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Phytochemicals Analysis, *In vitro* Anti-plasmodium Activity of *Delonix regia* Bark and *Carica papaya* Leaf Extracts in Combination with Chloroquine

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: The effectiveness of treating malaria may be jeopardized by artemisinin-resistant parasites. Pharmaceutical companies have been using plants for a very long time to generate the prototype molecules needed to treat *Plasmodium* infections.
Objective: To evaluate anti-plasmodial potency of extracts of *Delonix regia* and *Carica papaya* when used in combinations with chloroquine.
Methods: The plants were collected from Homa-Bay County in Kenya, dried under shade to a consistent weight, and then milled into a fine powder. Organic solvents were then used for extraction. After phytochemical screening of the extracts was conducted, *In vitro* tests were done

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on the extracts against *Plasmodium falciparum*, with a starting concentration of 100µg/mL. To prepare the test concentration ranges, the extracts were serially diluted 2-fold in growth medium. In a sealed gas chamber, the assay plates were incubated at 37^0C for 72 hours with 3% O_2 and 4% CO_2 with N_2 as the balance. By comparing each well's absorbance to that of a well that contained the drug-free control, the number of parasites still present at each concentration of the test substance were ascertained. Plotting survival against concentration enabled the determination of the IC50 values by a non-linear dose response curve fitting.

Results: In this investigation, the extracts from *Delonix regia* showed significant synergism (based on IC50 shifts) with CQ for both the ethyl acetate and methanol extracts with the exception of the highest combination dosage. *Carica papaya* extracts were much improved by the presence of CQ; however, this was not consistently reflected in the corresponding CQ IC50.

Conclusion: The results of this study which revealed synergism between the study plants extracts and chloroquine, support the reasons why some traditional healers have started combining medicinal herbs with chloroquine to boost its potency.

Keywords: Anti-plasmodium; Delonix regia; Carica papaya; synergistic effects; In vitro.

1. INTRODUCTION

In tropical and subtropical regions of the world, malaria is the most common parasitic disease affecting humans and ranks first in terms of socioeconomic impact and public health significance [1]. Children under the age of five are at highest risk of illness and mortality. This parasitic disease remains a serious threat to public health even after multiple efforts to eradicate the disease have been put into place in recent years [2]. Out of the 249 million cases reported in 2022, 233 million (about 94%) were in African Region; approximately 50% of all cases were in Nigeria (27%), the Democratic Republic of the Congo (12%), Uganda (5%), and Mozambique (4%). Kenya experiences 10,700 fatal cases and 3.3 million new clinical cases of malaria each year, despite multiple well-coordinated control initiatives [3–5]. Outside of Africa, the parasite is equally widespread; reports indicate that 2% of malaria cases worldwide occurred in South-East Asia Region, with 66% of those cases occurring in India alone [6]. Chemotherapy is essential for treating infected individuals who make a substantial contribution to the *Plasmodium* infectious reservoirs [7]. Nevertheless, the potentially fatal *Plasmodium* has demonstrated the ability to become resistant to almost all anti-plasmodial medications currently in use [8], motivating researchers to concentrate on developing novel antimalarial drugs [9]. Plant extracts have the potential to not only provide clues on new antimalaria medications, but also to shift the focus of drug research from discovering new, potent molecules to combining already-used chemicals in an effort to increase their sensitivity [10,11]. Many studies have previously examined medicinal plants extracts in an effort to produce substitutes for standard antimalarial medications in various parts of the world. Consequently, a number of antimalarial compounds were discovered. These consist of lignans, alkaloids, quinines, sesquiterpenoids, quassinoids, limnoids, and coumarins. These secondary metabolites have a strong antimalaria effect on *Plasmodium* [12,13]. Drug-resistant malaria has been traditionally treated with combinations of standard medications [14], although Little is known about how herbal medications would be potent if administered in combination with antimalarials drugs [15]. Chloroquine, an inexpensive, safe and once effective antimalarial, was a pillar of 20^{th} century malaria eradication and control efforts [16]. However majority of sub-Saharan African nations stopped using chloroquine (CQ) in the 1990s due to the development of resistance to the drug [17,18]. This study assessed the antimalarial efficacy of extracts from *Delonix regia* and *Carica papaya* in combination with chloroquine.

