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Antiplasmodial Activity of *Combined Bark Extracts* of *Vernonia amygdalina, Mangifera indica,* and *Carica papaya at a 1:1:1 Ratio* in a Murine Malaria Model

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ABSTRACT

Background: Malaria remains a significant global health burden, exacerbated by the rising prevalence of drug-resistant *Plasmodium* strains. This critical public health challenge highlights an urgent need for novel anti-malarial therapies, with traditional herbal medicine offering a promising avenue for drug discovery. This study evaluated the antiplasmodial efficacy of bark extracts from *Vernonia amygdalina* (BB), *Mangifera indica* (MB), and *Carica papaya* (PB), investigating both individual treatments and polyherbal combinations in an experimental parasitic infection model.

Methods: Parasitaemia was established in mice, followed by a 5-day curative treatment initiated on day 4 post-infection. Mice were administered 400 mg/kg of each extract, 10 mL of distilled water (negative control), or 5 mg/kg of Chloroquine (CQ) as a positive control. Parasite clearance was assessed on day 9 post-infection.

Results: Our findings demonstrated synergistic antiplasmodial activity in the polyherbal combinations for both aqueous and ethanol extracts, outperforming individual extract treatments. Notably, the triple ethanol combination (BB+MB+PB) exhibited the highest parasite clearance at 89.71%, closely approaching the standard drug CQ (97.89%), representing only an 8.18% difference in efficacy.

Conclusion: These results offer significant insights into the therapeutic potential of these plant extracts, particularly when combined, advocating for further dose-response investigations into their collective anti-malarial properties.

Keywords: Antiplasmodial, bark, *Carica papaya, Mangifera indica, Vernonia amygdalina*

1. INTRODUCTION

Malaria, an endemic tropical disease, is a life-threatening sickness particularly in sub-Saharan Africa and Southeast Africa. The female Anopheles mosquitoes transmit the Plasmodium species from the infected human red blood cells to uninfected humans resulting in a spectrum of clinical signs and symptoms, often starting with a seemingly mild fever and can rapidly progress to life-threatening conditions such as cerebral malaria and death [1]. *Plasmodium falciparum* is known to be responsible for the majority of malaria-related morbidity and mortality [2]. Even though there are numerous drugs for the treatment of malaria, the increasing prevalence of drug-resistant Plasmodium strains, particularly to artemisinin-based combination therapies, has made it imperative for research into non-conventional interventions [3]. One such intervention is exploring the potential of plants with antimalarial properties. Phytochemicals have been traditionally deployed in various cultures for their medicinal properties [4], including antiplasmodial activity in many plant species [5][6]. For instance, *Mangifera indica, alternatively called mango,* has

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been investigated for its antimalarial activity, identifying potential active compounds such as mangiferin, flavonoids, and terpenoids [7][8]. Research suggests that *M. indica* extracts may inhibit parasite growth and development through various mechanisms [8]. Similarly, extracts from *Carica papaya, also known as pawpaw,* have demonstrated antiplasmodial effects [9]. Studies have investigated the *in vitro* antiplasmodial activity of *C. papaya* extracts, identifying potential active compounds such as alkaloids and flavonoids [10]. Computational studies have also explored the potential of *C. papaya* compounds to inhibit malarial proteins [11]. In the same vein, *Vernonia amygdalina,* commonly known as bitter leaf, has demonstrated significant antiplasmodial activity both *in vitro* and *in vivo* [12][13]. Studies have identified several phytochemicals, including sesquiterpene lactones that may contribute to its antiplasmodial effects [14]. Research suggests that *V. amygdalina* extracts may inhibit parasite growth and development through various mechanisms [15]. While there is evidence for the individual antimalarial properties of these plants, research in the combination of the three plant species in combating malaria is limited. The therapeutic potential of plant extracts and their constituents may be amplified through synergistic combinations, potentially overcoming drug resistance [16]. Specifically, research suggests that combining extracts from different plant species with diverse mechanisms of action against plasmodium strains may be particularly beneficial in the treatment of malaria and in combating resistance to a single extract or isolated compound. Therefore, this study aims to assess the *in vivo* antiplasmodial potential of combined aqueous and ethanol bark extracts of *Mangifera indica, Carica papaya,* and *Vernonia amygdalina* in *Plasmodium berghei*-infected mice using a dose-response approach. By exploring the synergistic interactions of these traditionally used medicinal plants, this research seeks to identify novel antimalarial combinations with enhanced therapeutic profiles. However, based on the already established antiplasmodial activities of the plants, the objective of this study is to evaluate the *in vivo* antiplasmodial activity of aqueous and ethanol bark extracts of the selected plants individually and in combination to ascertain the potential synergistic effects of the combined extracts. This study aims to contribute to the development of novel antimalarial therapies by exploring the potential synergistic effects of plant-based extracts, which are both cost-effective and accessible in malaria-endemic regions [17]. Combining extracts from different plants may enhance their efficacy and reduce the likelihood of resistance development [18].

