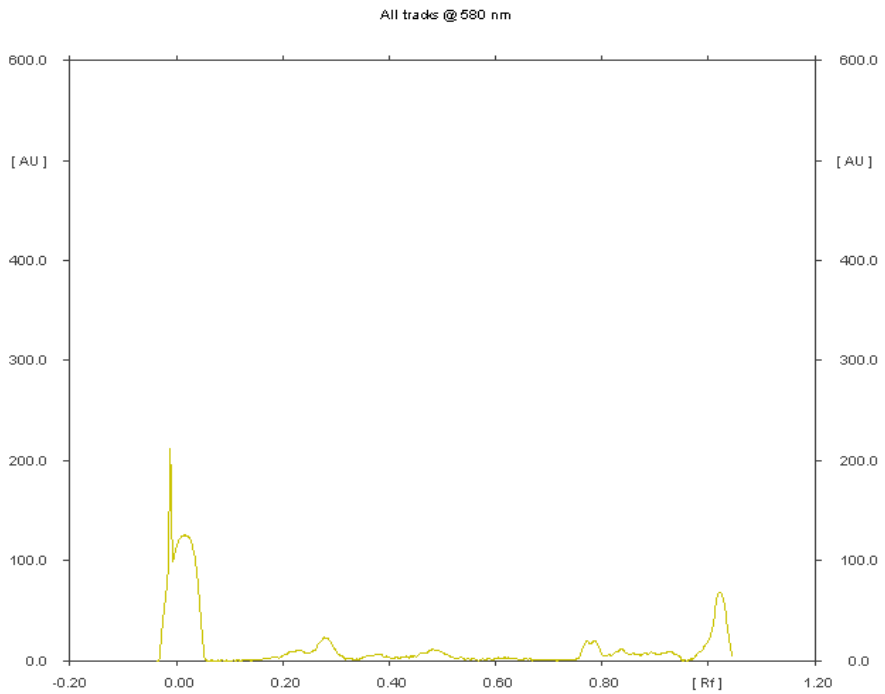

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Figure 2: Chemical Fingerprint of Aqueous Extract of *Jatropha tanjorensis* Leaves