2. MATERIALS AND METHODS

Delonix regia and *Carica papaya*, were collected from Homa-Bay County, Kenya in the month of April 2020. Identification of the plants were conducted by staff of the university of Nairobi herbarium. Voucher specimens: RO2019UON/002, and RO2019UON/003 for *Carica papaya*, and *Delonix regia* were assigned to the specimens respectively after which they were store at the University of Nairobi Herbarium.

2.1 Extraction of Plant Material

The plant samples were cleaned with distilled water and then dried to a constant weight under

shaded after which they were crushed into a fine powder using an electric blender. Then organic solvents of different polarity were used for extraction (hexane, ethyl acetate, and Methanol). As shown in Fig. 1 below, this was accomplished by immersing 150 g of each sample in 400 mL of the organic solvent for 72 hours at room temperature. The extract filtrate was subsequently dried using a rotary evaporator. The extract yields for methanol and ethyl acetate for *Carica papaya* were 7.3% and 5.8%, respectively, while those for *Delonix regia* were 8.31% and 6.81, respectively. The dried plant extracts (leaf and bark) were stored at -20°C until needed for use.

2.2 Phytochemical Screening of Extracts

Using the standard protocols, phytochemical screening of plant extracts was performed for each extract to identify the presence of different phytochemical [19-21].

2.2.1 Detection of alkaloids (mayer's test)

The test involved dissolving 1.36 g of mercury chloride and 5 g of potassium iodide in 60 ml and 10 ml of distilled water, respectively. Distilled water was used to mix and top off these two solutions to a volume of 100 ml. Plant extract (one milliliter) was put into a test tube. A milliliter of Mayer's reagent, potassium mercuric iodide solution, was then added to the test tube and gently shaken. The appearance of a light-colored, white, or cream precipitate signifies the presence of alkaloids.

2.2.2 Detection of Saponins (foam test)

A single drop of sodium bicarbonate was combined with fifty milliliters of aqueous plant extract. After shaking the mixture, it was allowed to standing for 3minutes. The formation of foam resembling honeycomb signifies the presence of saponins.

