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Synergism of *Azadirachta indica* Seed and *Nigella sativa* on *Plasmodium falciparum*: Study on Wistar Rats

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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

Aim: This study was carried out to determine the Phytochemical contents of *Azadirachta indica* seed and *Nigella. sativa* and their synergistic therapeutic effect on indices of clinical importance in malaria-induced male wistar rats.
Study Design: The animals that were used for this study where divided into 4 groups of 6 rats each. All rats in the 3 test groups were inoculated with 0.2 ml blood parasitized with *Plasmodium falciparum* and observed for 3 days for manifestation of signs of malaria. Treatment with *Azadirachta indica* seed extract only, *Nigella sativa* extract only and a combination of *Azadirachta indica* seed and *Nigella sativa* extracts was conducted for a period of 14 days. Changes in weight of the rats and hematological parameters RBC, WBC,PCV and Hemoglobin were assessed before commencement of treatment and through the 14 day period.

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Place and Duration of Study: This study was conducted at the National Veterinary Research Institute (NVRI), Vom in collaboration with the Department of Biological Sciences Abubakar Tafawa Balewa University, Bauchi, Nigeria between March 2022 and May 2022.
Methodology: *Azadirachta indica* seed and *Nigella sativa* seeds were extracted with distilled water and the lethal doses (LD_{50}) were determined on the rats. Qualitative phytochemical screening of *Azadirachta indica* seed and *Nigella sativa* extracts was performed. *Plasmodium* infected rats were divided into 3 groups of 6 rats each and a normal control group which was left uninfected. Symptoms of malaria infection were observed three days after infection. The treatment was commenced on day 4 post-infection and was continued for a period of 14 days. Each plant extract and the mixture of both extracts were administered at 100, 200, and 300mg/kg body weight of the rats. After first 4 days, 7 days, and at 14 days of treatment each rat's blood sample was taken for hematological analysis.
Results: The plant extracts lethal dose (LD_{50}) was considered safe for *Azadirachta indica* seed at 5000mg/kg and 2000mg/kg for *Nigella sativa* respectively. Qualitative phytochemical screening revealed the presence of saponins, alkaloids, flavonoids, resins, glycosides, steroids in both *Azadirachta indica* seed extract and *Nigella. sativa* extract. Treatment with a mixture of both extracts showed a high restoration of red blood cell count with a significant ($p>0.05$) increase in RBC with a non-significant decrease in PCV and WBC. There was no significant decrease in hemoglobin concentration observed across all treatment groups at the end of the 14 day study.
Conclusion; The combination of both *Azadirachta indica* seed and *Nigella sativa* is well tolerated and safe for *Plasmodium* parasites effects on wistar rats at concentration of 400mg/kg of body weight which showed highest values of restoration of RBC count as compared to the normal control group. Group A treated with only *Nigella sativa* also showed RBC count higher than that obtained in group B treated with only *Azadirachta indica* seed. PCV values compared with the normal control group showed an 8% increase at concentration of 400mg/kg at the end of the experiment in group C treated with the mixture of *Azadirachta indica* seed and *Nigella sativa.* At 200mg/kg concentration, the mixture of *Azadirachta indica* seed and *Nigella*. *sativa* gave a 25% increase in PCV values. This shows that the synergistic effect of *Azadirachta indica* seed and *Nigella sativa* has better therapeutic effects against *Plasmodium* parasites than either *Nigella sativa* or *Azadirachta indica* seed as a single therapy. This study provides a basis for the development of a cheaper plant-based antimalarial combination therapy.

Keywords: Phytochemicals; antimalarial; Azadirachta indica; Nigella sativa.