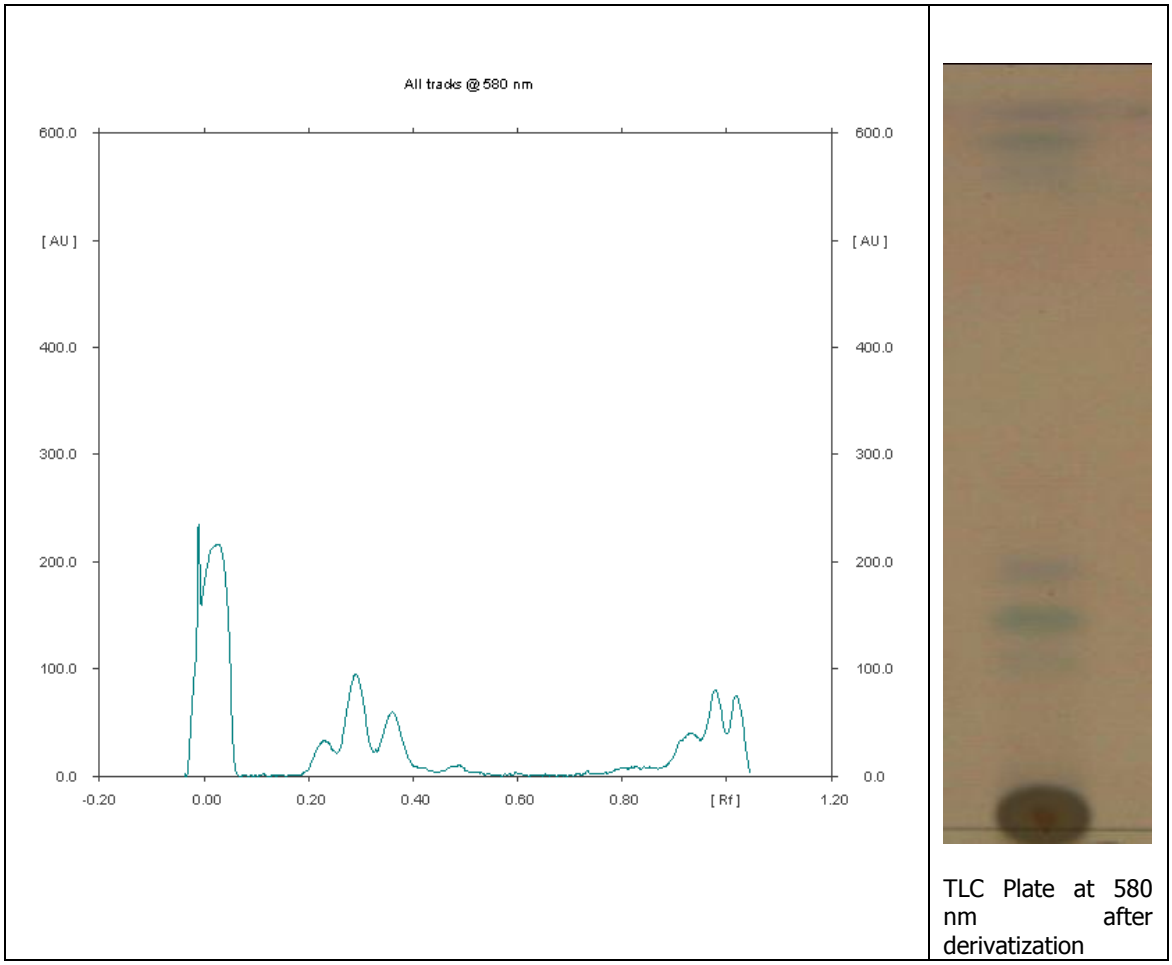

Figure 3: Chemical Fingerprint of Hydro-ethanolic (50:50) Extract of *Jatropha tanjorensis* Leaves

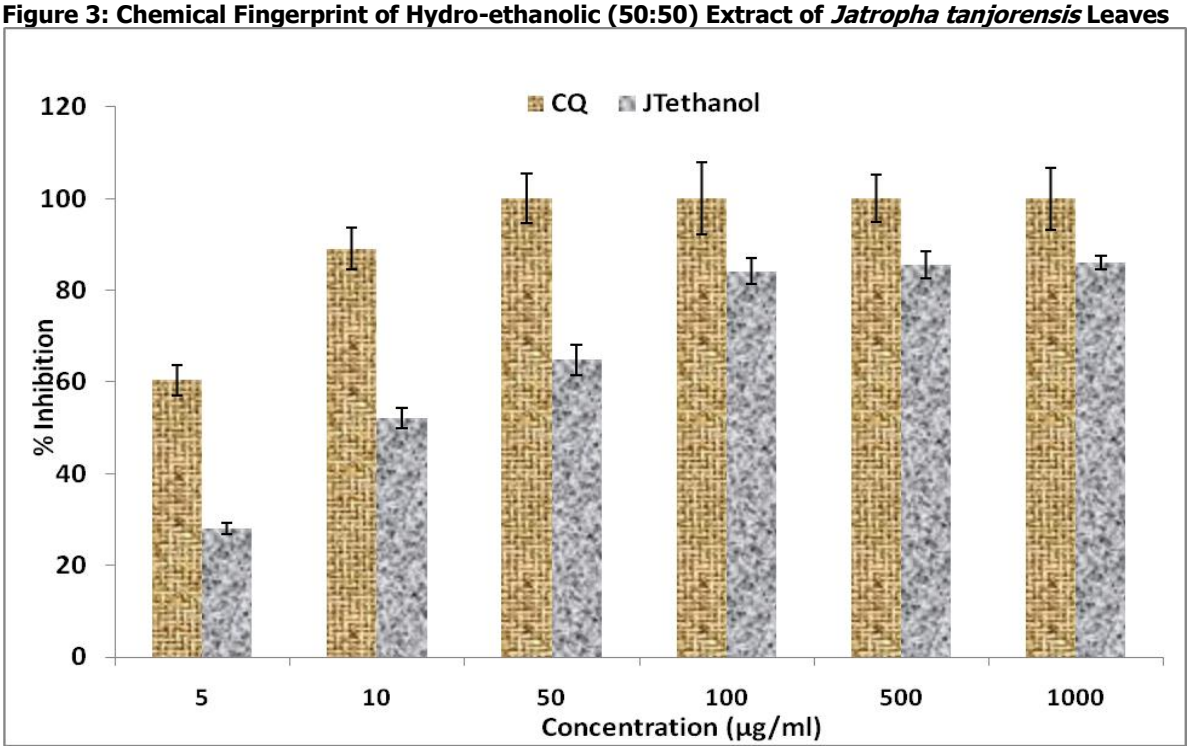

Figure 4: Effects of different concentrations of ethanolic extract of *Jatropha tanjorensis* and chloroquine on *in vitro* growth of *P. falciparum*

Values are Mean ± S.D. from two independent experiments performed in triplicates. $JT_{ethanolic}$ (*J. tanjorensis* ethanolic extract); IC_{50} chloroquine 0.087 µg/ml.