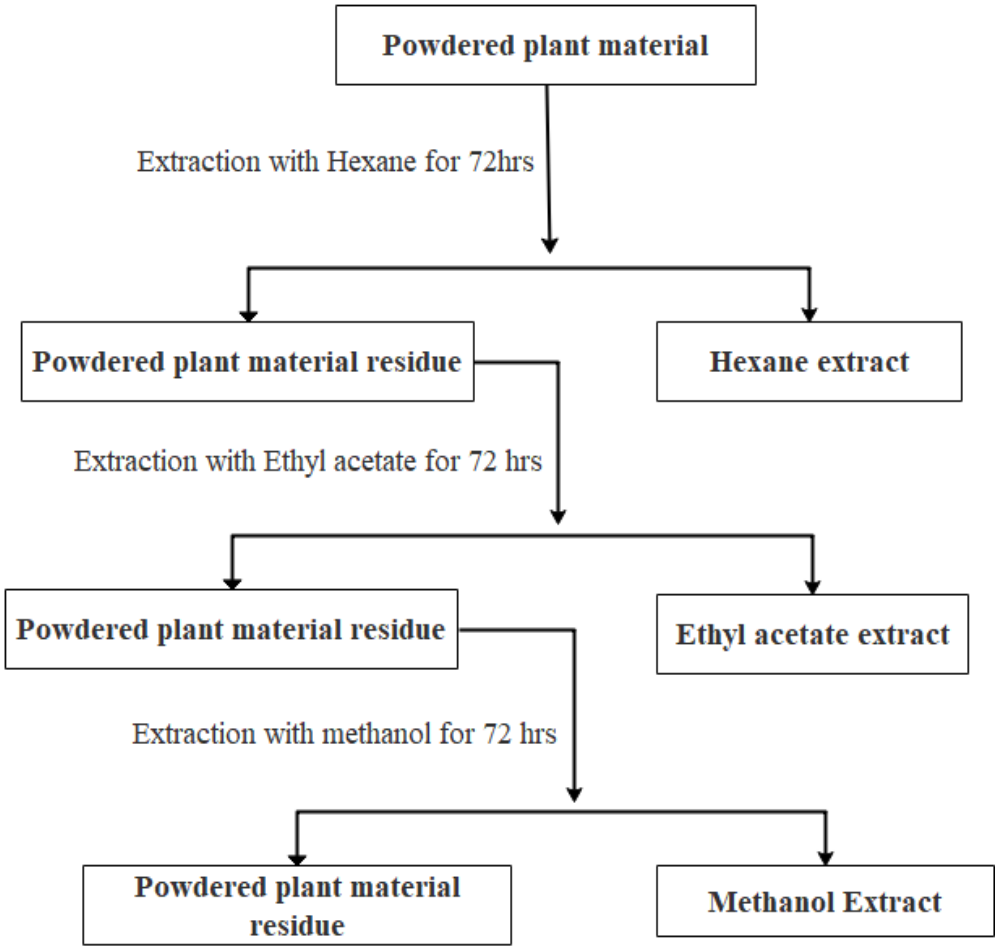

Fig. 1. Schematic representation of the extraction procedure of medicinal plants using organic solvent of different polarity at room temperature

2.2.3 Detection of tannins (lead acetate test)

Five grams of the plant extract were weighed, and ten milliliters of distilled water were added. The liquid was then stirred to dissolve the plant extract, and any remaining particles were filtered out. Next, 1% lead acetate solution was added to the filtered material. A yellow or red precipitate indicate tannins is present.

2.2.4 Detection of phenols (ferric chloride test)

One milliliter of an alcoholic plant crude extract solution was combined with two milliliters of distilled water in a sterile test tube. After that, 10% aqueous ferric chloride was added to the mixture. The presence of phenol is indicated by the development of a blue or green color upon the addition of ferric chloride.

2.2.5 Detection of Flavonoids (Shinoda's test)

Three milligrams of the plant extract were fully dissolved in five milliliters of ethanol. Ten drops of weak hydrochloric acid were then added, along with half a milligram of magnesium, and the mixture was allowed to boil for two minutes. A shift in color to a reddish-pink or brown hue signifies the existence of flavonoids in the plant extract being examined.

2.2.6 Detection of Terpenoid (Salkowskis Test)

In a test tube, 5 mg of plant extract, 2 ml of chloroform, and 3 ml of concentrated sulfuric acid were added dropwise to form a layer. The interface's reddish-brown coloration attests to the terpenoids' presence.

2.2.7 Detection of cardiac glycosides

Following a brief 3-minute hydrolysis of 5 mg of the extracts in a water bath with hydrochloric acid, the hydrolysate was subjected to the following tests.

2.2.8 Legal's test

The hydrolysate was made alkaline by adding sodium hydroxide, one milliliter of pyridine, and a few drops of a sodium nitroprusside solution. When glycosides are present, the pink color will change to red (pink to red color).

2.3 Preparation of Culture Reagents and Culturing of Malaria Parasite

2.3.1 Roswell Park Memorial Institute (RPMI) 1640 medium preparation

A standard procedure for *Plasmodium* cultivation was employed to prepare RPMI 1640 medium [22]. In a nutshell, 960 milliliters of demineralized autoclaved water were mixed with 10.4 grams of RPMI 1640 powder and 5.94 grams of HEPES, and the mixture was filtered through 0.2-micron pore size. The media was then dispensed into an aliquot portion of 100 milliliters and stored at 4 °C. Before using, 11 milliliters of human serum and 4.2 milliliters of a 5% $NaHCO_3$ solution were added.