1. INTRODUCTION

Malaria is a protozoal parasitic infection that is transmitted by the female *Anopheline* mosquito [1]. It is caused by the parasite *Plasmodium*, four strains of which infect humans; *Plasmodium falciparum, Plasmodium ovale, Plasmodium vivax,* and *Plasmodium malariae*. It is one of the world's most important public health issues, causing millions of deaths annually, especially in the developing countries. In sub-Saharan Africa, 2/5 of the global 300 million deaths caused by malaria occurs, which leads to poor health, poverty and obstructs social and economic development [2]. Malaria spread can be reduced using mosquito repellent, treated mosquito nets, clearing of bushes and stagnant water. Malaria has been treated using herbal drugs for thousands of years. The oldest and pioneer Antimalarial to be extracted from Cinchona tree is 'Quinine', after which 'Artemisinin' was later discovered. As at 2005, the Artemisinin Combination Therapy (ACT) was adopted in Nigeria. Over the last decade however, there has been increasing multi-drug resistance of the *Plasmodium* parasite [3].

The difficulty of creating efficient vaccines as well as the increasing multi-drug resistance of the *Plasmodium falciparum* strains has aggravated the problem of treatment failure. The high economic impact of the malaria infection is directed mostly towards high infant mortality, pregnant women and immunocompromised individuals. Adverse drug reactions posed by commercially available antimalarials also highlights the need for a novel and well-tolerated antimalarial drug [4]. Scientists all over the world have embarked on a journey to find an alternative antimalarial, testing different plants and plant parts for Antimalarial potency, some of which have been used as traditional remedy for many ailments. Amongst these plants are the Neem (*Azadirachta indica*) and Black Seeds (*Nigella sativa*) [3].

Azadirachta indica (*A. indica*) commonly known as 'Neem' or 'Darbejiya' (Hausa) is an evergreen plant abundant in Africa, America and parts of India. It has a long time history of traditional remedy against many ailments, due to its antibacterial, antifungal, anti-inflammatory and anti-parasitic properties. It is also rich in therapeutics like nimbin, nimbidin, gedunin, salannin, etc, but the most active ingredient is azadirachtin [5]. Different parts of the Neem plant have been tested for their antimalarial properties i.e. the leaves, seeds, stem bark, using aqueous, ethanolic and methanolic extracts. Several tests have proven the Neem extracts to be active against the chloroquine resistant malaria strains [6].

Nigella sativa which is also known as 'Black Seeds' is a plant of ancient traditional medicine, since the time of Prophet Muhammad (PBUH), who described it as 'the cure for all diseases except death' [7]. Its most active ingredient is thymoquinone and has antifungal, antiviral, antibacterial and anti-parasitic properties [8]. Studies using its ethanolic, aqueous and chloroform extracts have also demonstrated high percentages of parasitemia suppression [9] Since both plant extracts have proven to be effective against malaria infection, this research work focused on investigating the synergistic effect of a combination of these two (2) plant extracts against malaria parasite, the result of which will form a basis for further research study for a novel potent antimalarial.

In these trying times of increasing multi-drug resistant organisms, the ancient traditional healing methods are becoming more and more acceptable because people's perception towards traditional therapy has changed encouragingly. The World Health Organization (WHO) has estimated that about 80% of people living in the developing countries exclusively use traditional medicine for their health care, the use of which has been shown to be even safer as compared to modern allopathic medicines [10]. In many parts for Africa, particularly Nigeria, the use of herbal preparations as remedy for malaria is very common. Neem is one of such common therapies, and has proved to be effective against resistant *Plasmodium* strains. Due to high cost and unavailability of the commercial Antimalarial, this seems to be the only reliable form of treatment [11].

Antimalarial activity of Neem was reported by [12] to be equivalent to half the therapeutic dose of Chloroquine Sulphate on dry weight basis. Extracts from different parts of the plant showed inhibition of *Plasmodium berghei* and *Plasmodium falciparum* in mice [10]. Another study, using the Neem leaf extract (ethanolic) in Wister rats infected with *Plasmodium berghei*, revealed the safety of *A. indica* for use as an antiplasmodial. The results showed increase in total cholesterol, HDL and LDL ($p<0.05$) in both groups treated with *A. indica* leaves extracts and Lumartem. Increase in body weight of rats treated with *A. indica* was dependent on the concentration of extract administered [13].

