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Full Length Research Paper

In vitro antiplasmodial activities of extracts from five plants used singly and in combination against *Plasmodium falciparum* parasites

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Fresh leaves of *Citrus limon*, *Psidium guajava*, *Carica papaya*, *Cymbopogon citratus and Vernonia amygdalina* were investigated. These plants are used singly and in combination in the traditional treatment of malaria in Nigeria. The dried leaves of plants were sequentially extracted with solvents of different polarities. We investigated the *in vitro* activities of selected extracts singly and when combined against the chloroquine sensitive and resistant strains of *P. falciparum.* Cytotoxic activities as well as the fractional inhibitory concentration of extracts were further evaluated. Most of the single extracts showed equipotent activity against both strains of the parasite. However, it was apparent that there were slight increases in parasite survival in the resistant strain as compared to the sensitive strain. Interestingly, when the extracts were investigated in combination, we observed that the potency of most of the extracts was enhanced. In this study, *C. papaya* extract was demonstrated to enhance the activities of component extracts in the combination. The dominant effect of *C. papaya* activity could be traceable to its high selectivity index for the sensitive and the resistant strain of the parasites. We envisage that the escalating challenge posed by parasite resistance, to existing antimalarials could be averted by combination treatments.

Key words: *Citrus limon*, *Psidium guajava*, *Citrus papaya*, *Citrus citratus, Vernonia amygdalina, in vitro,* antimalarial, combination.

INTRODUCTION

Malaria remains one of the world's most debilitating infectious diseases (Prudencio et al., 2006). Forty percent of the world's population is at risk of infection in about 90 countries and in 2009 there were an estimated 225 million cases of malaria reported worldwide and an estimated 781 000 deaths (WHO, 2010). Globally, the two regions with the highest malaria transmission intensity are Oceania and sub Saharan Africa. The greatest brunt of the disease is felt in sub-Saharan Africa where more than 90% of morbidity and deaths occur (WHO, 2005). The most vulnerable population includes young children and pregnant women as well as non immune travellers/ immigrants. In sub-Saharan Africa, a child dies of malaria every 12 s (Snow et al., 2005). This death toll exceeds the mortality rate from AIDS and the situation has further been heightened due to concomitant infection of malaria and HIV. A child in sub-Saharan Africa (< 5 years) will experience on average between 1.6 and 5.4 episodes of malaria fever each year, and one in every five (20%)

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childhood deaths is due to the effects of the disease. In some regions, the situation is further aggravated due to relapsing liver-stage of *P. vivax* infections after treatment (Lin et al., 2011). Apart from the unacceptable loss of life, malaria also impacts significantly on economic development – Africa spends about US$1.2 billion dollars per year in malaria-related illnesses and mortality cost (Murray, 2006) and the disease robs African economies of US$ 12 billion a year. The WHO estimates that malaria decreases gross domestic product (GDP) by as much as 1.3% in countries with high disease rates (WHO, 2010).

Currently no effective vaccine against malaria is available but there are several candidates in development. Despite the progress made in malaria vaccine development there are several safety concerns with new candidates and since none is 100% efficacious, the reality of breakthrough infections (Mueller et. al*.,* 2005; Melissa et. al*.,* 2005) especially in immunocompromised individuals is real. Thus chemotherapy will remain the cornerstone of the multifaceted approach in malaria control, elimination and eradication in combination with available tools such as insecticide treated bed-nets and indoor residual spraying. Chloroquine (CQ) was the mainstay of malaria treatment for many decades, but development of drug resistance by the parasite led to therapeutic failure (Thanh et al., 2009). The replacement of CQ with Sulphadoxine-Pyrimethamine (SP) was short lived due rapid selection of resistant parasites (Scholte et al*.,* 2006; Willcox et al*.,* 2004; Talisuna et al*.,* 2003). Quinine is generally effective against CQ-resistant falciparum malaria, but its use is limited by its narrow therapeutic index and cardiotoxicity effects.

This lead to the adoption of combination therapy in malaria with the advent of artemisinin based combination therapy (ACT). The emergence of artemisinin resistance has raised concerns that threaten the potency of existing antimalarials (Bethell et al., 2011). The therapeutic effectiveness of artemisinins which have been the drugs of choice is limited by a number of factors such as short half-life, neurotoxicity, and low solubility which affects their bioavailability (Gordi et al*.,* 2004). Moreso, drug failure in candidates treated with artemisinin regimen has been recorded in some regions. A decrease in the sensitivity to artemisinin has now been confirmed in Cambodia (Anderson et al*.,* 2010; Noedl et al., 2008; Dondorp et al., 2009). Therefore, there is urgent need to protect and increase the available antimalarials by use of new well matched combination therapies.

