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ANTI-MALARIAL EFFECT OF *NIGELLA SATIVA* SEEDS (BLACK SEED) EXTRACT ON MICE INFECTED WITH *PLASMODIUM BERGEI* (NK 65).

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ABSTRACT

Nigella sativa have been used for thousands of years as a curative remedy for various disorders. It is known as a source of alkaloids, tannins, saponins, cardiac glycoside, flavonoids, carbohydrate, protein, terpenoids, fixed oil, thymoquinone, thymohydroquinone, and thymol. The immeasurable medicinal properties and therapeutic uses of *N.sativa* prove its importance as a valuable medicinal plant. Oral administration of methanol and aqueous seed extracts (25mg/kg, 50mg/kg and 100mg/kg) of *N. sativa* were screened in the 4-day suppressive assays for their anti-malarial properties against *Plasmodium bergei* in mice. The oral treatment of the seed extracts showed suppression activities in all groups of mice which significantly ($p<0.05$) decreased the parasitaemia. The results of this study reveals the antimalarial properties of this extracts, shows a correlation between anemia and malaria and open a new opportunity to further investigate the potentials of new plant based antimalarials in the future. However, the exact principles for this antimalarial action are yet to be identified, which need further studies to elucidate the antimalarial mechanism of their action.

KEYWORDS: Malaria, *Plasmodium bergei*, *Nigella sativa*.

INTRODUCTION

Malaria continues to pose a major public health threat in many African countries (WHO, 2005). The major obstacle to malaria control is resistance to antimalarial drugs. Anti-malarial drug resistance has become one of the greatest challenges against malarial control. The deployment of new classes of anti-malarial drugs has been relentlessly followed by the selection and spread of resistant parasites (WHO, 2001). As a result, anti-malarial resistance directly prevents the success of control program in many resources- constrained settings.

Anti-malarial drug resistance has also played an important role in the occurrence and severity of epidemics in some parts of the world. Population migration has introduced resistant parasites to areas previously free of drug resistance (Foster, 1991; Ridley, 1997). Antimalarial drug resistance, particularly *P. falciparum* resistance, has been a major setback in the fight against malaria and its attendant complications (Wongsrichanalai *et al.*, 2002). The deployment of new classes of anti-malarial drugs has been relentlessly followed by the selection and spread of resistant parasites (WHO, 2001). As a result, anti-malarial resistance directly prevents the success of control program in many resources- constrained settings. The reason behind increase in malaria-related mortality, and for the global resurgence of malaria in the last 3 decades lies in the continuous deployment of ineffective anti-malarial drugs in the face of increasing resistance (Marsh, 1998).

Due to the increasing incidence of resistance to anti-malarial agents there is a need to develop more effective new anti-malarial drug (Ridley, 2002) that is inexpensive, routinely available to people especially those in the developing countries and the curative must be short (Rosenthal, 2003).

The application of herbal drugs to different human and animal disease conditions dates back to human history. Plants have been the basic source of sophisticated traditional medicine systems, for thousands of years and were instrumental to early pharmaceutical drug discovery and industry (Elujoba *et al.*, 2005).

Nigella sativa (*N. sativa*) (Family Ranunculaceae) commonly known as black seed, have been used for thousands of years as a spice and food preservative, as well as a protective and curative remedy for several

disorders. The *N. sativa* seeds have great medicinal importance and are known to include many medicinal properties (Goreja, 2003; Salem, 2005). Recently, the seeds have been reported to exhibit many pharmacological effects including anti-parasitic/anti-helminthes (Agarwal *et al.*, 1979), anti-cestodal (Akhtar, *et al.*, 1991) and anti-schistosomal (Azza *et al.*, 2005)), anti-bacterial (Hanafy and Hatem, 2003), anti-fungal (Zeinab *et al.*, 2001), anti-viral (Salem and Hossain, 2000), anti-oxidant (Mahmood *et al.*, 2003), anti-inflammatory (Al-Ghamdi, 2001) activities and have been shown to enhance the T-cell mediated immune response (Haq *et al.*, 1999). *Nigella sativa* (known as habbatu sauda in Hausa) is a common medicinal herbs which are used in Nigeria locally to treat many ailments including parasitic infection. However, its anti-malarial properties are yet to be reported in Nigeria. An effort to discover the antimalarial potentials of these plants could make an immense contribution towards overcoming antimalarial resistance problems.

The aim of this study therefore, is to determine the antimalarial effect of methanolic and aqueous extract of *N. sativa* on mice infected with *Plasmodium bergei* NK 65 in vivo.