Fig. 1. *Azadirachta Indica* seed

Nigella sativa or Black Seeds is one of the plants of therapeutic potential that have recently drawn great attention for extensive studies in natural medicine due to its less toxic properties and having little or no side effects as compared to synthetic drugs [14]. It has been used to treat various ailments including parasitic infections [15]. Black Seeds comprises of 1.5% unstable oil and 37.5% fixed oil, nigellin, melanthin and Arabic acid, carvene, carvone, cymene, thymohydroquinone, and thymoquinone (which is the most active compound of its volatile oil, about 30-80%). It is also an immune system enhancer, and several scientific studies have confirmed the use of *N. sativa* oil and its extracts safe for use due to its minor/negligible toxological effects [16]. In 2018, [17], tested aqueous and methanolic extracts of *N. sativa* in *Plasmodium berghei* infected mice, which revealed parasitemia suppression significantly ($p<0.05$) in all groups treated with 25mg/kg, 50mg/kg and 100mg/kg. This result of antimalarial potency of *N. sativa* paves way for further investigation for a novel plant based antimalarial, as it indicated that higher doses of the extracts do not necessarily cause higher degree of parasitemia suppression.

Fig. 2. *Nigella Sativa* seed

Most antimalarials like Quinine and Artemisinin were all isolated from plants. Hence, investigating more traditional plants for anti-parasitic properties will serve as a basis for the development of a novel potent antimalarial. Usage of traditional medicine without a standard dosage has raised concerns about the possibility of toxicity. The lethal dose (LD_{50}) of the Neem extract has been estimated to be up to 5000mg/kg, hence qualifying it as safe, non toxic and well-tolerated at the administered doses [13].

2. MATERIALS AND METHODS

2.1 Plant Collection, Identification and Extraction

Neem seeds were collected from Neem trees in Abubakar Tafawa Balewa University, Bauchi, while Black Seeds of Egyptian origin were purchased from Al-Jazeera Bookshop, Anguwan Rogo, in Jos, Plateau state. Both seeds samples were taken to the NVRI Biology laboratory for authentication.

2.2 Seeds Extraction

The two seed samples were air-dried separately at room temperature in the laboratory and ground into powder using mortar and pestle. 100g of each powder was weighed on a weighing balance and placed into two different conical flasks. 500ml of distilled water was added to each conical flask and allowed to stand overnight (24hours). Each mixture was filtered separately using Whatman filter paper 1 and each of the filtrates was evaporated to dryness in a hot air oven at 40°C for 72hours to obtain the extracts, and then each extract was stored in a sterile airtight container until use.

2.3 Preliminary Qualitative Phytochemical Analysis

Phytochemical studies for the two extracts was carried out as follows:

Preliminary Qualitative Phytochemical Screening of Neem Seeds Extract: as described by [9];

Detection of Alkaloids: dilute hydrochloric acid was added to 1g of Neem seeds extract for Wagner's test. Four drops of Wagner's reagent (iodine in potassium iodide) was added to the prepared sample to test for the presence of alkaloids (appearance of a brown/reddish precipitate).

Detection of Terpenoids: 1g of Neem seeds extract was mixed with 2ml chloroform. 3ml concentrated sulphuric acid was added gradually to form a layer. A reddish brown interface indicated the presence of terpenoids.

Detection of Saponins: for thefoam test, 2ml of water was added to 1g of neem seeds extract and shaken well. The persistence of foam for about 10 minutes indicated the presence of saponins.

Detection of Sterols: 2ml chloroform and 2ml concentrated sulphuric acid were added to 1g of neem seed extract and shaken well. The appearance of greenish-yellow fluorescence at the red layer of chloroform and acid indicated the presence of sterols.

Detection of Glyosides: 1ml of water was added to 1g of neem seeds extract and shaken well. 2ml of aqueous solution ofsodium hydroxide was added. Appearance of yellow colour indicated the presence of glycosides.

