Original Research Article

In vitro anti-*Plasmodium*activity of combined extracts from *Toddalia Asiatica* and *Carica papaya.*

Abstract

Objective: To evaluate anti-plasmodial potency of extracts of *Toddalia asiatica* and *Carica papaya* when used in combinations.

Methods: The plants, were collected from Homa-Bay County in Kenya and dried under shade to constant weight then ground into fine powder. Extraction was then done using organic solvent. Extracts were tested against *Plasmodium falciparum* in vitro at a starting concentration of 100µg/mL, which was then serially diluted 2-fold in growth medium to generate the test concentration ranges. The assay plate was incubated at 37^0C for 72h in a sealed gas chamber under 3% O_2 and 4% CO_2 with the balance being N_2. The remaining population of parasites at each concentration of the test compound was determined by comparing the absorbance of each well to the absorbance of a well containing the drug-free control. Survival was plotted against concentration and the IC50-values were obtained using a non-linear dose response curve fitting analysis. Phytochemical screening of the extracts of the was also done.

Results: The study established that the plant extracts were reasonably active, yielding IC50 values between approximately 2 and 11 µg/mL. When the two plants extracts were used in combination with each other it was established that, addition of Carica papaya to *Toddalia**asitica* seems to improve its potency across most concentrations as reflected in the shift in IC50 of *Toddalia* . However, the opposite is true for *Carica*, Best results were however seen with higher ratios of the *Carica papaya* extract was paired with smaller levels of the *Toddalia* extract.

Conclusion: The shift in *Toddalia**asitica's*IC50 values indicates that adding *Carica papaya*extract to *Toddalia**asitica*extract generally seems to increase its potency.

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1 Introduction

Unicellular protozoans that cause malaria to humans are members of the *Plasmodium* genus [1]. *Plasmodium* is transmitted to humans beings by female anopheline mosquitoes[2,3].Malaria represents one of the most severe infectious diseases that affect humans. Because it is more common in less developed nations and significantly impedes socioeconomic growth, it presents therapeutic and economic challenges especially in third world countries where the disease is endemic. There were 247 million new cases of malaria reported in 2021, and an estimated 619 000 people died from the disease. In accordance with previous trends, African continued to carry the majority of the disease's burden in 2021, accounting for 95% of all malaria cases (234 million cases) and 96% of all fatalities (593 000). Children under five comprised the majority of the 80 percent of malaria mortality in the Africa[4]. Malaria morbidity and mortality are accompanied by treatment, control, and prevention expenses, creating a significant financial burden to people living in malaria endemic areas [5]. Antimalarial drugs constitute a key component of malaria control in Africa and other parts of the world. Currently, Artemisinin from a Chinese *artemisia annua* plant is considered the best treatment option for *Plasmodium* infection, however resistance to this new antimalarial drug has emerged, though the majority of the documented cases of Artemisinin-resistant variants of this parasite come from Southeast Asia[6,7].The development and spread of Artemisinin-resistant *P. falciparum* strains, remain a threat to the eradication of malaria. Since Artemisinin is a plant extract, enhanced research into the anti-plasmodial effects of plant metabolites is still crucial[8,9], to enable the development of new alternative safer and more effective antimalarial medications[10]. Plant extracts have the potential not only to provide clues for new drugs, but may also help to change

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the pattern of drug research from finding new potent compounds to blending chemical agents used currently with a view of enhancing drug sensitivity[11,12].Even though using herbs in combination is a frequent practice in traditional medicine, there are surprisingly few scientific research that explore the topic. It is important to determine scientifically whether combining two herbal extracts could result in therapeutic response that would be categorized as synergism [13,14]. Given the paucity of research on antimalarial medicinal plants especially when used in combinations as possible sources of effective and affordable pharmacotherapies against *Plasmodium*in Africa [15], this study attempted to find out anti-plasmodial potency of extracts from two Kenyan medicinal plants, namely, *Toddalia asiatica*and *Carica papaya*when used in combinations.