2. MATERIALS AND METHOD

2.1 Materials

2.1.1 Biological materials

Stem bark samples of *C. papaya, V. amygdalina* and *M. indica, Plasmodium berghei* NK – 65, Swiss albino mice and a donor mouse.

2.1.2 Chemicals and Reagents

Chloroquine phosphate tablets 250 mg (Natco Pharma Limited, India), Polysorbate 80 (Biochemical grade, Tokyo Chemical Industry Company Ltd., Japan), distilled water (Prepared in-house via distillation/deionization), 95 % methanol (Reagent Grade, Fisher Scientific, USA), 3% Giemsa solution (pH 7.2, Sigma Aldrich/Merck, Germany), normal saline (Fisher Scientific, USA).

2.1.3 Equipment and other materials

Hammer mill (HM 200, Retsch, Germany), weighing balance, JA 3103 (Mettler Toledo, USA)), 2.5 L volumetric flask (Pyrex/Corning, USA), Whatman No. 1 filter paper (Cytiva, USA), rotary evaporator (Büchi Rotavapor R-3, Büchi Labortechnik AG, Switzerland), Labconco FreeZone 4.5 lyophilizer (Labconco, USA), thermometer (Fisher Scientific, USA), Haier thermocool refrigerator (HR-135A), polypropylene cages with filter tops (Tecniplast, Italy), cannula (Becton Dickinson (BD), USA), forceps (Fine Science Tools, Germany) and scissors (Fine Science Tools, Germany).

2.2 Methods

2.2.1 Plant extraction

Stem bark samples of *C. papaya*, *V. amygdalina*, and *M. indica* were rinsed under running tap water to remove surface debris and then air-dried for 24 hours to reduce moisture content. The air-dried bark was then pulverized using a hammer mill to a uniform particle size of 2 mm. For each plant species, 500 g of the pulverized bark was weighed and transferred to separate, sterile containers and 2.5 liters of sterile distilled water was added to each container. The mixtures were then macerated for 24 hours at room temperature (25 ± 2°C) with occasional stirring. Following maceration, the extracts were filtered through Whatman No. 1 filter paper and the filtrates were collected. The filtrates

were concentrated using a rotary evaporator Buchi Rotavapor R-3 at 40°C and reduced pressure until a semi-solid extract was obtained. The resulting extracts were then lyophilized using a Labconco Free Zone 4.5 lyophilizer to remove residual water. The lyophilized extracts were stored at -20°C until further use. A 1:1:1 ratio of the *M. indica*, *C. papaya* and *V. amygdalina* extracts was chosen for initial evaluation based on preliminary testing.

2.2.2 Animal grouping

Swiss albino mice (both sexes, 22–25 g) were used in this study. Prior to experimentation, the mice were acclimatized for seven days under controlled laboratory conditions (25 ± 2°C, 50 ± 10% relative humidity, 12-hour light/dark cycle). During acclimatization and throughout the experiment, the mice were housed in sterile, individually ventilated polypropylene cages with filter tops and they all had access to pelleted growers mash and sterile water *ad libitum*. All procedures involving animals were conducted in accordance with the University of Uyo Animal Care and Use Committee guidelines and were approved by the same committee. Humane care was provided, adhering to the principles outlined in the National Research Council's Guide for the Care and Use of Laboratory Animals [19].