Combination therapies have the potentials to delay or prevent development of resistance by parasites as well as to enhance the effectiveness and extend the useful therapeutic lifespan of components in combination. Combination therapies have been approved for other multidrug resistant infections, such as HIV and tuberculosis. Combination of compounds for the treatment of malaria has been recommended (White, 1998; WHO, 2001). In this study we explored the *in vitro* antiplasmodial activities of extracts from *Citrus limon*, *Psidium guajava*, *Carica papaya*, *Cymbopogon citratus* and *Vernonia amygdalina,* investigated singly and when combined.

Majority of the African populace depend on plants used by traditional healers. African medicinal plants have demonstrated diverse potentials as possible sources of promising antiplasmodial compounds (Saidu et al., 2000; Okokon et al., 2006; 2007; Philippe et al., 2007). Traditionally, the plants investigated in this present study are used in treating malaria in Nigeria. They are accessible, cheap, affordable, available and sustainable. However, research output on the scientific information concerning the safety, and efficacy of these plants naturalized in Nigeria, in the traditional treatment of malaria, using *in vitro* systems is lacking. Moreso, since the activity of same species of plants could vary due to soil composition, geographical and climatic conditions (Massotti et al., 2003; Angioni et al., 2006), It is necessary therefore, that the species that occur in Nigeria be tested for activity. In this study we have investigated the antiplasmodial and cytotoxicity properties of *C. limon*, *P. guajava*, *C. papaya*, *C. citratus,* and *V. amygdalina* naturalized in Nigeria.

C. papaya L. belongs to the family Caricaceae. The fruits, leaves and latex of this species are traditionally used in various places in the treatment of asthma, rheumatism, fever, diarrhea, boils, hypertension and to increase the production of milk in nursing mothers (Zakaria et al., 2006). The fruit and seed extracts of *C. papaya* have shown significant bactericidal activity against *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Shigella flexneri* (Emeruwa, 1982). The unripe fruit contains glycine, phenylalanine and tryptophan, which have shown antisickling properties (Igbal and Kazi, 1980). *C. papaya* has also shown hypoglycaemic effects (Olagunju et al., 1995).

C. citratus Staph belongs to the family Poaceae. It is commonly known as lemon grass. It has a wide application in the traditional medicinal practices in tropical Africa (Tortoriello and Romero, 1992) with a characteristic pleasant aroma in their herbal teas (Cheel et al., 2005). The essential oil of *C. citratus* is documented to have antibacterial, as well as antifungal, activity (Suhr and Nielsen, 2003). *C. citratus* is widely used in Brazilian traditional medicine to ameliorate nervous and gastrointestinal abnormalities (Melo et al., 2001). A further study documented its use in Brazilian folk medicine as a tea to relieve anxiety, and this was shown by the activity of the essential oils in a mouse model (Blanco et al., 2009).

C. limon L. Burm. f. belong to the family Rutaceae. They have a characteristic spiny shoot and conspicuously green leaves. The species is commonly used in Nigerian

traditional practices to treat various ailments. *P. guajava* L. belongs to the family Myrtaceae and is a native to tropical America. In South Africa, infusions or decoctions of these species are used traditionally to control or treat ailments such as diabetes mellitus and hypertension (Oh et al., 2005; Ojewole, 2005). The anti-inflammatory and analgesic effects of this species have been reported (Ojewole, 2006; Murugananda et al., 2001). In Brazil, the ripe fruit, flowers and leaves are traditionally used as a decoction to treat anorexia, cholera, diarrhea, digestive problems, dysentery, gastric insufficiency, skin problems, sore throat, ulcers, vaginal discharges and laryngitis (Holetz et al., 2002; Cybele et al., 1995). In West Africa, Latin America and the Caribbean, decoctions of these species are used in the treatment of diarrhea and problems associated with indigestion (Mitchell and Ahmad, 2006b).