2 Materials and Methods

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2.1 Plants Collection and Extract preparation

Toddalia asiatica and *Carica papaya* plants, were collected from Homa-Bay County, Kenya. Voucher specimens of *Toddalia asiatica* and *Caricapapaya* were each deposited at the University of Nairobi herbarium under the identification numbers RO2019UON/001and RO2019UON/002, respectively. Distilled water was used to clean the collected plant samples before they were dried under shade to constant weight. Using an electric blender, plant parts were ground into fine powder. Extraction was then done using organic solvent of different polarity (Hexane, Ethyl acetate and Methanol). This was done by soaking 150 g of each sample in 400 mL of the organic solvent for 72

hours, as illustrated in Figure 1 below. Drying of the extract filtrate was then done using rotary evaporator. The dried plant extracts were preserved at -20°C until use.

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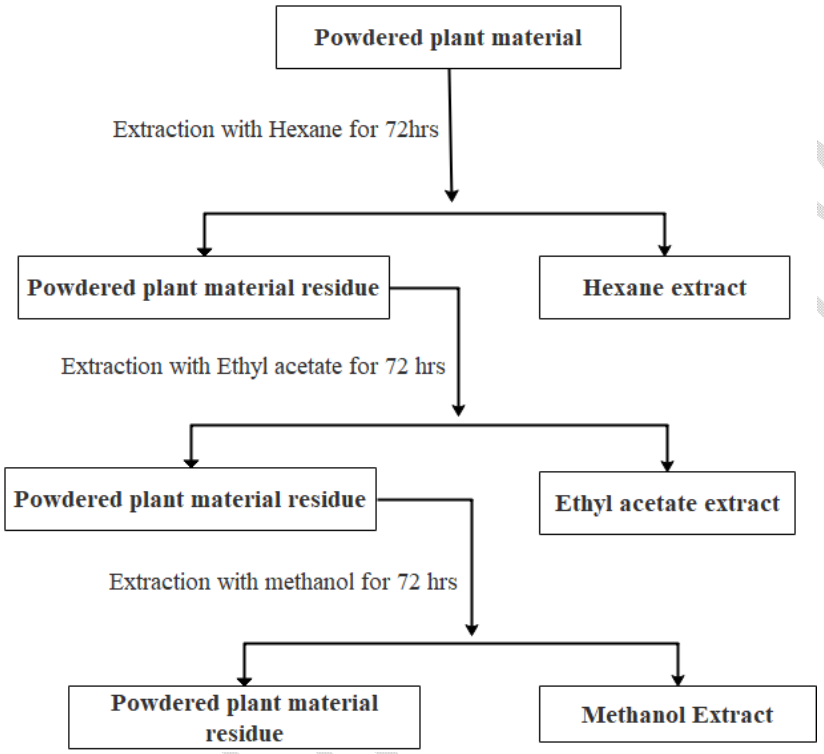

Figure 1 Diagram illustrating the process of extracting medicinal plants extracts using organic solvents.

2.2 Phytochemical screening

Phytochemical screening of plant extract to determine the presence of particular phytochemical constituents was carried out for all the extracts using the standard procedures[16–18].

2.2.1 Detection of alkaloids (mayer's test)

60 milliliters of distilled water were used to dissolve 1.36 grams of mercury chloride and 5 grams of potassium iodide, respectively. Using distilled water, these two solutions were combined and topped off to a volume of 100 ml. One milliliter of plant extract was put into a test tube, and to ensure proper mixing, one milliliter of potassium mercuric iodide solution (Mayer's reagent) was added and the test tube was gently shaken. The emergence of a light-colored, white, or cream precipitate signifies the existence of alkaloids.

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2.2.2 Detection of Saponins (foam test)

50 milliliters of aqueous plant extract were mixed with a single drop of sodium bicarbonate. After shaking the mixture, it was left to stand for 3 minutes. The formation of foam resembling honeycomb signifies the existence of saponins.