2.2.3 Inoculation procedure

A donor mouse, previously infected intraperitoneally with the chloroquine-sensitive NK-65 strain of *Plasmodium berghei*, was used after microscopic examination confirmed the desired level of parasitaemia. The donor mouse was anesthetized in a chloroform-saturated environment, and infected blood was collected via cardiac puncture. A standard inoculum of 0.3 mL, containing 10^7 parasitized erythrocytes suspended in normal saline, was then prepared from this blood sample [20].

2.2.4 Treatment protocol

In vivo antiplasmodial assay was adopted in the experimental design. Mice (n=5 per group) were randomly assigned to one of eighteen treatment groups, nine for aqueous extracts and nine for ethanol extracts. Extracts were prepared in 10% Polysorbate 80 (Biochemical grade, Tokyo Chemical Industry Company Ltd.) and 400 mg/kg [21] administered via oral gavage. The groups were as follows:

Aqueous extract groups:

Group 1: *M. indica* aqueous extract, 400 mg/kg
Group 2: *C. papaya* aqueous extract, 400 mg/kg
Group 3: *V. amygdalina* aqueous extract, 400 mg/kg
Group 4: *M. indica* and *C. papaya* aqueous extracts (1:1), 400 mg/kg total dose
Group 5: *M. indica* and *V. amygdalina* aqueous extracts (1:1), 400 mg/kg total dose
Group 6: *C. papaya* and *V. amygdalina* aqueous extracts (1:1), 400 mg/kg total dose
Group 7: *M. indica*, *C. papaya*, and *V. amygdalina* aqueous extracts (1:1:1), 400 mg/kg total dose
Group 8: Chloroquine phosphate B.P 250 mg (positive control), 5 mg/kg
Group 9: Distilled water (negative control), 10 mL/kg

Ethanol extract groups:

Group 10: *M. indica* ethanol extract, 400 mg/kg
Group 11: *C. papaya* ethanol extract, 400 mg/kg
Group 12: *V. amygdalina* ethanol extract, 400 mg/kg
Group 13: *M. indica* and *C. papaya* ethanol extracts (1:1), 400 mg/kg total dose
Group 14: *M. indica* and *V. amygdalina* ethanol extracts (1:1), 400 mg/kg total dose
Group 15: *C. papaya* and *V. amygdalina* ethanol extracts (1:1), 400 mg/kg total dose
Group 16: *M. indica*, *C. papaya*, and *V. amygdalina* ethanol extracts (1:1:1), 400 mg/kg total dose
Group 17: Chloroquine phosphate B.P 250 mg (positive control), 5 mg/kg
Group 18: Distilled water (negative control), 10 mL/kg

2.2.5 Parasitaemia assessment

Thin blood smears were fixed with 95 % methanol and stained with 3% Giemsa solution (pH 7.2) for 60 minutes. Parasitaemia was determined by microscopic examination (100x oil immersion) following WHO guidelines [22]. *Plasmodium berghei* parasites were counted per 200 leukocytes, and parasite density (parasites/µL of blood) was calculated using appropriate formula [22]. Parasitaemia was assessed every other day from day 1 to day 5 post-infection. The percentage parasitaemia was calculated and compared between treated groups and the control group [23].

2.3 Statistical Analysis

Data are reported as the mean ± SEM of triplicate determinations in all groups. Statistical analyses were performed using GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, CA, USA). Paired t-test was used to compare differences between control and pretreated groups. One-way ANOVA was used to test for variation in pharmacological activity among the treatment groups. Post hoc comparison of means was performed by Bonferroni's post hoc test, and $p < 0.05$ was considered to represent a statistically significant difference between test groups.

3. RESULTS

The results of this study compare the antiplasmodial efficacy of CQ with aqueous and ethanol bark extracts of *Carica papaya, Vernonia amygdalina* and *Mangifera indica* both singly and in various combinations.

Table 1: Comparison of the antiplasmodial effect of chloroquine with aqueous bark extracts of *Carica papaya*, *Vernonia amygdalina* and *Mangifera indica* administered singly and in combination in mice after 5 days treatment.