2.3.2 Human Erythrocytes preparation

To prepare the erythrocyte for the assay, a 50 ml centrifuge tube was aseptically filled with 30 ml of human blood type O containing acid dextrose (ACD) anticoagulant, and the tube was then spun for 10 minutes at 660g. The cells were again resuspended in incomplete medium (RPMI 1640, which includes 25 mM $NaHCO_3$, 25 mM $NaHCO_3$, 25 mM HEPES, 20 mM glucose, 2 mM l-glutamine, and 5 µg/mL gentamycin) and centrifuged twice after the buffy coat and supernatant were removed. The cells were resuspended in an equal volume of complete medium with serum to give a 50% haematocrit and stored for a maximum of 4 days at 4 ^{o} C after this washing process was repeated twice with 2 volumes of incomplete media [23].

2.3.3 Human serum preparation

In order to maximize the blood clot's shrinking, human blood of the serotype o was collected, let to coagulate at room temperature for ninety minutes, and then stored at forty degrees Celsius for the whole night. To separate undesirable cells from serum, the liquid component was then aseptically removed, put in centrifuge tubes, and centrifuged for 10 minutes at 500xg. After that, the serum was collected, bottled, and kept at -20^{0}C [23].

2.3.4 Culturing of malaria parasite

To culture strains of wild-types chloroquine-sensitive strain (Nf54) of the human malaria parasite *Plasmodium falciparum*, *Plasmodium* strains were cultured in complete parasite medium (CPM: RPMI 1640 supplemented with 2

mM l-glutamine, 25 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), and 24 mM $NaHCO_3$ supplemented with 0.1 mM hypoxanthine, 25 g/mL gentamicin, and 0.5% AlbuMAX® I). AlbuMAX® I was used as a serum substitute. The technique used to cultivate the *Plasmodium* strains was modified Trager and Jensen's technique. 2% haematocrit [24]. In an incubator set At 37^0C, O+ RBC was utilized to keep the parasites alive. The incubator's internal air gases were kept at 5% CO_2, 3% O_2, and 92% N_2. In accordance with standard protocol for continuous malaria parasite culture, parasite cultures were diluted every 48 hours to a parasitaemia of 0.4% using pre-warmed complete medium and uninfected erythrocytes media changes. This prevented the parasiteamia from rising above 5% [23].

2.3.5 Determination of Parasitemia

The infected red blood cells were resuspended in 10 milliliters of previously heated complete medium after the culture supernatant was removed. A 1.5 mL micro-centrifuge tube containing 100 µL of the suspension was centrifuged continuously at 800 × g for one minute. The remaining cell pellet was then resuspended in the 30 µL supernatant after 70 µL of the supernatant was removed. After that, a microscope slide was coated with 5 µL of the suspension to create a thin smear. The slide was allowed to air dry, the cells were then fixed to it by dipping the side into 100% Methanol. Each slide was stained for fifteen minutes with Giemsa staining solution after it had died, rinsed with water, and allowed to air dry. The number of infected erythrocytes per 1000 erythrocytes was calculated using the X 100 objective, and the level of parasitemia was computed using the following formula.

% Parasiteamia = (Number of parasitized RBC)/(Total number of RBC counted)×100 [25].

RBC= red blood cells.

2.3.6 Synchronization with sorbital

Prior to the scheduled assay, sorbitol synchronization was carried out at 5% parasiteamia. To do this, the culture was centrifuged at 200 g for five (5) minutes. The resulting RBC pellet was then resuspended in 2.5 ml of aqueous 5% D-sorbitol and allowed to stay at room temperature for five (5) minutes. Finally, an equal volume of RPMI-1640 (complete medium) was added to the pellet. After that, uninfected RBC was added to restore the culture at the proper hemoglobin concentration [25].