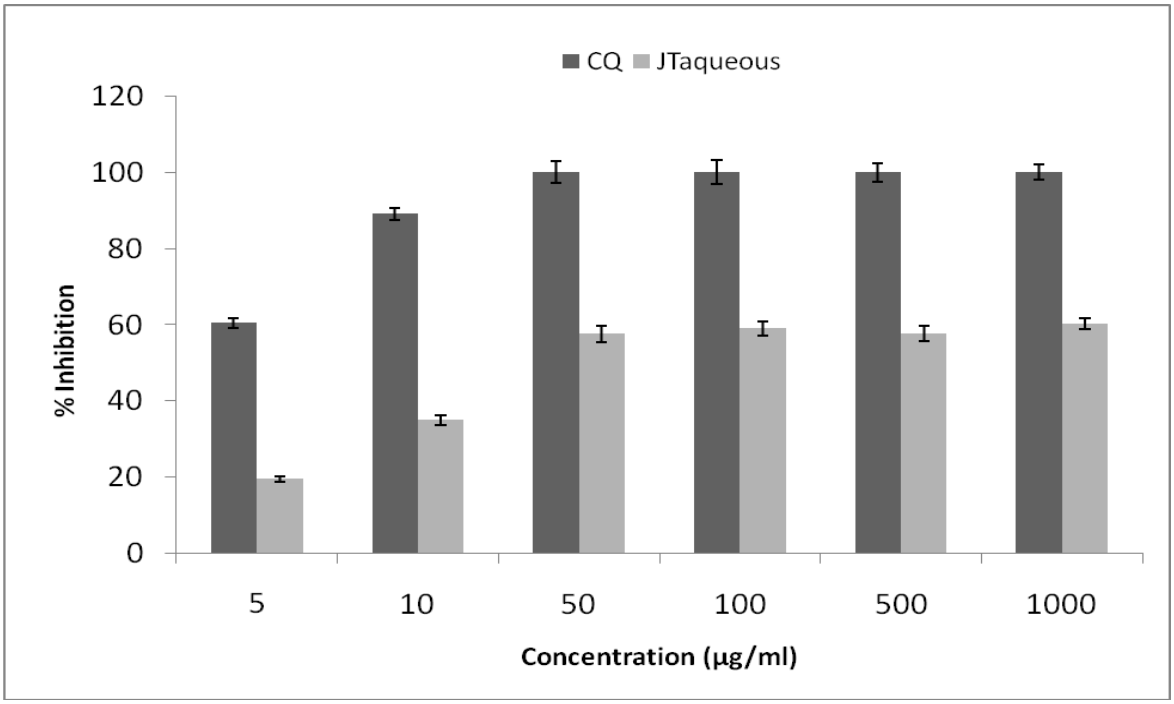

Figure 5: Effects of different concentrations of aqueous extract of *Jatropha tanjorensis* and chloroquine on *in vitro* growth of *P. falciparum*

All values are Mean ± S.D. from two independent experiments performed in triplicates. $JT_{aqueous}$ (*J. tanjorensis* aqueous extract); IC_{50} chloroquine 0.087 µg/ml.