Treatment Group	Baseline	Parasite Load					% Parasite Reduction	Minimum Survival Time
		Day 1	Day 2	Day 3	Day 4	Day 5		
CQ	134 ± 7.52	103 ± 5.21	64 ± 2.75	19 ± 3.33	6 ± 4.24	2 ± 0.85	97.89	29.75 ± 2.33
BB	122 ± 1.89	105 ± 3.06	82 ± 2.11	60 ± 2.26	39 ± 1.08	60 ± 0.88	44.82	9.61 ± 0.51
MB	125 ± 6.49	108 ± 1.18	81 ± 1.91	68 ± 1.03	47 ± 0.33	79 ± 0.67	45.80	11.16 ± 0.83
PB	112 ± 4.37	98 ± 3.67	85 ± 2.98	69 ± 1.33	57 ± 0.88	67 ± 2.60	40.18	10.40 ±0.98
BB+MB	116 ± 2.93	93 ± 3.11	78 ± 2.06	59 ± 3.37	58 ± 2.08	61 ± 6.16	56.31	13.51 ± 5.19
MB+PB	123 ± 3.79	95 ± 2.83	74 ± 2.45	50 ± 1.70	33 ± 1.11	46 ± 0.95	52.60	12.04 ± 3.38
BB+PB	114 ± 2.63	97 ± 2.06	71 ± 3.22	52 ± 4.21	39 ± 3.84	63 ± 1.67	54.74	13.03±6.89
BB+MB+PB	146 ± 1.93	98 ± 3.21	45 ± 1.13	23 ± 0.91	20 ± 0.33	28 ± 0.67	69.82	15.54 ± 1.76
Distilled water	130 ± 1.02	132 ±1.39	153±3.28	179 ± 1.75	203±2.67	230 ± 3.84	-77.45%	5.47 ± 0.75

MB = *Mangifera indica* bark, PB = *Carica papaya* bark, and BB = *Vernonia amygdalina* bark, CQ = Chloroquine phosphate

The aqueous extracts generally demonstrated moderate antimalarial activity, with individual treatments and various combinations showing initial parasite clearance but often experiencing parasite rebound by Day 5, resulting in modest parasite reductions (40.18 – 69.82 %) and survival times (9 – 15 days) compared to the negative control. The highest parasite reduction (69.82 %) among the treatment groups was achieved by the triple (BB+MB+PB) combination with a MST of 15.54 days.

Table 2: Comparison of the antiplasmodial effects of Chloroquine with ethanol bark extracts from *Carica papaya, Vernonia amygdalina*, and *Mangifera indica,* administered both singly and in combination to mice over a treatment period of five days.

Treatment Group	Baseline	Parasite Load					% Parasite Reduction	Minimum Survival Time
		Day 1	Day 2	Day 3	Day 4	Day5		
CQ	190 ± 3.14	123 ± 1.81	76 ± 2.56	41 ± 1.32	21 ±0 .78	4 ± 0.88	97.89	29.75 ± 2.33
BB	191 ± 0.87	180 ± 1.70	128 ± 1.72	89 ± 2.45	68 ± 2.80	60 ± 0.67	68.67	15.02 ± 2.03
MB	192 ± 1.27	155 ± 2.12	121 ± 2.33	67 ± 2.88	35 ± 1.11	21 ± 0.33	69.23	15.80±4.73
PB	194 ± 1.25	187 ± 1.23	136 ± 1.89	72 ± 3.07	47 ± 0.88	19 ± 0.36	62.33	14.64 ±1.23
BB + MB	186 ± 1.08	163 ± 2.58	122 ± 2.89	68 ± 2.67	34 ± 0.90	16 ± 1.53	81.40	16.51 ± 3.01
MB + PB	180 ± 0.48	130 ± 1.34	118 ± 2.63	69 ± 1.67	37 ± 0.29	27 ± 0.21	74.83	16.04 ± 2.63
BB + PB	193 ± 0.84	182 ± 1.66	152 ± 1.43	141 ± 2.00	69 ± 1.33	38 ± 5.28	71.23	16.03±3.78
BB+MB+PB	182 ± 2.31	131 ± 1.89	117 ± 2.07	58 ± 2.33	27 ± 0.33	19 ± 0.88	89.71	18.11 ± 0.91
Negative control	181±1.02	191 ± 1.39	214 ± 2.67	259 ± 1.75	275 ± 3.67	299 ± 3.84	-65.56	6.47 ± 1.23