V. amygdalina Delile, commonly known as bitter leaf, is a shrub or small tree belonging to the family Asteraceae. *V. amygdalina* is a popular African vegetable which grows in several parts of tropical and subtropical Africa (Oleszek et al., 1995; Farombi, 2003; Erasto et al., 2006). It is used to a large extent in tropical Africa for its culinary and medicinal purposes, it is also used in the traditional treatment of malaria, diabetes, diarrhea, veneral disease, hepatitis, gastrointestinal problems, skin disorders, cough, constipation and in the treatment of wounds (Bullogh and Leary, 1982; Akah and Ekekwe, 1995; Igile et al., 1995; John et al., 1995; Hamil et al., 2000; Otshudi et al., 2000; Kambizi and Afolayan, 2001; Ajebesone and Aina, 2004).

Traditionally, in Ethiopia the decoctions of *V. amygdalina* are used to control tick in cattle (Regassa, 2000). It is also used as an anthelmintic by a good percentage of livestock farmers in Nigeria (Nwude and Ibrahim, 1980; Kudi and Myint, 1999). In southwestern Uganda it is locally known as “omubirizi”, and is traditionally used for analgesia and in the treatment of malaria infections (Anoka et al., 2008). In Nigeria, it is locally known as “ewuru” in Yoruba, “onugbu /olugbu” in Igbo, and “shiwaka/chukwuaka” in Hausa (Ajebesone and Aina, 2004; Igoli et al. 2005).

Further studies documented its use in various folk medicines as an anthelmintic, antiprotozoal and antibacterial agent (Burkill, 1985; Tadesse, et al., 1993; Huffman et al., 1996a). The bitter pith from young shoots of *V. amygdalina* helps in the control of intestinal nematode infection when ingested by chimpanzees (Huffman, 2003). Hypoglycemic, antineo-plastic, antibacterial and antioxidant properties have been reported (Akah and Okafor, 1992; Izevbigie et al., 2004; Taiwo et al., 1999; Iwalewa et al., 2003). *V. amygdalina* has shown antiplasmodial properties (Masaba, 2000; Abosi and Raseroka, 2003; Tona et al., 2004). However *in vitro* antimalarial and cytotoxic properties with extracts from these plants when used in combination have not been reported.

MATERIALS AND METHODS

Collection of plants

Five plants, each from a different plant family, investigated in this study are commonly used by traditional healers in the diverse ethnic regions of Nigeria for the treatment of febrile illnesses and related ailments. Fresh leaves of plants were collected in June and identified by the Plant Science and Biotechnology Department of Abia State University. Voucher specimens PM/ABSU/06-32, PM/ABSU/06-52, PM/ABSU/06-63, PM/ABSU/06-72 and PM/ABSU/06-82 of the plants *C. limon*, *P. guajava*, *C. papaya*, *C. citratus and V. amygdalina* respectively were stored in the herbarium of the Plant Science and Biotechnology Department for future reference.

Plant extraction and preparation

The collected plants were air dried at ambient temperature (25 to 30°C) for 5 to 10 days. The dried plants samples were blended and each plant was sequentially extracted using petroleum ether, dichloromethane, ethyl acetate, methanol and water in accordance with the increasing polarity of these solvents. Each plant was exhaustively extracted in each of the five solvents. For a proper mixing plant material and the solvent were continuously shaken on a horizontal orbit shaker (Labcon, California, USA). The resultant mixture was filtered and the filtrate concentrated under pressure in a Büchi Rotavapor R-205 (Büchi Labortechnik AG Switzerland), at 24°C. Extracts were air dried in the hood and stored in - 20°C until use.

Parasite

The asexual erythrocytic stages of parasites were maintained in a continuous culture using a modified method of Trager and Jensen (1976). The chloroquine sensitive strain (D10) which was used for this experiment was donated by Dr A. Cowman, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia while the Chloroquine resistance strain (DD2) was derived from Indochina (Wiesner et al., 2001).