Detection of Phenols: for ferric chloride test, 1g of neem seeds extract was treated with 4 drops of ferric chloride solution. Formation of bluish-black colour indicated the presence of phenols.

Detection of Tannins: for gelatin test, 2ml of 1% gelatin solution encompassing sodium chloride was added to 1g of neem seeds extratct. Formationof white precipitate indicated the presence of tannins.

Detection of Flavonoids: alkaline reagent test; 1g of neem seeds extract was treated with 4 drops of sodium hydroxide solution. Establishment of intense yellow colour, which turned colourless on addition of diluted hydrochloric acid indicated the presenc of flavonoids.

Preliminary Qualitative Phytochemical Screening of Black Seeds Extract: following the procedure adopted by [18]**:**

Test for Alkaloids: Wagner's; 1.5ml of 1% HCL was added to 2ml of Black seeds extract. The solution was heated in a water bath, and then 6 drops of Wagner's reagent was added. An orange precipitate indicated the presene of alkaloids.

Test for Steroids: 5ml of chloroform and 2ml of acetic nhydride were added to 2ml of black seeds extract, followed by concentrated sulphuric acid. Reddish-brown coloration of the interface indicated the presence of steroids.

Test for Phenols: to 2ml of Black seeds extract, 5% ferric chloride solution was added.deep blue-black colour indicated the presence of phenols.

Test for Terpenoids: following the Salkowski's test procedure, 1ml of Black seeds extract was mixed with 2l of choloroform. 3ml concentrated sulphuric acid was added gradually to form a layer. A reddish-brown interface indicated the presence of terpenoids.

Test for Flavonoids: a few drops of ferric chloride solution was added to the test solution. Intense green colour showed the presence of flavonoids.

Test for Tannins: 2ml of Black seeds extract was dissolved in distilled water and then 2l of 5% ferric chloride solution was added. The formation of blue-green colour indicated the presence of tannins.

Test for Saponins: 2ml of Black seeds extract was diluted with distilled water and shaken in a graduated cylinder for 15 minutes. The formation of a layer of foam indicated the presence of saponins.

Test for Glycosides: to 2ml of test solution, 3ml of glacial acetic acid and 1 drop of 5% ferric chloride was added in a test tube. 0.5ml of concentrate sulphuric acid was carefully added by the side of the test tube. Formation of blue colour in the acetic acid layer indicated the presence of glycosides.

Animal Ethical Considerations, **Animals Used and Parasite Inoculation:** Animal care and use guideline was provided by the National Veterinary Research Institute (NVRI) Vom. Male wistar rats weighing between 110-125g were used as test animals. The rats were purchased from the NVRI Vom and kept under optimum conditions of temperature, and feeding in the laboratory in cages of 6 mice per cage at 25-28°C room temperature. Relative humidity was maintained at 60-70%.

Plasmodium falciparum infected blood was used to inoculate the mice intraperitoneally.

Infecting and Dosing of test Animals: To 2ml of parasitized blood was added 4ml of distilled water as diluent. Then each rat labeled from 1-18 was infected with 0.2ml of diluted parasitized blood (2×10^7 parasitized erythrocytes) intraperitoneally and divided into 3 groups of 6 rats each, and 1 normal control group of 6 uninfected rats were labeled rats 19-24.

Determination of Packed Cells Volume (PCV): Protocol highlighted by Mekonnen was followed to determine the PCV. It aided in evaluating the efficacy of the test extracts in inhibiting hemolysis due to malaria. Blood was collected from the vein of each mouse in heparinized capillary tube (¾ of its volume) before infection and on day 4 post treatment, centrifuged at 12,000 rpm for 10minutes. The PCV was determined using the formular:

$$\text{PCV} = \frac{\text{VOLUME OF RBCCs IN GIVEN VOL.OF BLOOD}}{\text{/TOTAL BLOOD VOLUME}} \times 100$$

2.4 Statistical Analysis

One way analysis of variance (ANOVA) was utilized to establish statistical significance for assessment of in vivo assay. A p-value of less than 0.05 was considered statistically significant.