MATERIALS AND METHODS

Plant Material

The *Nigella sativa* seeds were purchased from TASNEEM HERBAL STORES & GENERAL ENTERPRISES LTD, Guiwa low cost No. 1 Tanki mini Market area Guiwa, Sokoto. The seeds were identified and authenticated in the herbarium of Biological Science Department, Usmanu Danfodiyo University, Sokoto by Abdulazeez Salihu where voucher specimen of *N.sativa* seeds with a specimen number, UDUH/ANS/0106 were deposited. The seeds of *N.sativa* were milled to powder using mortar and pestle in the department of Pharmacognosy, Usmanu Danfodiyo University, Sokoto and stored at room temperature with plastic packaging until use.

Phytochemical screening of the crude extracts of the plants

The crude extracts labeled M (Methanolic extract of *N.sativa*) and A(aqueous extract of N.sativa) were tested for the presence of phenols, tannins, terpenoids, alkaloids, saponin, carbohydrate, flavonoid, anthraquinones, cardiac glycosides and protein using the method of Oyeleke and Manga (2008).

Seed extraction

Extraction of plant materials was done using maceration method. For the methanol extraction of *N.sativa*, 500g of the milled seeds powder was dissolved in 1 litre (1L) of absolute methanol. Similarly 500g of the same powder was dissolved in 1 litre of distilled water for aqueous extraction. The mixtures were allowed to stand for 24hours at room temperature and filtered using Whatman number 1 filter paper. The filtrates were evaporated to dryness at 50^{o}C in a water bath. However, after the complete dryness of the solvent, the methanol extract was found to be 100% oily. The resultant extracts were measured and expressed as a % extract yield of the original sample (Ncube *et al.*, 2008).

$$\text{Percentage (\%) yield} = \frac{E}{O} \times 100$$

Where E= weight of the extract and
O=weight of the original sample

The samples was then kept in a sterile bottle and labeled to prevent mix-up (Oyeleke and Manga, 2008). The crude extracts were dissolved in a sterilized distilled water (1:10) and then serially diluted to the desired concentration (25mg/kg, 50mg/kg and 100mg/kg).

Animals

Swiss healthy albino mice, 6 weeks old with average weight of 24g and 39g were obtained from the animal holding unit, Faculty of Pharmaceutical science, Ahmadu Bello University, Zaria. The animals were housed in well ventilated plastic cages under standard conditions and adequately fed with livestock feed to ensure their survival and optimum health. They were maintained under standard laboratory care, fed with standard animal feeds (Vital Feeds, Jos, Nigeria), and allowed access to water *ad libitum.* All experimental protocols were in accordance with Usmanu Danfodiyo University, Sokoto Research Policy; and ethics and regulations governing the care and use of experimental animals as contained in "Principles of Laboratory Animal Care" (1996).

Acute Toxicity Test

The safety of the extract was evaluated as described by (Lorke, 1983).

Phase I

In this phase, three groups of three each were given the following doses of the extracts; 10mg/kg, 100mg/kg and 1000mg/kg body weight of the extracts. Observation on the adverse effects of the extracts such as tremor, salivation, off feed, time of death was made at regular interval for 24 hours.

Phase II

In this phase, 4 mice were divided into 4 groups each and administered with the fraction at doses of 1,200, 1,600, 2,900 and 5,000 mg/kg, respectively. The animals were again observed for another 24 hours for toxic symptoms after which the LD50 was calculated as geometrical mean of the maximum dose producing no mortality and the minimum dose producing mortality using the formula by Lorke (1983).

$$LD50 = \sqrt{(Do \times D100}$$

Do = Highest dose that gave no mortality
D100 = Lowest dose that produced mortality

Malaria Parasite
Donor infected mice that was used for the experiment was obtained from department of Pharmacology, Faculty of Pharmacy, Ahmadu Bello University, Zaria. The parasitized blood was obtained from the donor infected mice by cardiac puncture into heparinized container and serially diluted with a sterile normal saline.

Animal Grouping
The first twenty mice were randomly divided into five groups of four mice each, four hours after infection. Group one, two and three were administered aqueous extract of *Nigella sativa* through oropharygeal canulla at a dosage of 25mg/dl, 50mg/dl and 100mg/dl respectively daily for 3 days. Group 4 (negative control group) was given appropriate volume of distilled water (10ml/kg). Group five was given 10mg/kg Chloroquine (Positive control). The last 20 mice were also randomly divided into five groups of four mice each, four hours after infection. Group one, two and three of this batch were administered methanol extract of *Nigella sativa* through the same route at a dosage of 25mg/dl, 50mg/dl and 100mg/dl respectively daily for 3 days. The dose of extracts that were used was determined from LD_{50}.