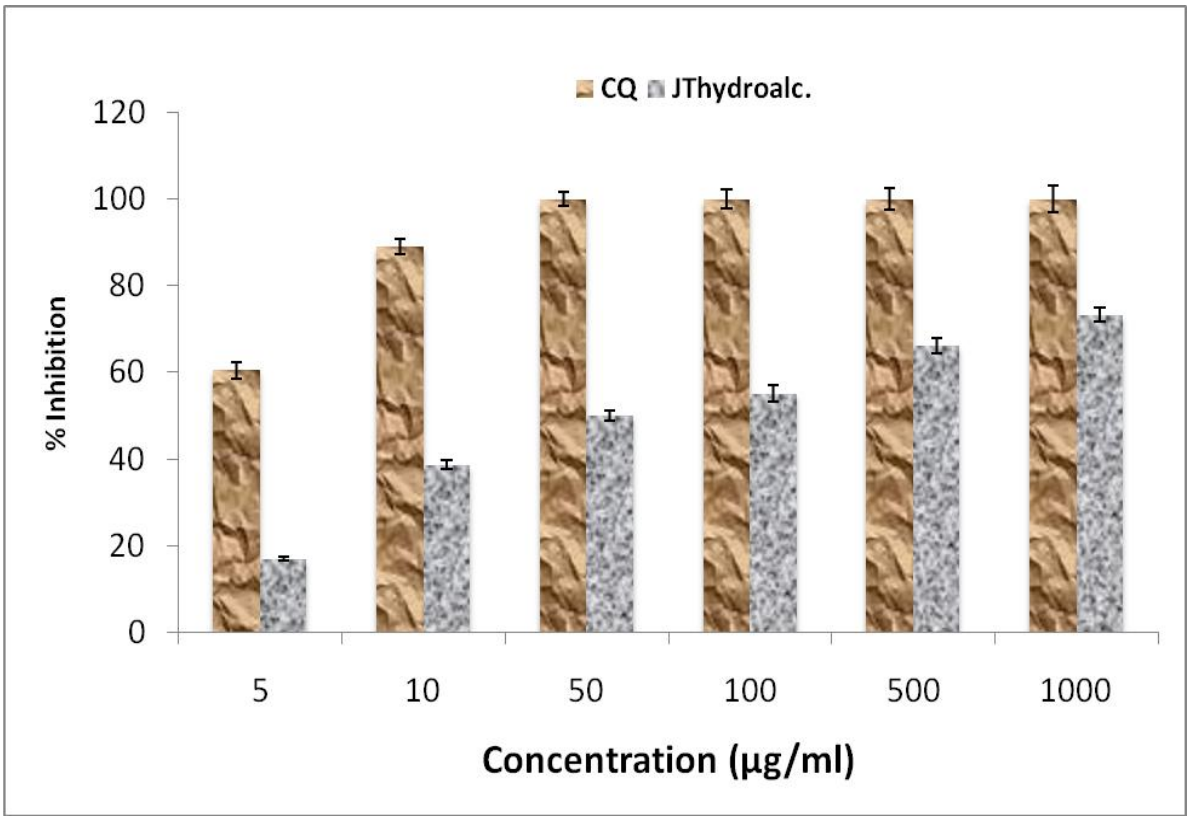

Figure 6: Effect of different concentrations of hydro-ethanolic extract of *Jatropha tanjorensis* and chloroquine on *in vitro* growth of *P. falciparum*

All values are Mean ± S.D. from two independent experiments performed in triplicates. $JT_{hydro\text{-}ethanol}$ (*J. tanjorensis* hydro-ethanolic extract); IC_{50} chloroquine 0.087 µg/ml.

Table 1: *In vitro* Antiplasmodial Activity, Cytotoxicity and Selectivity Index of various Leaf Extracts of *Jatropha tanjorensis*

Extracts	IC_{50}(μg/ml) Plasmodium	IC_{50} (μg/ml) Vero [a] Cells	Selectivity Index (SI) [b]
$JT_{ethanol}$	10.86 ± 1.52	86.8 ±4.8	8
$JT_{aqueous}$	44 ± 2.4	NC[c]	ND[d]
$JT_{hydro\text{-}ethanol}$	48 ± 1.34	547 ± 9.4	11.4

[a]Vero cell lines (C-1008) from African green monkey kidney fibroblast. [b] Selectivity Index = IC_{50} Vero Cells / IC_{50} Plasmodium. [c] Not cytotoxic at the highest test concentration of 1000 μg/ml. [d] Not determined. All values are Mean ± S.D. from two independent experiments performed in triplicates. $JT_{ethanol}$ (*J. tanjorensis* ethanolic extract), $JT_{aqueous}$ (*J. tanjorensis* aqueous extract); $JT_{hydro\text{-}ethanol}$ (*J. tanjorensis* hydro-ethanolic extract); IC_{50} chloroquine (standard reference drug) = 0.087 μg/ml; Doxorubicin hydrochloride (standard cytotoxic compound) = 1.8 ± 0.42 μg/ml.

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