3. RESULTS

Qualitative Phytochemical screening of the *Azadirachta indica* seed extract showed the presence of saponins, alkaloids, steroids, flavonoids, tannins, glycosides and terpens. Phytochemical screening of *Nigella sativa* extract also revealed the presence of of saponins, resins, alkaloids, steroids, flavonoids, glycosides and terpenes. This is in line with previous studies that revealed that alkaloids and terpenes in most medicinal plants have antimalarial activity.

Symptoms of malaria infection were observed three days after infection. One of the rats in group D died after day 8 but no death from the treatment groups was recorded after 8 days of treatment.

Table 1. Phytochemical analysis of the plants extracts

Test Parameters	Sample A: *A. indica* seed	Sample B: *N. sativa*
Saponins	+	+
Tannins	+	+
Resins	+	+
Alkaloids	+	+
Flavonoids	+	+
Glycosides	+	+
Steroids	+	+
Terpenes	+	+
Cardiac glycosides	+	+

**Data in tables are expressed as mean plus standard error in mean.*

Table 2. Report after first 4 days of treatment

Group		Weight(g)	RBC(10^12/L)	WBC (10^9/L)	PCV(%)	HB(g/L)
Group 1	Mean	132.8933 ±7.70854	5.9500 ±0.53213	12.5333 ±1.78562	36.1667 ±2.22736	12.0333 ±0.75085
	N	6	6	6	6	6
Group 2	Mean	132.7647 ±2.69409	5.7833 ±0.21042	11.8000 ±1.35769	33.0000 ±1.26491	11.8667 ±0.37387
	N	6	6	6	6	6
Group 3	Mean	146.8480 ±6.80675	5.6833 ±0.21042	12.4517 ±0.25613	31.5000 ±1.83938	11.5667 ±0.23190
	N	6	6	6	6	6
Group 4	Mean	127.9900 ±1.91256	6.5600 ±0.08083	10.2000 ±0.64161	37.7500 ±0.47871	11.6000 ±0.04082
	N	4	4	4	4	4
Total	Mean	135.7725 ±3.12667	5.9427 ±0.16974	11.8868 ±0.61319	34.3182 ±0.96112	11.7818 ±0.22588
	N	22	22	22	22	22

Table 3. Report after 7 days of treatment

Group		RBC	WBC	PCV	HB
Group 1	Mean	6.0667 ±0.45509	12.1500 ±1.75988	34.5000 ±2.04532	11.9500 ±0.65358
	N	6	6	6	6
Group 2	Mean	5.1833 ±0.22423	11.7167 ±1.35804	31.5833 ±1.28073	11.7333 ±0.31798
	N	6	6	6	6
Group 3	Mean	5.5333 ±0.17062	10.5000 ±2.10840	31.0833 ±1.98014	11.4833 ±0.21512
	N	6	6	6	6
Group 4	Mean	6.4700 ±0.07821	10.3000 ±0.67206	37.7500 ±0.47871	11.6000 ±0.04082
	N	4	4	4	4
Total	Mean	5.7536 ±0.17127	11.2455 ±0.80606	33.3636 ±0.96476	11.7000 ±0.19717
	N	22	22	22	22

Table 4. Report after 14 days of treatment

Group		RBC	WBC	PCV	HB
Group 1	Mean	7.3000 ±0.60992	11.8667 ±0.64377	37.5650 ±0.80334	13.0000 ±0.48922
	N	6	6	6	6
Group 2	Mean	6.1667 ±0.24313	11.6417 ±0.73801	39.1683 ±0.33456	12.5633 ±0.57629
	N	6	6	6	6
Group 3	Mean	6.5167 ±0.37275	12.0500 ±0.49783	38.9367 ±0.37134	13.3750 ±0.41585
	N	6	6	6	6
Group 4	Mean	8.0250 ±0.21747	14.3750 ±0.22500	40.4125 0.24291	14.1850 ±0.08302
	N	4	4	4	4
Total	Mean	6.9091 ±0.24529	12.3114 ±0.35474	38.8941 ±0.32181	13.1986 ±0.25119
	N	22	22	22	22