Inoculation of Experimental Mice
Albino mice were infected by intraperitoneal injection of standard inoculums (0.2ml of $1x10^7$infected erythrocyte) from a single donor mice previously infected with *plasmodium berghei* (29% parasitaemia). The blood schizonticidal activities of the seed extracts, Chloroquine (positive group) and normal saline (0.85% as a negative control), were assessed by the 4-day suppressive test (Peters and Robinson, 1992). After 4 hours post-infection, the experimental groups were treated orally with 25mg/kg, 50mg/kg and 100mg/kg doses of the methanolic and aqueous extracts. The positive control group was treated with Chloroquine diluted in distilled water (10mg/kg). All the treatments were repeated for the next 3 days. On the fourth day, blood smears were prepared and stained with Giemsa and parasitaemia were determined. The average percentage suppression of parasitamia was calculated.

Aseptic Method of Blood Colllection from Mice
The mice tail was sterilized with methylated spirit, warmed and placed in a restraining tube. Using a sterile sharp lancet, the tail of the mice was pricked. Blood was collected in a capillary tube as drops appear. Finger pressure was applied to the soft tissue to stop the bleeding (Hoff, 2000).

Preparation of Thick Blood Film and Staining
Thick blood film was prepared by placing two small drops of fresh blood collected from the mice without anticoagulant on a clean grease free glass slide. With a edge of another slide, the drops were mixed in a circular motion over an area about two centimeter (2cm) in diameter. The film was allowed to air dry at room temperature. Before staining, the thick films were laked by placing them in buffer solution to lyse the red cells and to remove haemoglobin so that the parasites can be easily seen (Ochei and Kohatkar, 2008).

The thick film slides were placed in a staining rack, flooded with the 3% Giemsa working solution and allowed to stain for 30 minutes. The film slides were individually dipped briefly in the buffer and excess stain was removed. The slides were allowed to air-dry in a vertical position (Ochei and Kohatkar, 2008).

3.12 Estimation of Percentage Parasitaemia Using Thick Film
Estimating parasite numbers/µl of blood by counting parasites against white cells.

A part of the thick film where the white cells are evenly distributed and the parasites are well stained was selected. Using the oil immersion objective, 100 white blood cells was systematically counted using two hand tally counters and the number of parasites (asexual) in each field covered was estimated at the same time. The counting was repeated in 3 areas of the film and average of the 3 counts was taken. The number of parasites/µl of blood was calculated as follows,

$$\% \text{ Parasitaemia} = \frac{8000 \text{ white cells}/\mu l \times \text{Parasites counted against 100 WBC}}{100}$$

(Cheesbrough, 2008)

Estimation of Percentage Chemosuppression
The percentage chemo-suppression of the parasite multiplication per days was calculated by using the formula,

$$\% \text{ Chemo-suppression } A = \frac{B-C}{C} \times 100$$

(Peters and Robinson, 1992)

Where B = Parasitaemia in study group
C= Parasitaemia in negative control group

Estimation of Packed Cell Volume Using Microhaematocrit Method
The capillary tubes were filled two-third full with a well-mixed blood obtained from the mice. One end of the capillary tube was sealed with plasticine and the filled tubes were placed in the microhaematocrit centrifuge and spun at 12,000 revolutions per minute (rpm) for 5 minutes. The spun tube was placed into a microhaematocrit reader and Packed Cell Volume was read as a percentage (Ochei and Kolhatkar, 2008).

Statistical Analysis
Statistical analysis was carried out using student's t-test to indicate significant level between groups. Results were expressed as mean ± standard error of mean (SEM) and presented as tables. Results considered as statistically significant at $p < 0.05$.

RESULTS

In the present study, the antimalarial activity of *Nigella sativa* seeds (black seed) extract against *Plasmodium bergei* (nk 65) infection in mice is reported. Maceration method was the technique employed in the extraction of the seed of *N. sativa*. The highest yield was obtained from the methanolic extract of *N. sativa* seeds (5.4%) followed by the aqueous extracts of *N.sativa* (4.8%). Preliminary phytochemical screening of the the methanol extract of *N. sativa* seeds showed the presence of alkaloids, tannins, cardiac glycoside, Anthroquinones, flavonoids, terpenoids, and fixed oil in high amount while saponins, carbohydrate, and protein are in trace amounts. The aqueous extracts showed presence of tannins, cardiac glycosides, flavonoids, carbohydrate, terpenoids, fixed oil and protein in high amount while alkaloids using Wagner's test, saponins, and anthroquinones were not detected (Table I).