2.3.7 *In vitro* assay of plant extracts to determine potency against malaria parasite

With some slight modification, the technique outlined by Trager and Jensen [23] was used to maintain cultures of *P. falciparum*'s asexual erythrocyte stages. Using the method outlined by Makler et al. (1993), which determines parasite viability colorimetrically using the breakdown of a dye by metabolic enzymes of the glycolytic pathway taking place in living parasites as a marker for survival, the quantitative assessment of anti-plasmodial activity *In vitro* was determined via the parasite lactate dehydrogenase (pLDH) method. The pLDH assay does not detect erythrocyte LDH since it is only specific for the enzyme of the malarial parasite. Its foundation is the principle that erythrocyte LDH cannot use 3-acetylpyridine NAD (APAD) as a NAD analogue, while malaria LDH can. Nitroblue tetrozolium (NBT), phenylethyl sulfate (PES), and Malstat reagent are the key assay reagents. The temperature of the reaction is set to 200C, and after about 20 minutes, each well receives 100 cc of 5% acetic acid to terminate the reaction. The absorbance is measured at 650 nM (A650) primarily because studies have demonstrated that it provides levels of parasitemia that are proportionate to the parasitemia found using the Giemsa-stained blood smear technique [26].

Briefly, small aliquots of the crude extracts were dried under nitrogen at 20°C for 72 hours. Samples of every dried extract were made into a stock solution containing 20 mg/mL of 100% dimethyl sulfoxide. The stock solutions were vortexed vigorously before use and stored in the refrigerator. If the samples weren't entirely dissolved, they were analyzed in suspension. In every experiment, the reference drug was chloroquine (CQ). To find the concentration that inhibits 50% of parasite growth (IC50–value), a full dose response was carried out for each preparation in a 96-well plate.Test samples were first examined at a concentration of 100µg/mL. To make the test concentration ranges, they were serially diluted twice in growth medium. For CQ a concentration of 1µg/mL was the starting concentration. For 72 hours, the assay plate was incubated at 37^0C in a sealed gas chamber with 3% O_2 and 4% CO_2 and N_2 as the balance.

Following a 72-hour incubation period, the assay plate's well contents were carefully reconstituted, and 15µL of each well was transferred to a duplicate plate that already contained 25µL of nitroblue tetrazolium solution and 100µL of Malstat reagent. After allowing plates to develop in the dark for 20 minutes, each well's absorbance was measured at a wavelength of 620 nM using a spectrophotometer. By comparing each well's absorbance to that of a well that contained the drug-free control, the number of parasites still present at each concentration of the test substance was ascertained. Plotting survival against concentration allowed researchers to determine the IC50 values. The Dotmatics software platform was utilized to perform a non-linear dose response curve fitting study.

2.3.8 Fixed ratio combination of plant extracts and their chloroquine potentiation effects

The pLDH assay, previously mentioned, was used to test combinations of preparations at various concentrations. Two-fold dilutions were carried after combinations were made just before use to ensure that both *extract*s and/or chloroquine were present at the maximum desirable concentration. To ascertain whether effects were additive or synergistic, IC50 values were obtained for each *extract* after the plates had developed during a 72-hour incubation period. These values were then compared to the IC50 of that *extract* when used alone.

3. RESULTS

3.1 Qualitative Phytochemical Screening of the Plant Extracts

The extract of *Carica papaya* and *Delonix regia* the presence of secondary metabolites such as alkaloids, flavonoids, polyphenols, tannins, and terpenoids during the phytochemical screening. Table 1 presents a summary of the findings from a qualitative investigation of the phytochemical content of *Delonix regia* and *Carica papaya*.

3.2 Baseline Anti-Plasmodial Activity of the Plant Extracts

All of the solvents' crude extracts showed some degree of activity against the malaria parasite *in vitro*, with IC50 values ranging from about 2 to 11 µg/mL (Table 2). *Carica papaya* extracts appear to be the most effective against the parasite. Chloroquine, the control drug, exhibited IC50 values of 10 nM and was comparatively very active.