Table 5. Body weights of rats before infection across treatment

Group		Wieght of rat before infection	Weight of infected rats	Weights of rats after treatment
Group 1	Mean	111.3140 ±23.53011	132.9457 ±7.66173	120.3197 ±5.75425
	N	6	6	6
Group 2	Mean	132.7647 ±2.69409	132.7528 ±2.69301	138.3095 ±14.54295
	N	6	6	6
Group 3	Mean	146.0030 ±7.14203	145.9893 ±7.14243	128.4700 ±6.77126
	N	6	6	6
Group 4	Mean	129.1997 ±2.09504	129.1997 ±2.09504	133.8110 ±1.45455
	N	3	3	3
Total	Mean	129.9090 ±7.24791	136.0822 ±3.23373	129.7156 ±4.82032
	N	21	21	21

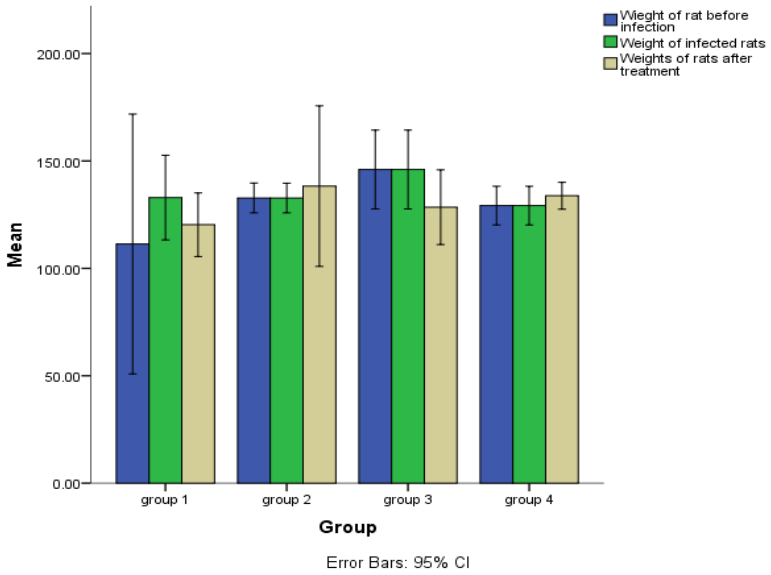

Fig. 3. Weights of the rats investigated

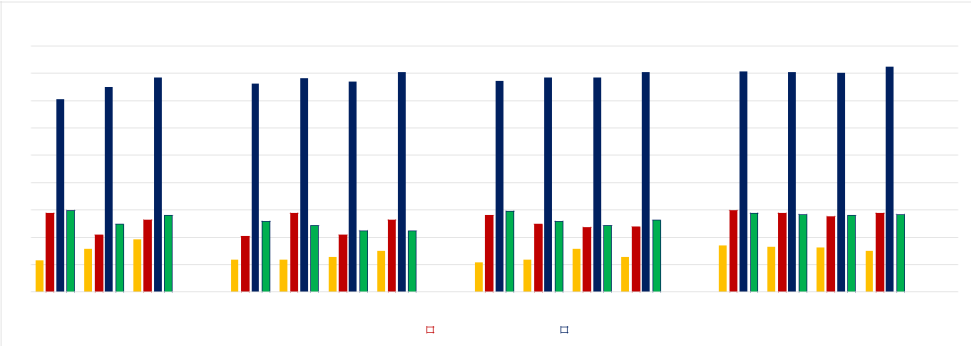

Fig. 4. Graph showing hematological changes during treatment

At the end of the treatment, the infected rats were still alive.

The body weights of the rats investigated in this study are shown in Table 5 and Fig. 3. There was no significant weight difference observed in all treatment groups.