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2.2.3 Detection of tannins (lead acetate test)

Weighed five grams of the plant extract were added to ten milliliters of distilled water, mixed to dissolve, and then filtered to get rid of any remaining particles. The filtered material was then mixed with 1% lead acetate solution. The presence of tannins is indicated by the formation of yellow or crimson precipitate.

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2.2.4 Detection of phenols (ferric chloride test)

One milliliter of an alcoholic solution containing crude plant extract was mixed with two milliliters of distilled water. The mixture was then mixed with 10% aqueous ferric chloride. The appearance of blue or green color following the addition of ferric chloride signifies the existence of phenol.

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2.2.5 Detection of Flavonoids (Shinoda's test)

5ml of ethanol was used to fully dissolve 3mg of the plant extract. After adding 0.5 of magnesium and ten drops of weak hydrochloric acid, the liquid was allowed to boil for two minutes. A shift in color to a reddish-pink or brown signifies the existence of flavonoids in the plant extract being examined.

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2.2.6 Detection of Terpenoid (Salkowskis Test)

A layer was created by combining 5 mg of plant extract with 2 ml of chloroform and 3 ml of concentrated sulfuric acid, added dropwise. The interface's reddish-brown coloration attests to the terpenoids' existence.

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2.2.7 Detection of cardiac glycosides

Following a brief hydrolysis of a small portion of the extracts in a water bath with hydrochloric acid, the hydrolysate underwent the subsequent testing (Legal's test).

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).

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2.3 Parasite cultivation

Cultures of asexual erythrocyte stages of *P. falciparum* were maintained using the method described by Trager & Jensen (1976)[19], with minor modifications. The strain of *Plasmodium* that was employed in this study was D6, which is susceptible to chloroquine. The host cells used for the parasite cultivation were uninfected O^+ RBCs. The parasites were kept in RPMI-1640 media supplemented with 2 mg/mL $NaHCO_3$, 10 µg/mL hypoxanthine, 2 mg/mL glucose, 1 percent albumax II, and 10 µg/mL gentamicin. The culture was kept in a CO_2 incubator at 37 °C. Levels of parasitemia was determined and monitored using light microscopy technique

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2.4 In vitro anti-plasmodial assay

Quantitative assessment of anti-plasmodial activity *in vitro* was determined via the parasite lactate dehydrogenase (pLDH) assay using the method described by Makler et al (1993)[20], in which parasite viability is determined colourimetrically using the breakdown of a dye by metabolic enzymes of the glycolytic pathway taking place in living parasites as a marker for survival.The pLDH assay does not detect erythrocyte LDH since it is only specific for the enzyme of the malarial parasite. It is based on the principle that malaria LDH can utilize 3-acetylpyridine NAD (APAD) as an NAD analogue, whereas erythrocyte LDH cannot. This method was selected for this study because the pLDH assay's inhibitory profiles and IC50 are identical to those obtained when using the microscopic and radioactive uptake methods [20,21].

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Briefly to analyse the anti-*Plasmodium* activities of the plant extracts in this study, small aliquots of the crude extracts were dried under nitrogen at 20°C for 72h. Samples of each dried extract were prepared to a 20 mg/mL stock solution in 100% dimethyl sulfoxide, Stocks were kept refrigerated until use, and vortexed vigorously prior to use. Samples were tested as a suspension if not completely dissolved. The standard antimalarial drugs chloroquine (CQ) was used as the reference drug in all experiments. A full dose response was performed for all preparations in a 96-well plate to determine the concentration inhibiting 50% of parasite growth (IC50–value). Test samples were tested at a starting concentration of 100µg/mL, which was then serially diluted 2-fold in growth medium to generate the tested concentration range. The same dilution technique was used for all samples. CQ were tested from a starting concentration of 1µg/mL. The assay plate was incubated at 37^0C for 72h in a sealed gas chamber under 3% O2 and 4% CO_2 with the balance being N_2. After 72h, the wells contents in the assay plate were gently resuspended, and 15µL from each well was transferred to a duplicate plate containing 100µL of Malstat reagent and 25µL of nitroblue tetrazolium solution in each well. Plates were left to develop for 20 minutes in the dark and then absorbance of each well was quantified using a spectrophotometer at 620nM wavelength. The remaining population of parasites at each concentration of the test compound was determined by comparing the absorbance of each well to the absorbance of a well containing the drug-free control. Survival was plotted against concentration and the IC50-values were obtained using a non-linear dose response curve fitting analysis via the Dotmatics software platform.