Table 1. Qualitative phytochemical analysis of the plant extracts

Phytochemistry name	Test	*D. regia*	*C. papaya*
Glycosides	Legal test	-	+
Saponin	Foam test	-	-
Flavonoid	Shinoda test	+	+
Alkaloid	mayer's test	+	+
Triterpenoid	Salkowskis Test	+	+
Tannin	Lead acetate test	+	+
Phenols	Ferric chloride test	+	+

Key: + = present; - = Absent

Table 2. Anti-plasmodial activity of single plant extract and chloroquine

Extract	Sample Number	Test 1 IC50 (µg/mL)	Test 2 IC50 (µg/mL)	Mean	SEM°
DR_M	RO-02	4.910	3.945	4.428	0.482
DR_EA	RO-07	5.221	8.889	7.055	1.834
CP_M	RO-06	2.114	3.248	2.681	0.567
CP_EA	RO-08	1.616	2.421	2.019	0.403
CQ	H3D-006879-01-01	9.711*	10.469*	10.090*	0.379

*Anti-plasmodial activity of the crude Methanol and ethyl acetate extracts. Data shown are the mean of two evaluations carried out in duplicate. *nmol/L °Standard error on the mean.*
NB: DR = D. regia; CP= C. papaya. EA= ethyl acetate and M= Methanol

3.3 Chloroquine Potentiation Effects of the Study Plants Extracts

The predetermined IC50 of the plant extracts and chloroquine, respectively, were used to determine the quantities of both substances to be used in the test for the evaluation of the chloroquine potentiation effects of the plant's extracts. The potentiation effect of each study plant, as indicated by the IC50 shift, was then established by calculating the cumulative IC50 of the chloroquine-plant extract combination.

3.4 Chloroquine Potentiation Effects of *Delonix regia*

The concentrations of each extract and chloroquine in growth media were adjusted to be 20× the respective IC50 values for *D. regia* and chloroquine. Tables 3 and 4 illustrate the various combinations that were produced by adding fixed volumes of each preparation to a fixed volume of chloroquine. Each partner's IC50 in the combination was determined and is shown as a *extract* shift with respect to the extract's or CQ's IC50 alone. With the exception of the highest combination (9:1), The ethyl acetate and Methanol portions of the extracts showed a considerable synergism with CQ.

3.5 Chloroquine Potentiation Effects of *Carica papaya*

Table 5 indicates that the presence of CQ considerably increased the potency of extracts from *C. papaya*. The extract's IC50 was greater when used at higher ratios (9:1 and 7:3) than when used alone; there was also some improvement in the activity when CQ was added at 5:5, 3:7, and 1:9. However, these were not always reflected in the corresponding CQ IC50, with shifts usually not being significant based trends of shifts of CQ IC50. This implies that *C. papaya* has no effect on CQ's ability to combat the malaria parasite.

Table 3. Chloroquine potentiation effects of *Delonix regia* Methanol extract

Preparation	Extract:CQ	Extract IC50	CQ IC50	Extract shift IC50	CQ Shift IC50
DR_M (IC50 = 4.4) and CQ (IC50 = 0.01nM)	9:1	8.41	0.0027	1.87	0.27
	7:3	1.73	0.0045	0.38	0.45
	5:5	2.68	0.0053	0.60	0.54
	3:7	0.94	0.0048	0.21	0.48
	1:9	0.36	0.0065	0.08	0.65

IC50 of combinations of plant DR with chloroquine, each at extracts of 20X the parent IC50, showing the relative shift in the IC50 for each component of the combination.
NB: DR=D. regia

Table 4. Chloroquine potentiation effects of *Delonix regia* Ethyl acetate extract

Preparation	Extract:CQ	Extract IC50	CQ IC50	Extract shift IC50	CQ Shift IC50
DR_EA (IC50 = 7.0) and CQ (IC50 = 0.01nM	9:1	11.17	0.0018	1.60	0.18
	7:3	2.49	0.0036	0.36	0.36
	5:5	2.20	0.0032	0.31	0.31
	3:7	0.68	0.0023	0.10	0.23
	1:9	0.33	0.0042	0.05	0.42

IC50s of combinations of plant DR with chloroquine, each at extracts of 20x the parent IC50, showing the relative shift in the IC50 for each component of the combination.