A reduction in all hematological values was observed in the treatment groups after administration of plant extracts compared to the normal control. Malaria infection caused a non-significant decrease in PCV and WBC with a significant ($p>0.05$) increase in RBC. There was no significant decrease in hemoglobin concentration observed across all treatment groups.

4. DISCUSSION

Malaria is characterized by anemia which is as a result of the destruction of RBCs both infected and non infected which leads to a decrease in erythroid precursors and erythropoiesis inhibition resulting in the death of the patient [13]. Anemia level is directly proportional to the severity of the infection [9]. PCV levels are inversely proportional to anemia due to malaria infection. The onset of anemia results from decrease in hemoglobin i.e. decrease in RBCs. The administration of *A. indica* extract in combination with *N. sativa* extract was practically safe and well tolerated at the administered doses. The results indicate increased PCV values in the test groups, with highest values in group C treated with mixture of *Azadirachta indica* seed and *Nigella sativa*, which shows the extracts endure hemolysis of RBC due to infection causing anemia. This agrees with [17] who reported that *Nigella sativa* extracts elevate PCV values [3].

Medicinal plants with anti-anemic properties are used to manage severe anemia by restoration of

the RBC count [13]. The hematological values in this study were restored with the administration of the plant extracts.during the treatment, rats in group C treated with a mixture of *Azadirachta indica* seed and *Nigella sativa* extracts exhibited higher restoration of the RBC count. The study also proved that the rats treated with only either of the extracts responded to treatment as only 2 deaths were recorded at the end of the experiment. The prevention of weight loss by both the *Azadirachta indica* seed and *Nigella sativa* extracts in the treated rats was also observed as compared to the weights of the normal control group.

Various medicinal plants that have antimalarial activity such as chloroquine, artemisinin products and derivatives were discovered using the rodent models. With such drug discovery investigations, in vivo tests are considered to be more practical, faster and cheaper than the invitro experiments. A potential antimalarial drug exhibits therapeutic activity without having any toxic effects on the host animal. Acute toxicity test for both *Azadirachta indica* seed and *Nigella sativa* proved no death at doses as high as 5000mg/kg and 2000mg/kg respectively for the duration of the 14 days period. The successful completion of the 4-day suppressive test without any death records indicates the safety of the two extracts [19-24].

Nigella sativa has been found to be active against various strains of *Plasmodium*, with high parasite clearance and restoration of altered hematological parameters. In this study*, Nigella sativa* showed higher RBC count at concentrations of 200mg/kg and 400mg/kg. These results indicated that *Azadirachta indica* seed and *Nigella sativa* extracts are safe for use as a form of malaria treatment.

5. CONCLUSION

This study revealed that the combination of both *Azadirachta indica* seed and *Nigella. sativa* extracts is well tolerated and safe for *Plasmodium* parasites effects on wistar rats at concentration of 400mg/kg of body weight which showed highest values of restoration of RBC count as compared to the normal control group. Group A treated with only *Nigella sativa* also showed RBC count higher than that obtained in group B treated with only *Azadirachta indica* seed. PCV values compared with the normal control group showed an 8% increase at concentration of 400mg/kg at the end of the experiment in group C treated with the mixture of *Azadirachta indica* seed and *Nigella sativa.* At 200mg/kg concentration, the mixture of *Azadirachta indica* seed and *Nigella*. *sativa* gave a 25% increase in PCV values. This shows that the synergistic effect of *Azadirachta indica* seed and *Nigella sativa* has better therapeutic effects against *Plasmodium* parasites than either *Nigella sativa* or *Azadirachta indica* seed as a single therapy. This study provides a basis for the development of a cheaper plant-based antimalarial combination therapy.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Animal care and use guideline was obtained from the National Veterinary Research Institute (NVRI) Vom, Plateau state.

NOTE

The study highlights the efficacy of Herbal Medicine, which is an ancient and still a common tradition in most parts of Nigeria. This traditional concept should be carefully evaluated in the light of modern science and can be utilized if found suitable.

COMPETING INTERESTS

Authors have declared that they have no known competing financial interests or non-financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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