KEY: BB= *Vernonia amygdalina,* MB=*Mangifera indica,* PB=*Carica papaya,* CQ = Chloroquine phosphate

The ethanol extracts consistently exhibited high antimalarial efficacy, with most individual and combination treatments achieving over 60 % parasite reduction and sustaining parasite suppression throughout the 5-day period, without significant rebound. This led to considerably longer survival times (14 – 15 days) for monotherapy (BB, MB, PB) and 16 days for dual combinations (BB+MB, BB+PB, MB+PB) compared to the negative control. The triple ethanol combination (BB+MB+PB) also showed excellent performance, achieving 89.71% reduction and an MST of 18.11 days.

4. DISCUSSION

Our findings provide compelling insights into the antiparasitic potential of *Vernonia amygdalina* (BB), *Mangifera indica* (MB) and *Carica papaya* (PB) bark extracts, both as monotherapies and in polyherbal combinations. A consistent observation across all treatment groups was the superior performance of ethanol extracts compared to their aqueous counterparts in achieving higher percentage parasite reduction and longer minimum survival times. This disparity is likely attributable to the differential solubility of bioactive phytochemicals in solvents of varying polarities; ethanol, being a more versatile solvent, is capable of extracting a broader spectrum of secondary metabolites, including those with known antiparasitic activities such as alkaloids, flavonoids, and terpenoids, which may be less soluble in water [24, 25]. Detailed analysis of individual plant extracts revealed distinct efficacies. The ethanol extract of *V. amygdalina* bark achieved a parasite reduction of 68.67 % (Table 1), significantly outperforming its aqueous counterpart (44.82 %) (Table 2). This observation aligns with existing literature reporting substantial anti-malarial activity from *V. amygdalina* leaves and root-bark; for instance, ethanolic leaf extracts have been shown to suppress parasitemia in mice by 63.70 % and 77.00 % at varying doses [26, 27], while root-bark extracts demonstrated 53.50 % suppression [28]. The rich phytochemical profile of *V. amygdalina*, including saponins, tannins, alkaloids, terpenoids, and flavonoids, is well-documented and contributes to its diverse medicinal properties, including its observed antiparasitic effects [29]. Similarly, the ethanol extract of *M. indica* bark yielded a 69.23 % parasite reduction, marginally superior to *V. amygdalina* ethanol extract and considerably more effective than its aqueous form (45.80 %). Previous research supports the anti-malarial potential of *M. indica*; aqueous stem bark extract has been reported to significantly suppress *Plasmodium berghei* multiplication and alleviate anemia in mice [30], and crude ethanol leaf extract demonstrated notable antiplasmodial activity against *P. berghei* in a dose-dependent manner [31]. The efficacy of *M. indica* bark is likely attributed to its abundant content of flavonoids, particularly mangiferin, and other phenolic compounds recognized for their antioxidant and anti-inflammatory properties, which may mediate antiparasitic actions. Among the individual extracts, *C. papaya* bark exhibited the lowest antiparasitic activity, with ethanol and aqueous extracts achieving 62.33 % and 40.18 % parasite reduction, respectively. While our study focused on the bark, *C. papaya* leaves are more extensively characterized for their anti-malarial properties, with studies reporting substantial parasite suppression often linked to the presence of alkaloids and other phytochemicals [32, 33]. Although bark studies are less prevalent, the broader medicinal applications of *C. papaya* imply that its bark also contains active compounds, albeit potentially in different concentrations or compositions than its leaves [34]. A pivotal finding of this study is the observed augmentation of antiparasitic activity in the polyherbal combinations when compared to individual extracts. All polyherbal ethanol combinations demonstrated improved antiparasitic activity compared to their single-extract counterparts. There was a significant improvement in the parasite clearance in the double combinations compared to monotherapy in both aqueous and ethanol extract groups. The aqueous double combinations (BB+MB, BB+PB, MB+PB) showed more effective and somewhat sustained daily parasite clearance (52.60 – 56.31 %) compared to individual treatment groups with the BB+MB combination proving to be the most promising within the category. A similar trend was also observed within the ethanol extract group with the double combinations (BB+MB, BB+PB, MB+PB) proving to be more effective, achieving a more sustained daily parasite clearance (71.23 – 81.40 %) with respect to the individual treatment groups. Again, BB+MB combination proved most promising within the category. There was also an improved MST (12 – 13 days) among the dual aqueous combination and about 16 days among the dual ethanol combination suggesting a more promising interaction compared to their respective monotherapies. However, the triple combination (BB+MB+PB) was notably superior in both aqueous and ethanol groups, achieving 69.82 % and 89.71 % parasite reduction respectively. Notably, the aqueous triple combination (BB+MB+PB) performed best among aqueous extracts (69.82 % parasite reduction and 15.54 ± 1.76 days survival time), though its efficacy remained lower than the ethanol triple combination and the standard drug (Table 1). Similarly, the triple combination of ethanol extracts (BB+MB+PB) exhibited the most potent effect among all herbal formulations, achieving 89.71 % parasite reduction with the longest survival time of 18.11 ± 0.91 days (Table 2). This level of efficacy represents a substantial improvement over the best performing individual ethanol extract (MB at 69.23 %) and the difference is statistically ($p \leq 0.05$) significant. The noticeable increases in parasite reduction and survival time in the