In vitro antiplasmodial assay of individual extracts against *P. falciparum* strains

The method used to measure parasite viability was the parasite lactate dehydrogenase activity (pLDH) method of Makler et al. (1993). Each extract was screened for *in vitro* antiplasmodial activity using the chloroquine sensitive (CQS) D10 strain. Antiplasmodial activities of extracts were investigated against the chloroquine sensitive (CQS) and chloroquine resistant (CQR) strain of *P. falciparum*. Extracts with *in vitro* activities of ≤10 µg/ml were selected. A stock solution of 2 mg/ml of extract was prepared in 10% methanol (MeOH) (in deionised water). This was further diluted in complete medium to attain a final concentration of 200 µg/ml and 1% MeOH. The stock solutions were prepared on the assay day. Chloroquine (CQ) (Sigma) was used as the standard reference drug (positive control). Two mg/ml stock of CQ (Sigma) was constituted in deionised water and further diluted in complete medium to a concentration of 200 µg/ml. Extracts were serially

diluted two-fold in complete medium up to a concentration of 0.195 µg/ml using a flat-bottomed 96-well microtitre plate (Greiner Bio-One). CQ was taken as a positive control drug and was tested at a starting concentration of 100 ng/ml or 1000 ng/ml for the sensitive (D10) and resistant (DD2) strains of *P. falciparum* respectively. Unparasitised erythrocyte (RBC) was added to column 1 (blank) which had no drugs, while parasitized red blood cells (pRBC) were added to columns 2 to 12.

The plate was gassed for 2 min (93% N_2, 4% CO_2 and 3% O_2) and incubated for 48 h. A final hematocrit and parasitemia of 2% was used for all experiments. Parasite growth in the wells containing different concentrations of extract was compared to control wells. The IC_{50} recorded in this study is the mean of 3 independent experiments.

Plate preparation for drug combination assay

The checkerboard method used in this combination experiment was adapted from Berenbaum (1978). The *in vitro* combination studies involved two extracts a and b. One extract (component a) with a predetermined concentration needed to inhibit parasite growth by 50% (IC_{50}) was kept constant, while the other extract also with a pre-determined IC_{50} (component b) was added at different concentrations to determine the antiplasmodial effect of the two extracts in combination.

The same experiment was repeated, this time with component b constant while component a was varied. The combination experiment used a 96-well microtitration plate. The control drug was chloroquine. The first column was the blank with complete medium and unparasitised erythrocytes (100 µl complete medium (CM) +100 µl RBC), while the second column contained complete medium with parasitized erythrocytes (100 µl CM + 100 µl pRBC). The wells in columns 4 to 12 contained 100 µl of complete medium. Wells in column 3 contained 100 µl of 50 µg/ml of component a in CM, except for the first duplicate rows (3A and 3B) which had the concentration of component a alone (200 µl).

The rest of the rows in duplicate (rows C to H) containing 100 µl of CM received additional 100 µl aliquots of 50, 25 and 5% of the IC_{50} of component b, respectively. Serial dilutions of these concentrations were made from columns 3 to 12 using a multichannel pipette with a volume of 100 µl transferred after resuspending thoroughly. The last 100 µl from column 12 was discarded. A volume of 100 µl pRBC was added to wells in columns 3 to 12 halving the drug concentrations. The final volume in each well was 200 µl.

Plates were gassed for 2 min (93%N_2, 4% CO_2 and 3% O_2) and incubated for 48 h, as carried out in the *in vitro* monotherapy experiment. Viability of parasites was measured using the parasite lactate dehydrogenase activity (pLDH) method of Makler et al. (1993). Plates were developed, read and analyzed using the same procedure as was carried out in the *in vitro* experiments with individual extract.

Statistical analysis and data evaluation

To ascertain the absorbance of each well from the *in vitro* antiplasmodial experiment, plates were read when the colour changes from yellow to purple, using a microplate reader at 590 nm. The percentage parasite survival and the concentration that inhibits the growth of parasites by 50% were determined by measuring the conversion of NBT by *P. falciparum*. This was achieved by analyzing the readings from the microplate reader using Microsoft Excel® 2002, and the IC_{50} value was determined using a non-linear dose response curve fitting analysis in Graph Pad Prism version 4.

***In vitro* cytotoxicity assay**

The cytotoxicity assay for extracts singly and when combined was carried out using the modified method described by Mossman (1983). The cell survival for each well was determined using a microplate reader at 540 nm wavelength. The IC_{50} values were given as a mean of 3 independent experiments.