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3 Results

3.1 Preliminary phytochemical screening

In the preliminary phytochemical screening, the extract of the two study plants showed the presence of secondary metabolites such as alkaloids, flavonoids, polyphenols, saponin, tannins, and terpenoids. The results of a quantitative analysis of the phytochemical content of *Toddalia asiatica and Caricapapaya,*are summarized in Table 1.

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Table 1 Qualitative phytochemical analysis of the plant extracts.

Phytochemistry name	***Test***	***C. papaya***	***T. asiatica***
Glycosides	Legal test	+	+
Saponin	Foam test	-	+
Flavonoid	Shinoda test	+	+
Alkaloid	mayer's test	+	+
Triterpenoid	Salkowskis Test	+	+

Tannin	Lead acetate test	+	+
Phenols	Ferric chloride test	+	+

-: Absent; +: Present

3.2 Baseline anti-plasmodial activity of the most reactive plant extracts

A baseline study was conducted to ascertain the potency of each plant's extract against the malaria parasite before the plants were subject to the combination assay. This test established that the plant extracts from all the solvents used were reasonably active against the parasite *in vitro,* yielding IC_{50} values between approximately 2 and 11 µg/mL Table 2. The extracts from the plant *Carica papaya* seem to be the most active against the malaria parasite. The control compounds chloroquine was extremely active and showed IC_{50} values of 10 nM.

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

Extract	Sample Number	Test 1 IC_{50} (µg/mL)	Test 2 IC_{50} (µg/mL)	Mean	SEM°
CP_M	RO-06	8.26	9.781	9.021	0.670
CP_EA	RO-08	1.616	2.421	2.019	0.403
TA_M	RO-09	9.509	12.768	11.139	1.630

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TA_EA	RO-10	9.767	10.431	10.099	0.332
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: TA= *T. asiatica* and CP= *C. papaya*. EA= ethyl acetate and M= Methanol

3.3 Combination studies for the plant extracts

The extracts*Toddaliaasistica* and *Carica papaya* were combined with each other in the ratios shown in Table 3 bellow, to determine whether or not they could be used synergistically to enhance their potency. Generally, addition of *Carica papaya* to *Toddaliaasitica* seems to improve its potency across most concentrations as reflected in the shift in IC50 of *Toddalia*. However, the opposite is true for *Carica*, Best results were however seen with higher ratios of the *Carica papaya* extract was paired with smaller levels of the *Toddalia*extract.

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Table 3. Toddalia asiatica*and* Carica papaya *combination best*

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Ratio *Toddalia*:*Carica*	*Toddalia* IC_{50}	*Carica* IC_{50}	*Toddalia* shift (vs 10.1 µg/mL)	*Carica* shift (vs 2.1 µg/mL)
1:1	**2.25**	**1.75**	0.22	0.83
1:3	**1.08**	**2.47**	0.11	1.18
1:4	**1.14**	**3.46**	0.11	1.65

1:5	**0.65**	**2.49**	0.06	1.19
3:1	**7.24**	**1.8**	0.72	0.86
4:1	**8.34**	**1.09**	0.83	0.52
5:1	**10.59**	**1.63**	1.05	0.78

