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In vitro and in vivo antimalarial activity of Nigella sativa L. extracts

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The Arabs, Asians and, Traditional Health Practitioners in Mombasa county found in Kenya have been using Nigella sativa L. seeds to traditionally manage malaria associated symptoms that is, headache, fever, chills, loss of appetite among others. The present study investigated in vitro antiplasmodial, in vivo antimalarial activities and safety of different extracts of N. sativa. Five extracts obtained via aqueous extraction and sequential extraction using hexane, dichloromethane, ethyl acetate and methanol were tested against in vitro cultures of Plasmodium falciparum. The most active extracts (methanolic and ethyl acetate) were assessed for cytotoxicity and toxicity. The two active extracts were evaluated in vivo against Plasmodium berghei ANKA strain at 500, 250 and 125 mg/kg/day. On in vitro assay, methanolic and ethyl acetate extracts showed good activity with IC50 of 80.48±12.29 and 69.81±5.24 µg/ml against W2 strain and 31.93±4.31 and 53.79±6.02 µg/ml against D6 strain, respectively. The extracts exhibited weak cytotoxicity on Vero cells and high parasitemia suppression of 75.52 and 75.30% at 500 mg/kg dose of methanol and ethyl acetate extracts respectively. Notably, there was significant decrease (p<0.001) in activity with lower doses of the extracts. The results explain the traditional use of this plant in the Middle East and Mombasa County.

Key words: Nigella sativa L. seeds extracts, Plasmodium, antimalarial activity.

INTRODUCTION

Malaria is one of the most vital parasitic infections common in many developing countries mostly affecting sub-Saharan Africa (Nkumama et al., 2017). Amongst the Plasmodium species affecting human, Plasmodium
Plasmodium falciparum is the chief cause of morbidity and mortality (Maier et al., 2018). Approximately 219 million episodes of malaria were recorded in 2017 globally in comparison to 239 million occurrences in 2010 and 217 million cases in 2016. Notably, about 435, 000 malaria deaths were witnessed in 2017 worldwide, a decrease from 451, 000 approximated deaths in 2016, and 607, 000 in 2010 (WHO, 2018). In Kenya, malaria transmission is prevalent in the western region with the target population being children under the age of 5 years and pregnant women (Kepha et al., 2016). There has been significant reduction in morbidity and mortality due to control programs and scientific interventions (Mogeni et al., 2016). However, malaria remains an essential public health problem globally and in developing countries.

Chemotherapy plays a crucial role in malaria control (Mvango et al., 2018). Effective antimalarial drug should have therapeutic activity and no toxicity on human host (Na-Bangchang and Karbwang, 2009). However, the central drawback to treatment has been the rise of parasite resistance to most antimalarial drugs (Menard and Dondorp, 2017). Chloroquine (CQ) has been used since 1940s until resistance challenged its use (Ecker et al., 2012). However, it has remained the drug of choice for Plasmodium malariae, Plasmodium ovale, Plasmodium vivax, and the uncomplicated P. falciparum in some parts of the world where resistance to CQ is relatively low (Torres et al., 2013). A combination therapy of sulphadoxine and pyrimethamine was later introduced (Horn and Duraisingh, 2014). Afterwards, it incurred resistance associated with dhfr, dhps and pfmdr genes hence ineffective for plasmodium management (Jelinek et al., 1998; Jacques Le Bras and Durand, 2003). Artemisinin-based combination therapies (ACTs) remain the most effective antimalarial medicines available today (Sugiarto et al., 2018; White, 1999). Tolerance of the malaria parasite to ACTs is currently increasing in South-East Asia. Resistance associated with point mutations in the “propeller” region of a P. falciparum kelch protein gene on chromosome 13, also known as k13 or kelch 13 has been established (Ariey et al., 2014). There is a risk of artemisinin resistance spreading to Africa allowing resistant parasites to go global. Therefore, new products for malaria management are necessary to outcompete resistance (Ashley and Phyo, 2018).