The formula used in calculating the sum of the fractional inhibitory concentrations (FIC) is given below;

$$\frac{IC_{50}\ (\mu g/ml)\ \text{of extract (a) in combination}}{IC_{50}\ (\mu g/ml)\ \text{of extract (a) alone}} + \frac{IC_{50}\ (\mu g/ml)\ \text{of extract (b) in combination}}{IC_{50}\ (\mu g/ml)\ \text{of extract (b) alone}}$$

RESULTS AND DISCUSSION

In vitro antiplasmodial activity of extracts singly

The sequential extraction of each plant using the five solvents gave rise to five extracts with antiplasmodial activities as shown in Table 1. The chloroquine (CQ) used during this experiment showed an IC_{50} of 8.55 ± 2.81 ng/ml in the chloroquine sensitive (CQS) D10 strain while the chloroquine resistant (CQR) DD2 strain showed IC_{50} value of 98.5 ± 26.1 ng/ml.

With the exception of *C. papaya* which showed stronger activity in ethyl acetate extract, all others showed promising activity in the dichloromethane extracts. Poor activity was recorded for the water extracts with IC_{50} values >50µg/ml. A recent study reported the poor activity exhibited by the aqueous extract of *C. papaya* (Onaku et al. 2011) Similar results have been recorded in a previous study with 14 plants (Irungu et al., 2007). Bhat and Surolia (2001) recorded no activity of the water extracts of *C. papaya.* The petroleum ether extracts of the rind and pulp of the unripe fruit of *C. papaya* showed IC_{50} values of 15.19 µg/ml and 18.09 µg/ml respectively against FCK 2 (a local strain of *P. falciparum* from Karnataka state, India) (Bhat and Surolia, 2001). Their findings with petroleum ether extract compares well to the activity of petroleum ether extract from the present study (Table 1). Previous studies reported that compounds isolated from the methanol and ethanol fractions of *C. papaya* seeds (MCP1 and ECPI), respectively showed contraceptive activities in male albino rats (Nirmal et al., 2005). *C. papaya* ameliorates vaginal disturbances due to *Trichomonas vaginalis* (Calzada et al., 2007). It has been reported to show anti-inflammatory and immunomodulatory properties (Mojica-Henshaw et al., 2003). Previous studies have shown the potency and cost effectiveness of the fruit when applied topically in the treatment of chronic ulcers in Jamaica (Hewitt et al., 2002). Another study reported the use of the fruit from

Table 1. The *in vitro* antiplasmodial activity of the five plants extracted with the various solvents using the CQS D10 strain.

Plant botanical name	PET IC_{50} µg/ml	DCM IC_{50} µg/ml	EA IC_{50} µg/ml	MEOH IC_{50} µg/ml	H_2O IC_{50} µg/ml
C. limon	37.2	5.0	>50.0	12.0	>50.0
P. guajava	15.5	6.0	21.6	>50.0	>50.0
C. papaya	16.4	12.8	2.6	10.8	>50.0
C. citratus	9.1	7.6	12.1	15.9	>50.0
V. amygdalina	14.2	4.1	10.7	>50.0	>50.0

PET= petroleum ether; DCM=dichloromethane; EA= ethyl acetate; MEOH= methanol; H_2O= water

this species in the treatment of burns, as investigated using a mouse model (Gurung and Škalko-Basnet, 2009). *P. guajava* is commonly used to treat malaria and diarrhea in Nigeria. The aqueous stem bark of this plant showed IC_{50} values of 10 to 20 µg/ml against the D10 strain of *P. falciparum* (Nundkumar and Ojewole, 2002). In the present study, the activity of this plant in the petroleum ether, dichloromethane and ethyl acetate extracts were found to be 15.5, 6.0l, and 21.6 µg/ml, respectively (Table 1).

Previous studies reported that *P. guajava* species have promising antibacterial, antimicrobial (Coutino et al., 2001, Arima and Danno, 2002; Chah et al., 2006), and antifungal activity against *Arthrinium sacchari* M001 and *Chaetomiu funicola* M002 strains (Sato et al., 2000). Its potency against *Propionibacterium acnes* has also been reported (Qadan et al., 2005). The work of Mukhtar et al. (2004) has shown the potent hypoglycaemic activity of the water extract at a dose of 250 mg/kg. Anti-diabetic activity of ethanol fractions of this species has been reported in a further study (Mukhtar et al. 2006). Other researchers previously reported the hypoglycaemic activity in mice and humans (Cheng and Yang, 1983). An anti-hyperglycemic effect of the butanol-soluble fraction from *P. guajava* leaves was shown in mouse models with type 2 diabetes (Oh et al., 2005). *P. guajava* has shown antiplasmodial properties (Nundkumar and Ojewole, 2002).