Table I: Preliminary phytochemical screening results of Methanol and Aqueous extracts of *N.sativa.*

Constituents	Types of tests	ME	AE
Alkaloids	Meyer's test	+++	+
	Wagner's test	++	-
Tannins	$Fecl_3$	++	++
	Lead Acetate test	++	++
Saponins	Froth test	+	-
Cardiac glycoside	Kella-Killiani's test	++	++
Anthroquinones	Borntrager's test	++	-
Flavonoids	NaoH test	+++	++
	Schinoda's test	++	++
Carbohydrate	Mollisch's test	+	++
	Fehling's test	+	++
Terpenoids	Liebermann-Buchard's test	+++	++
Fixed oil	Paper test	+++	+++
Protein	Xanthoproteic test	+	++

Key:
- means absent
+ means trace
++ and +++ means present
ME= Methanol extract
AE= Aqueous extract

There was no mortality recorded on the administration of the *N. sativa* seeds extract at doses up to 5,000 mg/kg. The oral median lethal dose value of the *N. sativa* seeds extract in mice was thus estimated to be greater than 5, 000 mg/kg. According to Lorkes's (1992) toxicity scale, the *N. sativa* seeds extract can thus be classified as relatively non-toxic (Table II).

Table II: Results of the Acute Toxicity study of the Methanol seed Extract of *N.sativa*.

Phase	Groups	no. of animals	Weight (g)	Dose(mg/kg)	O.P	B.C	Mortality
I	I	3	i. 31 ii. 37 iii. 28	10	24hrs	No	No
	II	3	i. 28 ii. 34 iii. 30	100	24hrs	No	No
	III	3	i. 39 ii. 34 iii. 23	1000	24hrs	No	No
II	I	1	34	1600	24hrs	No	No
	II	1	29	2900	24hrs	No	No
	III	1	32	5000	24hrs	No	No

Key: O.P= Observation period, B.C= Behavioral changes

The rodent malaria parasite model was used to investigate the antimalarial activity of the *N. sativa* extract as it is a reliable and highly reproducible *in vivo* model for evaluating new antimalarial agents (Otto *et al.*, 2014). Table III and IV shows that the Packed Cell Volume (PCV) values for study group (25mg/kg, 50mg/kg and 100mg/kg) increased significantly when compared with Negative control but not statistically significant when compared with the positive control. The percentage chemosuppression obtained after

administration of methanol extract of *N sativa* were 91.8, 92.99 and 90.44% at the doses of 25, 50 and 100 mg/kg respectively while the percentage chemosupression after oral administration of the aqueous extract of *N. sativa* obtained were 66.36, 72.73 and 88.18% at the doses of 25, 50 and 100 mg/kg respectively. Both methanol and aqueous extracts showed suppression activities in all groups of mice with the highest values were noted by the 50mg/kg dose for the methanol extract (92.99% suppression) and by the 100mg/kg dose for the aqueous extracts (88.18% suppression) which significantly ($p < 0.05$) decreased the parasitaemia in the infected mice.

Table III: Packed Cell Volume (PCV) and Anti-malarial activity of the methanol extracts of *N.sativa* seeds treated orally against *Plasmodium bergei* (NK 65) in mice.

Treatment Groups	Dose (mg/kg)	PCV (%)	Average Parasitemia	Percentage Chemosuppression
DW	10ml/kg	29.75±3.77**	31400.00±9338.09	00.00
N. sativa	25	47.50±4.20	2800.00±1032.79	91.08
N. sativa	50	53.25±5.73	2200.00±1306.26	92.99
N. sativa	100	43.50±4.67*	3000.00±1366.26*	90.44
CQ	10	55.25±7.27	600±765.94	98.09

Values are presented as Mean ± SEM; Data analysed by one-way ANOVA; * = $p < 0.05$ and ** = $p < 0.001$ versus control; DW = Distilled Water; CQ = Chloroquine; Route of administration = oral.

Table IV: Packed cell volume and Anti-malarial activity of the Aqueous extracts of *N.sativa* seeds treated orally against *P.bergie* (NK 65) in mice.

Treatment Groups	Dose (mg/kg)	PCV (%)	Average Parasitemia(cells/µl)	Percentage Chemosuppression
DW	10ml/kg	29.75±3.76	22000.00±9340.95	00.00
N. sativa	25	40.25±6.85	7400±3829.71	66.36
N. sativa	50	41.00±10.23	6000.00±2732.52	72.73
N. sativa	100	47.25±9.18	2600.00±1366.26	88.18
CQ	10	55.25±7.27	600±765.94	97.27

Values are presented as Mean ± SEM; Data analysed by one-way ANOVA; * = $p < 0.05$ and ** = $p < 0.001$ which are statisticaall significan with positive control (CQ) per column; DW = Distilled Water; CQ = Chloroquine; Route of administration = oral.