N. sativa, also well-known as Black seed belongs to the family of Ranunculaceae. The plant is extensively distributed especially in Middle Eastern Mediterranean region, South Europe, India, Pakistan, Syria, Turkey and Saudi Arabia. There has been rising concern to this plant since its wide discovery of therapeutic potential against many ailments (Ahmad et al., 2013; Kooti et al., 2016). Among the eminent clinical importance, N. sativa has been shown to possess antidiabetic, anticancer and anti-inflammatory activities (Heshmati and Namazi, 2015; Heshmati et al., 2015; Periasamy et al., 2016). It has also been demonstrated to possess antiparasitic activity in the Middle East folk medicine (Razavi et al., 2018).

The previous study conducted using ethanol, aqueous and chloroform extracts of N. sativa demonstrated higher percentages of parasitemia suppression against P. berghei PZZ1/00 strain. Parasitemia reduction of 86.19, 76.94 and 66.92% was recorded upon use of 100, 400 and 100 µL/kg doses of ethanol, aqueous and chloroform extracts respectively (Abdelelah and Zainal-Abidin, 2007). In Nigeria, melantholic and aqueous extracts’ activity of N. sativa against P. berghei (nk 65) infection in mice have been reported (Ashcroft et al., 2018). However, antiplasmodial and antimalarial potential of N. sativa extracts is yet to be documented in Kenya.

Mombasa County located in the Coast of Kenya, which principally was our geographical site is rich in ethnomedical knowledge. The region is highly inhabited by Arabs and Asians who apparently migrated from Asian countries where N. sativa plant originates (Silberman, 1950). This important group of society (Arabs and Asians) together with the Traditional Health Practitioners (THPs) have for a long time used N. sativa to traditionally treat malaria associated symptoms that is, headache, fever, chills, loss of appetite amongst others (Bahekar and Kale, 2013). Lack of affordability, limited access to pharmaceutical treatment and modern treatment amenities are the key reasons (Cunningham, 2001; Katz and Kimani, 1982). This study determined the effect of N. sativa seed extracts on Plasmodium parasite as a crucial step towards seeking effective drug for malaria. In addition, cytotoxicity and toxicity of the bioactive N. sativa extracts were evaluated in order to determine their selective indexes and lethal doses correspondingly. This will offer valuable information towards establishing lead compounds responsible for N. sativa antimalarial activity.

MATERIALS AND METHODS

Plant collection and authentication

N. sativa seed samples of Saudi Arabian origin were collected from Mwembe Tayari market in Mombasa County. The plant was positively identified by a taxonomist and indigenous Mombasa residents who use it traditionally. 2 kg of N. sativa seeds were packed in plastic bags (voucher specimen No; PAS0023) and transported to KEMRI Centre for Traditional Medicine and Drug Research, Nairobi for processing.

Extraction

The seeds were air dried for 72 h and ground using an electric laboratory mill (Christy and Norris Ltd., Chelmsford, England). 400 g of the ground seed material was used for aqueous extraction whereas 1 kg of the material underwent sequential extraction method with different solvents of increasing polarity; hexane, dichloromethane, ethyl acetate and methanol. Briefly, ground plant material (400 g) was extracted in 1.2 L of double distilled water in a water bath at 60°C for 1 h, cooled and filtered using Whatman No 1 filter paper (Whatman, England). The filtrate was then concentrated to dryness by lyophilization.
One kilogramme (1 kg) of the plant was extracted using 1.5 L of the solvents in an order of increasing polarity by maceration at room temperature for 48 h. The soaked plant material was first filtered using gauze followed by Whatman’s No 1 filter paper (Whatman, England). The residue was re-macerated for another 48 h and filtered. The filtrates were then pooled together and concentrated to dryness under reduced pressure using rotary evaporator. The filtered plant residue was dried and the next solvent introduced as per the order of increasing polarity. Finally, the collected crude extracts were further dried in a vacuum drier to remove any possible traces of solvents and later stored at -20°C until use.