In the present study, stronger antiplasmodial activities with IC_{50}s of <10 µg/ml were seen mostly in the dichloromethane extracts, except *C. papaya* which showed the highest activity in the ethyl acetate fraction. The greater activity in the dichloromethane and ethyl acetate extracts suggests the presence of active lipophilic agents.

The high activity recorded in the dichloromethane (DCM) extracts over extracts from other solvents, like water and methanol, was also reported by Koch et al. (2005). This activity could be traceable to the absence of tannins, polysaccharides and other water- soluble molecules which do not have antiplasmodial properties. This may help explain the inability of these polar solvents to effectively extract the active lipophilic constituents from these plants. The essential oils from *C. citratus* have demonstrated antioxidant and radical scavenger properties (Menut et al., 2000).

The chloroform/ethanol extract of *Vernonia* species recorded 57.9% growth inhibition of malaria parasites *in vitro* (Bidla et al., 2004). *Vernonia amygdalina* in this study showed IC_{50} values of 3.98 ± 1.21 µg/ml and 4.12 ± 0.39 µg/ml against the D10 and DD2 strains, respectively in the dichloromethane (DCM) extracts. This correlates well with the activity of *V. colorata* Drake, *V. myriantha* Hook.f. and *V. oligocephala* Gardner naturalized in South Africa which showed IC_{50} values of 4.7, 3.0 and 3.5 µg/ml, respectively against D10 (Clarkson et al., 2004). *V. amygdalina* leaves have previously been reported to show *in vitro* antiplasmodial activity (Masaba, 2000; Alawa et al., 2003; Tona et al., 2004). These reports further emphasize the antimalarial potential of *Vernonia* species. The hexane extract of *C. limon* was active against *Trichophyton mentagrophytes* and *Microsporum canis* (Johann et al., 2007). Previous studies on the flavonoids from *Citrus* species have described their anti-hemorroidal, anti-oxidant, anti-inflammatory and anti-lipid peroxidation properties (Galley and Thillet, 1993). The oil extracted from *C. limon* effectively killed mosquito larvae (Zayed et al., 2009). It has been recorded that most of the natural limonoids are from *citrus* plants of the family Rutaceae (Ohta et al., 1992). Further studies reported that limonin and nomilin are the most abundant *Citrus* limonoids and have shown anti-carcinogenic properties in rodent models (Karim and Hashinaga, 2002; Kelly et al., 2003). We are yet to come across literature data on the antiplasmodial properties of *C. limon*.

The IC_{50} values of the five selected extracts which had IC_{50} <10 µg/ml compare favourably with those reported for extracts of *Artemisia annua* (3.9 µg/ml) and *Azadirachta indica* (≤10 µg/ml), against *P. falciparum* (Maria do Ceu et al., 2002). Methanol, as well as

Table 2. *In vitro* antimalarial activity of the selected extracts on *Plasmodium falciparum* cultures and toxicity towards the CHO cell line.

Crude extract/Drug	Solvent	IC_{50} D10 (µg/ml)	IC_{50} DD2 (µg/ml)	IC_{50} CHO (µg/ml)	(SI) D10	(SI) DD2	RI
C. limon	DCM	5.01 ± 0.32	5.99 ± 0.39	247 ± 2.94	49.30	41.23	1.19
P. guajava	DCM	3.38 ± 1.16	4.60 ± 1.00	85.64 ± 2.14	25.33	18.61	1.36
C. papaya	EA	2.96 ± 0.14	3.98 ± 0.42	737.8 ± 0.28	249.25	185.37	1.34
C. citratus	DCM	6.85 ± 0.56	9.44 ± 1.02	331 ± 0.70	48.32	35.06	1.37
V. amygdalina	DCM	3.98 ± 1.21	4.12 ± 0.39	38.54 ± 0.97	9.68	9.35	1.03

IC_{50} values are given as the mean of three independent experiments. **Key**: **DCM** = Dichloromethane **EA** = Ethyl acetate, **SI** = selectivity index=Cytotoxic antiplasmodial ratio (IC50 CHO/IC_{50} *P.falciparum*, **RI** = Resistance index=IC_{50} DD2/IC50 D10, **CHO** = Chinese Hamster Ovarian cell line.

