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Antimicrobial Activities and Malaria Parasite Clearance of Crude Extract of *Carica papaya* Seeds in Mice Infected with *Plasmodium berghei*

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ABSTRACT

Medicinal plants are known for a number of health benefits. This study investigated the *in-vitro* antimicrobial activities and *in-vivo* malaria parasite clearance rate using respectively the methanol and aqueous extracts of *Carica papaya* seeds. Soxhlet and Cold-maceration extraction techniques were used to obtain the methanol and aqueous extracts, respectively. The phytochemical constituents were quantitatively/qualitatively analyzed. The antimicrobial activities of the extracts were tested *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* using the agar-well-diffusion method. The minimum inhibitory concentration (MIC) of the extracts were determined using the agar-dilution method. Five groups of animals (mice) were used in this study, each having five animals each. The animals in group two to five were induced with *Plasmodium berghei* after which, those in group two, four, and five were treated with 25 mg/kg bw of artemether/lumefantrine and 200 and 400mg/kg bw of the plant aqueous extract respectively. Group three were not treated, while group one was neither induced nor treated. The phytochemical analysis showed presence/quantities of steroids, saponins, tannins, flavonoids, terpenoids, glycosides, reducing sugars, proteins, phenolic compounds, and alkaloids. With varying preliminary antimicrobial effects, the MIC of the methanol extract against *E. coli* and *S. typhi* were 62.5mg/ml and 125 mg/ml, respectively. The aqueous extract showed MIC of 125mg/ml against S. *aureus*. The antimalarial activity of aqueous extract increased in a dose-dependent manner. The aqueous extract at 400 mg/kg bw dose had a significant therapeutic ($P<0.05$) response. The study shows that the plant seeds have agents with both antimicrobial and antimalarial effects.

***Keywords*:** *Carica papaya*, Antimicrobial activity, Malaria parasite clearance, Extracts, *Plasmodium berghei* .

Introduction

The rapid in microbial resistance to traditional antibiotics has caused major concern in treating infectious diseases. In 2013, 9.2 million deaths were reported to have been caused by infections.[1] Antibiotics have grown less effective and some completely ineffective due to emerging and remerging resistant microorganisms. Currently, various strategies are being employed to combat this challenge. Combining other molecules with the failed antibiotics to restore the desired antimicrobial activity is one of the strategies and the use of plants and plants-based products remains a relevant option.

Plants and their various derivatives have been in use for both the prevention and treatment of different diseases since time immemorial. The oldest documentation on the use of medicinal plants to prepare medicine was found on a Sumerian clay slab from Nagpur; dating approximately 500 years back.[2] Various studies have also been conducted globally to ascertain the efficacy and toxicity of these plants, some of which have led to the discovery and development of some plant-based drugs including; digoxin, atropine, quinine morphine, artemisinin, colchicine, vinblastine, vincristine, tubocurarine, and morphine.[3] Medicinal plants contain a wide variety of secondary metabolites such as tannins, saponins, flavonoids, alkaloids, terpenoids, and phenols. These metabolites are greatly responsible for their therapeutic efficacy especially their antimicrobial properties. Many countries, especially the developing countries like the African states depend on traditional herbal medicine for their primary health care. This is due to their better cultural acceptability, easier accessibility, lesser side effects, better adaptability, and compatibility with the human body.[4] Also, the plants Phytochemicals have been shown potent antimicrobial activity and can be used alone or in combination with other antibiotics to boost antimicrobial activity against various microorganisms. Oftentimes, most of the therapeutic plants are not fully exploited due to inadequate knowledge if their potentials. Thus, the need for various plant studies in order to facilitate the discovery and development of newer agents to support the fight against antimicrobial resistance. *Carica papaya* commonly known as papaw or pawpaw is a succulent fruit produced by a huge plant belonging to the *Caricaceae* family. It originated from Central America, but is now grown in all tropical countries and many subtropical regions of the world. Studies have the presence of minerals, carotenoids, vitamins, locopherol, flavonoids, vitamins, enzymes, gucosinolates, tocopherols, benzyl isothiocyanate, alkaloids, saponins, and tannins in the various parts of the plant.[5-7] They also contain lycopene and β-carotene which are good free radical scavengers. Also, the plant seeds contain fatty acids, crude protein, crude fiber, papaya oil, carpaine, papain, caricin, glucotrapaeolin, benzylglucosinate, benzylisothiocyanate, benzylthiourea, hentriacontane, β-sitostrol and myrosin.[8] Several therapeutic effects of the plant parts have been reported[6], including but