DISCUSSION

The packed cell volume (PCV) obtained from this study indicates that there is anemia in malaria by the mechanisms of red cell destruction which is thought to result from hemolysis of parasitized red cells. On treatment with the extracts, the study groups showed an increase packed cell volume (PCV) when compared with the negative control group but is not statistically significant when compare with the positive control group. Anaemia in malaria was supported with the facts that, anaemia correlates with the severity of the malaria infection (Jandl, 1996; Das and Nanda, 1999). Delayed parasite clearance was highlighted by Price *et al.,* (2001) as a significant independent risk factor for anemia; it was also identified as a risk factor for persistent anemia (Price *et al.,* 2001; Obonyo *et al.,* 2007). In this preliminary evaluation and screening of anti-malarial activity of methanol and aqueous extracts of *N.sativa* seeds, the results showed that each of the two extracts have some degree of anti-malarial activity against *P.berghei* infection in the mice. The results revealed that higher doses of the extracts not necessarily caused higher degree of suppression although in case of aqueous extracts the 100mg/dl dose gave considerable degree of suppression when given orally.

The low toxicity observed suggests a wide margin of safety for therapeutic doses of these extracts, which means that they might be relatively safe even at higher doses. These findings are in conformity with the work of Zaoui *et al*., (2002) who reported low toxicity, high LD_{50} values, stabilized hepatic enzymes and organ integrity associated with *N. sativa* extracts.

The chemosuppression produced by the *N. sativa* extract in the current study was in agreement with the previous work of Rakhshande *et al* (2011), who reported an antimicrobial activity of methanol extract of *N.sativa* against a wide range of micro-organisms. The anti-malarial activities exhibited by those extracts were perhaps due to the possible presence of active compounds.

The *N.sativa* seeds have great medicinal importance and are known to include many medicinal properties (Goreja, 2003; Salem, 2005). Secondary metabolites such as alkaloids, saponins, flavonoids, tannins and phenols which were found present in the *N. sativa* extract have been reported to possess antimalarial actions. Alkaloids have been reported to produce antimalarial properties by blocking protein synthesis in *Plasmodium* parasites (Olorukooba *et al*., 2018). Saponins, flavonoids and tannins have been suggested to act as primary antioxidant or free radicals scavengers that can counteract the oxidative damage induced by the malaria parasite (Ezenyi *et al*., 2014). The anti-oxidant effect of

the *N. sativa* oil and its components may represent another mechanism that contributes to its anti-malarial activity. *N.sativa* seeds components inhibit nitric oxide (NO) production in macrophages (Mahmood *et al.*, 2003). Nitric oxide is a potent intracellular parasite killing mechanism in macrophages, and macrophages are crucial in innate immune response. These inhibitory mechanism assist in providing a favourable environment to multiplication of intracellular parasite; however, the inhibition of one killing mechanisms can cause the up regulation of secondary mechanisms which the parasite cannot protect itself against (Antony, 2005). The inhibition of Nitric oxide production causes an increase in tryptophan degradation through indolamine deoxygenase induction in human peritoneal macrophages; this starves the parasite of an essential amino acid, leading to its death. A further anti-inflammatory effect of aqueous extract of *N.sativa* were reported by Al-Ghamdi (Al-Gamdi, 2001) and has been found to be hepatoprotective when liver injury is induced in mice by carbon tetrachloride (Turkdogan *et al.*, 2001). It has also been shown to help to protect against chromosomal aberrations induced as a results of *Schistosoma mansoni* infection.

The anti-malarial activities of *N.sativa* seed extracts observed in this study could have resulted from a single or combined action of these mechanisms, or may be even through a yet to be identified mechanism.

CONCLUSION

The results obtained from this study suggest that *N. sativa extracts* possesses significant antimalarial activity providing the scientific basis for the use of the plant in traditional medicine for the management of malaria. Further research on the phyto-constituents isolation, purification and testing of the bioactive component of this *Nigella sativa* seeds should be carried out as this could lead to the development of more effective broad spectrum antimalarial medication which can be used to treat malaria.

Authors Contributions

This work was carried out in collaboration between all authors. Author OFA and OFS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors KM, THIS and MLU managed the analyses of the study. Authors MKG, SUN and UMI managed the Literature searches. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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