petroleum ether, extracts recorded activities of >10 µg/ml in most of the plants. In the present study, work with extracts with IC_{50} ≥10 was not taken further. Cytotoxic activities of selected extracts were determined on Chinese Hamster Ovarian (CHO) cells (Table 2). The *in vitro* antiplasmodial activity of the DCM extract of *C. citratus* was not significantly different from *C. limon* DCM extract. Similarly, there was no significant diffe-rence between the *in vitro* antiplasmodial activities of the DCM extracts of *V. amygdalina* and *P. guajava*. The strongest antiplasmodial activity was observed in *C. papaya* ethyl acetate extract, which also recorded a good selectivity index to the D10 and DD2 strains of Plasmodium parasite (Table 2).

In vitro antiplasmodial activity of extracts in combination

Combination therapy, which has been a strategy approved for other multidrug resistance infections such as HIV and tuberculosis, is widely recommended for malaria treatment (White, 1998; WHO, 2001). Over the past decades combination therapy has gradually replaced single drug treatment due to the rapid spread of drug resistance by *Plasmodium* parasites globally (Martinelli et al., 2008). Recently, artemisinin combination therapy (ACT) has been the main therapeutic treatment for malaria, but has been met with treatment failures in some regions. This emphasizes the urgent need for the development of new drugs and combination treatments. Work done by Iwalokun (2008) showed that *V. amygdalina* dose dependently enhanced the efficacy of CQ against *P. berghei*. This suggests that extracts of plant species may be good candidates in fighting resistance by parasites. In this study, five extracts selected from five different plants were investigated for antiplasmodial activity when combined. Several combinations of these plants were tested and this was confined to combinations of two extracts (Table 3). Each combination was tested with one component being kept constant while the other is varied and vice versa. Some combinations such as that in *C. Papaya* + *C. citratus* and *C. limon* + *P. guajava* do not show significant differences in their activity when either extract was kept constant and the other varied at different concentrations. This suggests that their activities were not dose dependent.

Combinations of *P. guajava* + *V. amygdalina* showed no enhancement of the activity of either component in combination. However significant enhancement of activity was observed between the extracts *C. papaya* + *C. limon* as well as *C. papaya* + *P. guava.* This suggests that some of the components in this combination may have acted synergistically (Berenbaum, 1978). In most cases increased activity was noted whenever *C. papaya* ethyl acetate extract was one of the components used in combination. The increase in activity tends to be more obvious whenever the *C. papaya* ethyl acetate was kept constant and the other component varied (Table 3). In this work *in vitro* antiplasmodial activities of ≤10 µg/ml were chosen for further investigation hence, combinations with activities ≤ 10 µg/ml were further tested against CQS (D10) and CQR strain (DD2) (Table 4). The results given are the mean of three independent experiments. The activities of these combinations showed no significant difference in the D10 and DD2 strains of *P. falciparum*, and the combinations were not cytotoxic to the CHO cell lines at the concentrations tested (Table 4).

Seven combinations showed promising *in vitro* antiplasmodial and cytotoxic activities. The strongest activity was recorded in the combination of the ethyl acetate fraction of *C. papaya* and the dichloromethane fraction of *C. limon*. This combination was not cytotoxic to the CHO cell lines and had a high selectivity index (Table 4). In order to ascertain the relationships between the selected combinations, their fractional inhibitory concentrations (FIC) were determined (Table 5) as described by Berenbaum (1978). This method was used initially for studying drug interactions with bacteria (Hall et al., 1993). However, the principles are easily applied to *P.*

Table 3. IC_{50} values (µg/ml) of two extracts in combination (a+b) when extract a is kept constant while extract b is the variable component using CQS D10 strain of *P. falciparium.*