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not limited to antimicrobial, anti-oxidative, anti-cancer, anti-ulcer, anti-inflammatory, hepatoprotective, and antidiabetic effects. Specifically, the flower has been reported to have antibacterial activities against *Escherichia coli* and *Bacillus subtilis*. Also, Aruljothi *et al*.[9] reported antibacterial effects of the acetone leaves extract against wound implicated bacteria including *Staphylococcus aureus, Proteus vulgaris, Klebsiella pneuomoniae*, ad *Pseudomonas aeruinosa*. Okpe *et al*.[10] demonstrated synergistic ability of *Vernonia amygdalina* and *C. papaya* in the reduction of Plasmodium infection in mice. Teng *et al*.[11] also revealed antiplasmodial activity against two *Plasmodium falciparum* strains. This present study is aimed at investigating the *in vitro* antibacterial and *in vivo* antimicrobial activity of *Carica papaya* seeds against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Salmonella typhi* and *Plasmodium berghei*, respectively.

Materials and Methods

Ethical Approval
Ethical approval (number: FPSRE/UNN/22/0022) for methods and appropriate use of animals were duly obtained from the Research Ethics Committee, Faculty of Pharmaceutical Sciences, University of Nigeria Nsukka.

Confirmation and preparation of test isolates
Already characterized clinical isolates including *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella typhi,* and *Plasmodium berghei* were used in the study. They were obtained from the Department of Microbiology, Faculty of Veterinary Medicine, University of Nigeria, Nsukka. The stock bacterial cultures were maintained in nutrient agar slants at 4^{o}C in a refrigerator. Standard biochemical tests including indole, citrate, and catalase were used to confirm the bacterial isolates. The bacterial isolates were activated by successive sub-culturing onto nutrient agar and incubation. The 24 hours distinct colonies were prepared in normal saline to match 0.5 McFarland's standard. The *P. berghei* was obtained in liquid nitrogen, thawed prior to introduction into the animals.

Plant collection and identification
Carica papaya (Pawpaw) fruits were collected from a local market in New Market Enugu, Enugu state, Nigeria in January 2021. It was properly identified and authenticated (voucher number: INTERCEDD/025) by Mr Alfred Ozioko, a certified taxonomist at International Centre for Ethno-medicine and Drug Development (INTERCEED), Nsukka, Enugu State, Nigeria.

Plant preparation and Extraction
The *C. papaya* fruit pulp was cut open and the seeds removed after which they were thoroughly rinsed to get rid of any adhering flesh. The seeds were then air-dried at room temperature and ground into a fine powder. The powder was divided into two parts and were respectively subjected to standard soxhlet and cold maceration extraction methods using methanol and water as solvents

Qualitative and quantitative Phytochemical analysis
The qualitative and quantitative phytochemical analysis (tannins, glycosides, alkaloids, reducing sugars, saponins, flavonoids, phenolic compounds, terpenoids, steroids, proteins, and phenols) of the extracts were conducted using protocols as described by Harborne[12] in the Department of Pharmacognosy, University of Nigeria Nsukka.

Agar-well diffusion method
The test bacteria from the stock culture were sub-cultured onto a nutrient agar and incubated for 24 hours at 37 ºC. A 400 mg of the extracts was weighed out and transferred into a sterile test tube containing 2 ml of dimethylsulfoxide (DMSO) to form the stock solution. A two-fold serial dilution was then carried out to obtain four different concentrations (200 mg/ml, 100 mg/ml, 50 mg/ml, and 25 mg/ml) of the extracts. The sterile petri dishes containing equal volumes of molten Mueller Hinton agar were allowed to solidify. A 0.1ml each, of the test organisms were smeared on the surface of the Mueller-Hinton agar in the different petri dishes with aid of a sterile glass rod. The plates were allowed to stand for 10 minutes in aseptic conditions. With the aid of a sterile cork-borer, 5 wells were made in the agar. Three drops of each concentration was transferred into four out of the five wells while the fifth well was filled with DMSO as a negative control. Ciprofloxacin (5 µg) antimicrobial disc was then placed on the agar as a positive control. The plates were properly labeled and a pre-diffusion time of 30minutes was allowed before incubation. They were incubated for 24 hours at 37 ºC temperature and the zones of inhibition obtained using meter rule.