polyherbal combinations, particularly the triple blend, strongly suggest potential additive or synergistic interactions among the plant constituents. Synergism in herbal medicine, where the combined therapeutic effect of multiple compounds or extracts surpasses the sum of their individual effects, can manifest through various mechanisms, including multi-target action, improved bioavailability, modulation of resistance, and attenuation of adverse effects. Previous scholarly work on polyherbal anti-malarial formulations substantiates this concept, with numerous studies reporting superior efficacy compared to single-plant extracts [35, 36]. For instance, reviews on polyherbal anti-malarial formulations have indicated that combinations could serve as promising leads for developing new antiplasmodial drugs, with some formulations achieving over 50 % inhibition of parasitaemia [37, 38]. The notable performance of the triple combination in this study, particularly with ethanol extracts, aligns with these established findings and underscores the considerable potential of traditional polyherbal remedies. The side-by-side analysis of the result with the positive and negative controls clearly shows that chloroquine (CQ), serving as an effective positive control, achieved nearly complete parasite reduction (97.89 %), alongside a significant extension in survival time (29.75 days). While no single or binary herbal extract matched CQ's full efficacy, the ethanol triple combination (BB+MB+PB) surprisingly showed a promising 89.71 % parasite reduction. This represents only an 8.18 % difference compared to CQ's 97.89 %, indicating that this polyherbal blend, though not as potent as the gold standard, possesses substantial antiparasitic activity. Conversely, the negative control group consistently demonstrated parasite proliferation and minimal survival time, effectively validating the experimental model and confirming the active nature of the tested herbal extracts. Although the results of this study are promising, it is worthy to highlight on its major limitation. The study investigated a single concentration of extracts, necessitating future dose-response studies to establish optimal therapeutic concentrations and safety profiles.

5. CONCLUSION

The findings of this study provide compelling evidence for the antiparasitic potential of *Vernonia amygdalina*, *Mangifera indica*, and *Carica papaya* bark extracts, particularly when integrated into polyherbal combinations. The ethanol triple combination (BB+MB+PB) notably demonstrated substantial parasite reduction and prolonged survival time, closely approaching the efficacy of Chloroquine. This underscores the synergistic potential inherent in these traditionally utilized medicinal plants and highlights their promise as natural reservoirs for the development of innovative, effective, and potentially safer anti-malarial therapies. Further rigorous scientific investigation is unequivocally warranted to fully explore and leverage their therapeutic benefits.

DECLARATIONS

We declare that this is an original work and is not being considered for publication anywhere else.

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Conflict of interest

None to declare.

Contribution of the authors

Aniekan Ebong conceptualized, designed the work, analyzed the data and drafted the manuscript. Goodnews Emana, Festus Esenam conducted all experiments and handled daily monitoring of survivals. Victor Anah and Victor Attih provided reagents, materials and involved in the experimental analysis. Olorunfemi Eseyin revised and finalized the manuscript.

Ethics approval

This study was approved by the University of Uyo Animal Care and Use Committee in line with the Guide for the Care and Use of Laboratory Animals.

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