Combination of extract a + extract b	50%a + 50%b	50%a + 25%b	50%a + 5%b
C. limon + *P. guajava*	3.39	3.91	4.17
P. guajava + *C. limon*	3.09	4.89	6.76
C. limon + *C. papaya*	2.73	4.47	4.4
C. papaya + *C. limon*	1.83	2.28	2.65
C. limon + *C. citratus*	4.22	5.62	6.02
C. citratus + *C. limon*	6.6	8.1	11.4
C. limon + *V. amygdalina*	24.26	25.17	30.83
V. amygdalina + *C. limon*	17.03	13.5	19.01
P. guajava + *C. papaya*	2.72	39.90	48.30
C. Papaya + *P. guajava*	2.31	2.59	6.32
P. guajava + *C. citratus*	9.68	10.02	10.23
C. citratus + *P. guajava*	6.08	10.30	13.89
P.guajava + *V. amygdalina*	>50	>50	>50
V. amygdalina + *P. guajava*	43.35	44.05	>50
C. Papaya + *C. citratus*	3.01	3.8	3.98
C. Citratus + *C. Papaya*	4.07	4.51	4.46
C. Papaya + *V. amygdalina*	8.87	15.84	16.03
V. amygdalina + *C. Papaya*	4.42	8.74	23.01
C. citratus + *V. amygdalina*	7.23	6.82	10.37
V. amygdalina + *C. citratus*	9.1	9.5	11.2

Table 4. *In vitro* activity of selected combination of crude extracts on Plasmodium falciparum D10 and DD2 strains and toxicity towards Chinese Hamster Ovarian (CHO) cell lines.

Extract a+b Each at 50 µg/ml	D10 IC_{50} (µg/ml)	DD2 IC_{50} (µg/ml)	CHO IC_{50} (µg/ml)	SI
C. limon +*P. guajava*	3.39 ± 1.85	3.78 ± 0.95	82.60	24.32
C. Papaya +*C. limon*	0.83 ± 0.56	0.86 ± 0.62	>100	ND
C.Papaya + *P. guajava*	3.71 ± 0.22	4.02 ± 1.12	>100	ND
C. Papaya + *C. citratus*	3.01 ± 0.55	2.95 ± 0.78	>100	ND
C. limon +*C. citratus*	5.01 ± 1.21	4.98 ± 1.86	>100	ND
C. citratus + *P. guajava*	5.01 ± 0.74	5.28 ± 1.03	>100	ND
C.citratus + *V. amygdalina*	4.43 ± 0.21	3.82 ± 0.72	>100	ND

NB a+b = extract a combined with extract b, IC_{50} values are given as the mean of 3 independent experiments. ND= Not determined. In this study the extract of interest for *C. papaya* which is the ethyl acetate extract, while dichloromethane was the extract of interest for the remaining four plants

falciparum (Fivelman et al., 2004). It has been further explored by other researchers including Canfield et al. (1995) and Fivelman et al. (2004)). The extracts whose activity was significantly enhanced in the combination experiment showed an FIC value of 0.76 (Table 5). This was recorded in the combination between the ethyl acetate extract of *C. papaya* and the dichloromethane extract of *C. limon*. When the sum of the FIC values is less than 0.5 this indicates a clear case of synergism while values between 1 and 0.5 indicate a low grade synergism (Gupta et al., 2002). Values between 0.5 and 2 are generally regarded as indeterminate, while those above 2 indicate anta-gonism (Gupta et al., 2002).

FIC values for the seven most active combinations are shown in Table 5. The combination between the ethyl acetate extract of *C. papaya* and the dichloromethane extract of *C. limon* is tending towards synergism while the others show either an antagonistic or

Table 5. The selected combinations and their effect when extract a is kept constant using the D10 chloroquine sensitive strain.

Combination a+b	IC_{50} of (a) in combination	IC_{50} of (b) in combination	FIC value
C. limon + *P. guajava*	3.39	4.97	1.99
C. papaya + *C. limon*	0.83	2.43	0.76
C.papaya + *P. guajava*	3.71	3.82	2.39
C. papaya + *C. citratus*	3.01	3.87	1.56
C. limon + *C. citratus*	5.01	5.63	1.85
C. citratus + *P. guajava*	5.01	6.26	2.58
C.citratus + *V. amygdalina*	4.43	9.61	3.09

indifferent effect

Conclusion

Of the plants investigated in this study, seven combinations showed promising antiplasmodial properties.The strongest activity was recorded in the combination of the ethyl acetate fraction of *C. papaya* and the dichloromethane fraction of *C. limon*. There was an enhancement of activity whenever *C. papaya* ethyl acetate is one of the extracts tested in combination. The fractional inhibitory concentration (FIC) of 0.76 recorded between the ethyl acetate extract of *C. papaya* and the DCM extract of *C. limon* suggests that, the components of these extracts may have acted synergistically. It is therefore recommended that researchers should exhaustively explore the *in vitro* properties of extracts in combination since it has the potentials of enhancing their therapeutic efficacy.

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