Minimum inhibitory concentration (MIC)
The minimum inhibitory concentration of *C. papaya* seed extract against *E. coli, S. aureus*, and *S. typhi* was determined using the agar dilution method. The methanol extract was prepared by dissolving 1000 mg of the extract in 4 ml of DMSO to obtain the stock solution which was subsequently diluted to following seven different concentrations; 250 mg/ml, 125 mg/ml, 62.5 mg/ml, 31.25 mg/ml, 15.625 mg/ml, 7.81 mg/ml, and 3.91 mg/ml. The different concentrations of the extract were aseptically transferred into the petri dishes containing double strength molten Mueller-Hinton agar. The agar was gently swirled for even distribution and then allowed to solidify. Using a sterile wire loop, inoculums of microbial cultures of *E. coli* and *S. typhi* were streaked on the surfaces of the agar plates with the different concentrations of the methanol extract. The plates were properly labeled and incubated at 37ºC for 24 hours. The procedure was repeated for *S. aureus* using the aqueous extract. The minimum inhibitory concentrations of the extracts being the least concentration that inhibited growth were recorded following incubation.

Animals and experimentation
Twenty-five mice (15 – 20 kg) of both sexes were obtained from the animal facility of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka. The animals were acclimatized for two (2) weeks. They were fed optimally and had access to air and clean drinking water. Following acclimatization, the animals were placed into five groups. All the groups except Group 1 were injected intraperitoneally with 0.2 ml suspension *P. berghei* and were left for four (4) days to establish infection, after which the administration of the different treatments began and happened once per day for three consecutive days. The groupings and administrations are as follows, with Group 2 serving as standard control group:

Group 1: Normal control (No infection, no treatment)
Group 2: *P. berghei* + 25mg/kg body weight of standard control (artemether/lumefantrine (80/480mg))
Group 3: *P. berghei* + positive control (untreated malaria-induced mice)
Group 4: *P. berghei* + 200 mg/kg body weight of aqueous extract of *C. papaya* seeds
Group 5: *P. berghei* + 400 mg/kg body weight of aqueous extract of *C. papaya* seeds.

At the end of the treatments, blood sample were collected and used in the determination of parasitaemia, red blood cell (RBC) count, white blood cell (WBC) count, and pack cell volume (PCV) using standard protocols as described in the following sections. Studies were in line with the recommendations of The National Institute of Health Guide for Care and Use of Laboratory Animals.[13]

Measurement of parasitaemia
A thin blood film stained with Giemsa stain was prepared after infection and treatment for each mouse and allowed to dry completely. The percentage of red blood cells (RBCs) infected with malaria parasites were determined microscopically using the x100 objective with immersion oil in 10 different fields on each slide. The percentage (%) parasitemia and suppression were calculated respectively.[14]

Red blood cell (RBC) count
4ml of RBC diluting fluid was added to 20 µl of well mixed anti-coagulated blood and was allowed to stand for 5 minutes for WBC destruction. The counting chamber and the cover slip were assembled making sure they are completely clean and dry. The blood sample was

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remixed with capillary and the grid filled with the blood sample. It was left undisturbed for 2 minutes to allow the red blood cell to settle. It was covered with the slid, placed in the microscope stage and counted within the clean and dry counting chamber in a microscope at 10x objective in 5 different boxes.

White blood cell (WBC) count
Similarly, 0.38ML of WBC diluting fluid was added to 20 µl of well mixed anti-coagulated blood and was allowed to stand for 5 minutes RBC destruction. The blood sample were remixed with capillary and the grid filled with the blood sample. It was left undisturbed for 2 minutes to allow the white blood cell to settle. It was covered with the slid, placed in a microscope stage and viewed with 10x objective. The cells in the four large corner squares of the chamber were counted.

Determination of pack cell volume (PCV)
The PCV was determined by taking blood sample with a heparinized capillary tube, cleaned and sealed with plasticine. The filled cubes were placed in the micro haematocrit centrifuge and spun at 10, 000 rpm for 5 minutes after which the tubes were placed into a specially designed scale and the packed cell volume was read as a percentage as shown below.[15]

$$PVC\ \% = \frac{Packed\ RBC\ column\ height}{Total\ blood\ column\ height} \times 100$$

Statistical analysis
T–test and One-Way Analysis of Variance (ANOVA) were employed in the data analysis using the Statistical Package for the Social Sciences (SPSS).