4 Discussion

Every year, malaria claims the lives of about 500,000 individuals, mostly in Southeast Asia and Africa. The threat of *Plasmodium* parasite spread remains, despite significant advancements in malaria research in recent years. Reports of Artemisinin-resistant variants of this parasite have come from Southeast Asia, underscoring the pressing need to create safer and more effective antimalarial medications[22].The discovery of leads for the development of medications to treat human diseases has primarily involved natural products, and this suggests that new antimalarial leads may again come from tropical plants[23].Thus, using medicinal plants that have been used to treat malaria in Africa in the past is a realistic and affordable method for developing new potent antimalarial medications because they are easy to get, have few side effects, and might cost less [15],the main challenge to this approach is that though traditional medicine practitioners have employed a variety of plants for the treatment of malaria for many years, their effectiveness if used in combination has not yet received a lot of scientific attention as evidence by limited number of publications. For the purpose of creating novel, highly effective antimalarial drugs, it is vital to assess and document this information. In this study, the anti-*Plasmodium* effectiveness of two indigenous medicinal plant extracts, *Toddaliaasiatica* and *Carica papaya*, was examined when

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used both singly and in combination. When tested individually, the extracts were categorized as highly active, active, moderately active, and inactive at IC50 values of 5µg/ml, 5-15µg/ml, 15-50µg/ml, and > 50µg/ml, respectively, according to world health organization (WHO) criteria for categorization of anti-plasmodial activity [24,25]. With IC50 values ranging between roughly 2 and 11 g/mL, the crude extracts of the two study plants were considered effective against the malaria parasite *in vitro*. Ethyl acetate extracts of Carica papaya yielded the best mean IC50 value of 3.0 µg/ml. The results of this investigation align with other studies on *C. papaya* extracts, which have indicated a higher level of anti-plasmodial action, with an IC50 of 2.96 µg/ml. As documented in previous studies [26],the ethyl acetate extract treatment greatly slowed the parasites' ability to proliferate, this findings substantiated the usage of leaf decoctions of *Carica papaya* in some regions of Indonesia, India, Latin America and Africa to treat and prevent malaria [27–29]. For example, Nefang is a multi-herbal remedy used in Cameroon to cure malaria. It is made up of several plant extracts [30]. Carica papaya's potency, as shown by this study's findings, explains why it has been added to Nefang.*Toddalia asiatica* with an average IC50 of 10 µg/ml when tested against D6 strain of *Plasmodium* can be considered moderately active. The findings of this studies is almost similar to other studies on crude extracts of *T. asiatica* against on*Plasmodium falciparum* which revealed an IC50 value of 12 µg/ml [31],the impacts of the geographic factors from where the plants under examination were obtained can be used to explain the small discrepancy in IC50 values between the two studies[32] .

Drug combination therapy has many benefits over monotherapy, such as potentiation and synergism[33].Considering the drug-interaction assay results of this study, most

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combinations of Carica papaya and *Toddaliaasitica* had synergistic effects. The shift in *Toddaliaasitica's* IC50 indicates that adding Carica papaya to *Toddaliaasitica* generally seems to increase its potency across most concentrations. For Carica, on the other hand, the opposite is true: the best outcomes were obtained when larger ratios of Carica papaya extract were combined with lower concentrations of *Toddalia* extract. It appears therefore that adding Carica papaya to *Toddalia*asitica generally increases its potency across most concentrations, based on the shift in the IC50 of *Toddaliaasitica*.This potentiation/synergism effects of *Carica papaya*extracts has also been reported in other studies[34] . The results of this investigation and other related studies show that the two plants extracts might be taken into consideration for formulation into novel cheap affordable polyherbal antimalarial in future especially if used in combination.

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5 Conclusion

In conclusion, this study has shown promising anti-plasmodial activity for the treatment of malaria using the two study plants extracts when used in combination. Traditional healthcare users' practice and usage of mixed medicinal plant extracts in the therapy of malaria is remotely explained by the improved efficacy of combinations of plants extracts in this study.The shift in *Toddaliaasitica's*IC50 indicates that adding Carica papaya to *Toddaliaasitica* generally seems to increase its potency across most concentrations. For Carica, on the other hand, the opposite is true: the best outcomes were obtained when larger ratios of Carica papaya extract were combined with lower concentrations of *Toddalia* extract.

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