Table 5. Chloroquine potentiation effects of *Carica papaya* Methanol and ethyl acetate extract

Preparation	Extract:CQ	Extract IC50	CQ IC50	Extract shift IC50	CQ Shift IC50
CP_M (IC50 = 2.7) and CQ (IC50 = 0.01nM	9:1	5.83	0.0024	2.24	0.24
	7:3	4.76	0.0076	1.83	0.76
	5:5	1.84	0.0069	0.71	0.69
	3:7	0.59	0.0051	0.23	0.51
	1:9	0.27	0.0093	0.11	0.93

Preparation	Extract:CQ	Extract IC50	CQ IC50	Extract IC50 shift	CQ IC50 Shift
CP_EA (IC50 = 2.0) and CQ (IC50 = 0.01nM	9:1	2.96	0.0022	1.48	0.22
	7:3	4.31	0.0094	2.16	0.94
	5:5	4.93	0.0070	2.47	0.70
	3:7	0.75	0.0110	0.37	1.10
	1:9	0.13	0.0054	0.07	0.13

IC50 of combinations of plant CP with chloroquine, each at extracts of 20x the parent IC50, showing the relative shift in the IC50 for each component of the combination. EA= Ethyl acetate= Methanol

4. DISCUSSION

A feasible and cost-effective approach to develop novel, highly effective antimalarial drugs is to utilize medicinal plants that have historically been used to treat malaria in Africa. These plants are easy to obtain, are believed to have minimal side effects, and may be less expensive [27], The main obstacle facing this strategy is that while traditional medicine has utilized a range of plants to treat malaria, there is a dearth of scientific research supporting the plants' efficacy when combined with conventional antimalarial medications. Even though chloroquine appears to have already failed in most parts of the world as a first line antimalarial, efforts are currently being made to reverse the parasite's resistance to this drug. Chloroquine is an inexpensive, fast-acting, and well-tolerated antimalarial that may be revived when combined with effective resistance reversers [28,29]. Records also show that some native African communities are already combining CQ with medicinal plants extracts to boost its efficacy [30]. In this investigation, the extracts from *Delonix regia* showed significant synergism with CQ for both the ethyl acetate and Methanol extracts with the exception of the highest combination dosage. While the reason behind *Delonix regia* inability to potentiate CQ at high dosages remains unclear, it may have something to do with the *extract* decreased solubility at such high concentrations. At the high ratios (9:1 and 7:3), *Carica papaya* extracts potency were much improved by the presence of CQ; however, this was not consistently reflected in the corresponding CQ IC50. This implies that *C. papaya* does not increase CQ's effectiveness in fighting the parasite. The findings of this study, however, generally support the reasons why some traditional healers have started mixing medicinal herbs with chloroquine to boost its potency. For instance, studies on the Madagascar plants *Mundulea antanossarum* Baill and *Ocotea racemosa* (Danguy) Kosterm have shown that they can overcome chloroquine resistance in both *In vitro* and mouse models [31].

5. CONCLUSION

Except for the highest combination, the extracts from *Delonix regia* demonstrated strong synergism with CQ for both the ethyl acetate and Methanol *extract*s. The inclusion of CQ at greater ratios somewhat enhanced the extracts of *Carica papaya*; however, this was not always reflected in the corresponding CQ IC50. This suggests that CQ's capacity to fight off the malaria parasite is largely unaffected by *C. papaya* extracts addition.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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