Results and Discussion

Phytochemical tests
Plants are a valuable reservoir of bioactive compounds of substantial importance.[16] This study has demonstrated the antimicrobial activities of extracts from C. *papaya* seeds, a plant known for its use in traditional medicine for the treatment of infectious disease amidst other ailment. The phytochemical analysis demonstrated the presence of a number of important phytochemicals and also revealed that both the methanol and aqueous extract contains similar compounds. The phytochemicals found to be contained in the extract includes saponins, tannins, flavonoids, phenols, alkaloids, terpenoids, steroids, reducing sugars and glycosides all of which are all well known to have antimicrobial activity.[6] This helps to explain why both extracts had antibacterial action against the isolates tested. However, the quantitative assay revealed that the methanol extract has high contents of phenols, flavonoids, tannins, steroids, alkaloids, glycosides and reducing sugars. This may be responsible for the very high activity observed with the methanol extract in table 4 when compared to the aqueous extract. This also implies that the phytochemicals are more soluble in organic solvent than in aqueous solvent

Antibacterial effects of the extracts
The aqueous extracts of *C. papaya* seeds has significantly ($P < 0.05$) shown high antibacterial activity against *S. aureus* growth (compared to the standard drug (ciprofloxacin)) as indicated in Table 4, while that of methanol extract produced the highest inhibition zone diameter of 15 mm against *S. typhi* (Table 5). The susceptibility test shows that the aqueous and methanol extract at various concentrations, have activity against *S. aureus, S. typhi and E. coli* with the water extract exhibiting its highest activity against *S. aureus*. However, it was observed that both extracts exhibited poor activity against *P. aeruginosa*. The difference in the activities of the extract may be due to the differences in the structural cell wall of the bacteria, the nature of the media used and the solubility of the extract in the media used.[17] The extracts have activities against both the gram positive and the gram negative bacteria, implying that it has broad spectrum antibacterial property. This knowledge can be exploited in the development of a novel drug with a wide spectrum of antimicrobial activity.

Table 1: phytochemical composition of *Carica papaya* seeds

Phytochemicals	Methanol extract	Aqueous extract
Tannins	+	+
Glycosides	+	-
Alkaloids	+	+
Reducing sugars	+	-
Saponins	-	+
Flavonoids	+	+
Phenolic compounds	+	+
Terpenoids	-	+
Steroids	+	-
Proteins	+	+

Key: '+' = Present, '-' = Absent

Table 2: phytochemical composition of *Carica papaya* seeds

Phytochemicals	Methanol extract mg/g	Aqueous extra mg/g
Phenols	8.48	0.137
Tannins	4.22	0.200
Flavonoids	14.95	0.175
Steroids	28.23	0
Terpenoids	0	0.093
Saponins	0	0.103
Alkaloids	11.65	0.164
Glycosides	12.93	0
Reducing sugars	10.52	0

Table 3: Biochemical test results

Isolates tested	Catalase	Indole	Citrate
E. coli	+	+	-
S. typhi	+	-	-
P. aerugenosa	+	-	+
S. aureus	+	-	+

Key: '+' = Positive, '-' = Negative

Minimum inhibitory concentration
The MIC of the methanol extract were 62.5 mg/ml and 125 mg/ml for *E. coli* and *S. typhi*, respectively while that of aqueous extract was 125 mg/ml for *S. aureus.* The MIC of the methanol extract against *S. typhi* and *E. coli* were found to be 125 mg/ml and 62.5 mg/ml, respectively while that of the aqueous extract against *Staphylococcus aureus* was found to be 125 mg/ml. The low MIC observed for *E. coli* (62.5 mg/ml) is a good indication of high efficacy of the plant extract against the bacterium. This outcome is significant considering that *E. coli* is a multi-drug resistant organism responsible for many common bacterial infections. The activity of the extract against the organisms indicates that the plant part can be used in medicine upon isolation and purification of the active compounds.

Effect of aqueous extracts of C. papaya seeds on malaria-passaged mice
The effect of aqueous extract of *C. papaya* seeds on percentage of parasite load in malaria-passaged mice shows that on day 4 after infection, significantly ($p < 0.05$) higher percentage of parasite was observed in all groups except Group 1 (normal control group).

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Table 4: Inhibition Zones (mm) of the test bacteria to the aqueous extracts of *C. papaya* seeds

Bacteria	Extract concentrations (mg/ml)				Control	
	200	100	50	25	Ciprofloxacin	DMSO
P. aeruginosa	7	0	0	0	17	0
S. aureus	17	12	10	7	12	0
E. coli	4	3	0	0	20	0
S. typhi	5	0	0	0	16	0

Table 5: Inhibition Zones (mm) of the test bacteria to the methanol extracts of *C. papaya* seeds

Bacteria	Extract concentrations (mg/ml)				Control	
	200	100	50	25	Ciprofloxacin	DMSO
P. aeruginosa	7	0	0	0	22	0
S. aureus	4	0	0	0	10	0
E. coli	7	5	4	3	18	0
S. typhi	15	12	7	4	16	0

On day 1, 2, and 3 post treatment, significant ($p < 0.05$) reduction were observed in the parasite in all groups except Group 3 passaged (untreated group). There were no significant ($p > 0.05$) differences between Groups 4 and 5 malaria infected mice administered low (200 mg/kg b.w) and high (400 mg/kg b.w) doses of the extract, respectively (Figure 1). The result on the effect of aqueous extract of *C. papaya* seeds on packed cell volume of the malaria-induced mice shows that no significant difference was observed in the packed cell volume (PCV) of all the groups before the treatment period. Dose dependent significant ($p < 0.05$) reduction was observed in all groups except in Group 1 (normal control), on day 3 after infection. Results obtained after the treatment period showed significantly ($p < 0.05$) higher PCV in all groups except Group 3 (untreated malaria-passaged mice). On the other hand, Group 5 mice had PCV that was found to be significantly ($p < 0.05$) higher than the PCV of Group 2 mice as presented in figure 2. The effect of aqueous extract *C. papaya* seeds on red blood cell volume of malaria-passaged mice and dose-dependent decrease in red blood cell (RBC) count were observed in day 3 after infection and after treatment periods in Groups 4 and 5 administered 200 and 400 mg/kg b.w. of the extract, respectively as shown in Figure 3. After the treatment period, it was observed that the RBC counts in Groups 4 and 5 exhibited significant ($p < 0.05$) elevation compared to the RBC counts of Group 3 mice that were malaria passaged but untreated. On the other hand, Group 5 mice had significantly ($p < 0.05$) higher RBC count than the RBC counts of Groups 2 representing treated with artemether/lumefantrine (80/480 mg).

The effect of aqueous extract *C. papaya* seeds on white blood cell count of malaria-passaged mice and the total white blood cell (WBC) on day 3 after infection recorded the highest count across the groups except Group 1 which represented positive control group, while the lowest WBC count was recorded after treatment in all the groups except Group 3 (untreated malaria - passaged mice). After treatment, significant ($p < 0.05$) reduction was found in the total WBC counts of Groups 4 and 5 mice compared to the total WBC counts of Group 3 mice that represented normal control mice, malaria passaged untreated (Figure 4). The aqueous extract of *C. papaya* seeds *at* 400 mg/ kg b.w showed a high percentage of malaria parasite clearance rates in sensitive strain of *P. berghei* comparable to the standard drug (artemether/lumefantrine). Initially, percentage of parasite clearance was very low during treatment but lower during the final stage of treatment. Importantly, our results indicated that the inhibition by the extract of *C. papaya* seeds at 400 mg/kg b.w was effective than the positive control (untreated malaria-passaged mice) and standard control (malaria-passaged mice treated with artemether/lumefantrine) used for treatment of malaria. Group treated with 200 mg /kg b.w had similar clearance rate with group treated with standard drug.

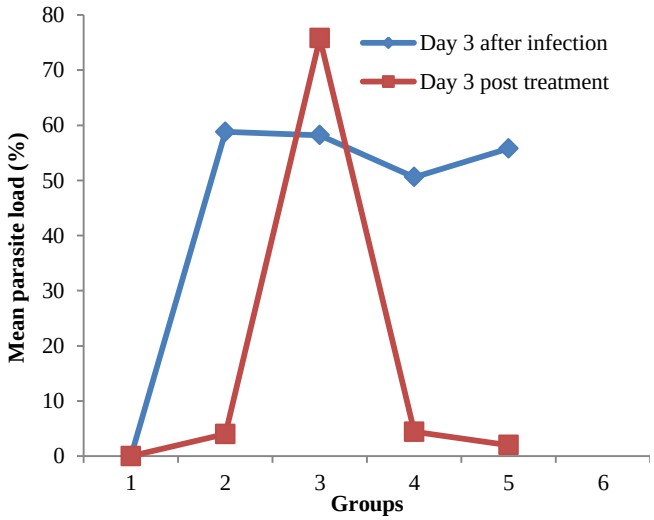

Figure 1: Effect of aqueous extracts of *C. papaya* seeds on percentage of parasite load in malaria-passaged mice

Remarkably, the parasite clearance by the extract at 400 mg/kg b.w was comparable to the control (artemether/lumefantrine) commonly used for treatment of malaria. The results showed that the percentage of parasite load in malaria passaged mice was found to reduce significantly in animals treated with high doses (400 mg/kg body weight of extract) of the extract when compared to the standard control groups which only has equal effect with group 4 (treated with 200 mg/kg b.w). This suppressive antiplasmodial effect of the aqueous extract of *C. papaya seeds* on the multiplication of *P. berghei* portends that these extracts are a potential source for new antimalarial drugs. The effect of aqueous extract of *C. papaya seeds* on various blood parameters such as pack cell volume (PCV), red blood cell count (RBC) and white blood cell count (WBC)) of malaria passaged mice were evaluated. A reduction in concentration of PCV and RBC are useful indicators of clinical malarial anemia and frequently monitored as drug efficacy against Plasmodium infection.[16] In the present study, PCV was measured to assess the efficacy of the extract and artemether/lumefantrine in preventing haemolysis due to increasing parasitemia level. There was observable increment in the packed cell volume (PCV) of the groups treated with 200 and 400mg/kg b.w (Group 4 and 5) of aqueous extract when compared with the control though greater effect was seen in group treated with 400 mg / ml of the aqueous extract (Group 5).

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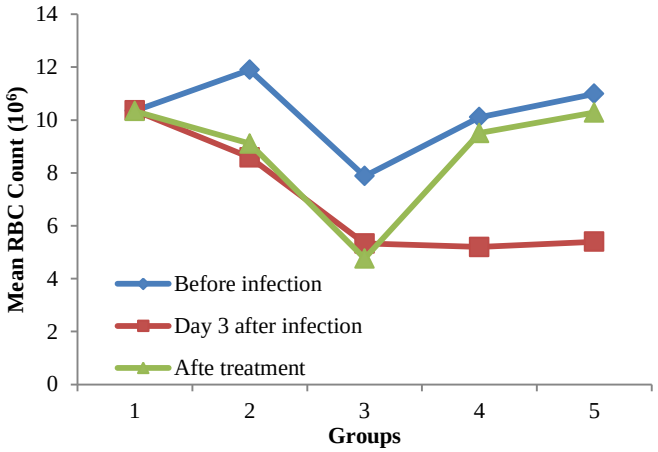

Figure 2: Effect of aqueous extract of *C. papaya* seeds on packed cell volume of malaria- passaged mice.

Group treated with 200 mg/kg of aqueous had similar effect with the group treated with artemether/lumefantrine (group 2). Plasmodium parasite strains have high affinity for RBCs and feeds on it. Initially, a dose-dependent decrease in red blood cell (RBC) count were observed at day 3 after infection but gradual increase was recorded after treatment periods in Groups 4 and 5 administered 200 and 400 mg/kg b.w. of the extract respectively and changes in RBC is typical features of malarial infections.[18] At day 3 after infection, a significant elevation was observed in WBC in all groups except group 1. The results of the present study imply that the *C. papaya* seeds extracts enhanced the normal status of the WBC.

Conclusion

The aqueous and methanol extracts of *C. papaya* seeds were found to contain a variety of phytochemicals ranging from glycosides to flavonoids, saponins, tannins, alkaloids, reducing sugars, phenolic compounds, proteins, terpenoids and steroids. These phytochemicals may be responsible for the antibacterial and antimalarial property of the extracts. From the experimental outcomes, the extracts had inhibitory actions against the tested isolates. The aqueous extracts of *C. papaya* seeds showed antimalarial activity against *P. berghei* infection in mice as evidenced by the percentage of mean parasite load and the observed ability of re-establishing the immune cells by boosting and stabilizing the blood parameters.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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