**In vitro anti-plasmodial studies using P. falciparum strains**

Two different isolates of laboratory adapted *P. falciparum* cultures were used in the study. These were; Chloroquine Sensitive Sierra Leone 1 (D6) and Chloroquine-resistant Indochina 1 (W2) strains. *In vitro* anti-plasmodial study with *P. falciparum* strains was performed according to Trager and Jensen (2005) method with slight changes. The parasites were maintained in a continuous culture in RPMI 1640 medium supplemented with 10% human serum and hematocrit of O+ red blood cells under a gas mixture of 92% nitrogen, 5% carbon dioxide and 3% oxygen.

All crude extracts of the plant were initially diluted in DMSO (Sigma Chemical Co., St Louis, MO, USA) and then dilution to a concentration of DMSO was reduced to <1% in the final working solution. Distilled water was used to dissolve CQ (positive control). Semi-automated micro-dilution technique that measures the ability of extract to inhibit the incorporation of [3H]hypoxanthine into the parasite was utilized (Desjardins et al., 1979; Le Bras and Deloron, 1983). Briefly, 200 µl of 2% (v/v) suspension of parasitized erythrocytes composed of 1-2% parasitemia was added to all wells except the last well H that was the negative control having uninoculated parasitized erythrocytes. The contents in the plate were then incubated at 37°C supplied with a gas mixture of 3% CO2, 5% O2, and 92% N2 for 48 h. After 48 h, 25 µl of culture medium comprising 0.5 µCi of [3H]hypoxanthine (Amersham International, Buckinghamshire, UK) was added to each well and incubated further for 18 h. Harvesting of the plates was done onto glass fibre filters, washed using distilled water and dried. The glass fibre filters were smeared with 25 µl liquid scintillation fluid. The filters were measured for radioactivity using micro-beta plate scintillation counter (Wallac MicroBeta TriLux).Counts per minute (CPM) were obtained and then used to compute the IC50 values (Sixsmith et al., 1984). Non-linear regression analysis enabled calculation of the concentration of test samples that inhibited 50% (IC50) growth. This calculation was carried out using Chemosensor Program 2 according to the following formula;

\[
\text{IC}_{50} = \text{antilog} \left( \text{log}X1 + \left[ \text{log}Y50 - \text{log}Y1 \right] \times \text{log}X2 - \text{log}X1 \right) / \text{log}Y2 - \text{log}Y1
\]

Where, Y50 is the Counts per minute (cpm) value midway between parasitized and non-parasitized control cultures, and X1, Y1, X2, Y2 are the concentrations and cpm values respectively for the data points above and below the cpm midpoints (Sixsmith et al., 1984).

**In vivo assay**

**Host, environment and parasites**

Male Swiss albino mice, 6-8 weeks old, weighing 20±2 g bred in the KEMRI animal facility Nairobi were used as subjects. Test mice were housed in experimental rooms in standard microlon type I cages, well labeled with experimental details in air-conditioned rooms at room temperature and 60-70% relative humidity. They were well nourished with commercial rodent feed and water ad libitum. Five mice were used for every cage per test sample. *P. berghei* ANKA parasites were used to determine parasite reduction in mice.

**Four days parasite suppression test**

Four days parasite suppression test study was conducted according to Peters (1975) protocol with slight adjustments. *P. berghei* strain ANKA infected blood was obtained by heart puncture from donor mouse and mixed with 1% (w/v) heparin. The infected blood was diluted in physiological saline to approximately 10⁸ parasitized erythrocytes per ml. The test animals were infected by intraperitoneal injection with 0.2 ml (2×10⁸ parasitized erythrocytes) and randomly grouped. The experimental groups were treated orally with 0.2 ml single dose of 125, 250 and 500 mg/kg of the test sample, 2 h post infection (D0) (Gessler et al., 1995). Only, extracts active as per in vitro assay were used (methanolic and ethyl acetate extracts). Two control groups comprising five mice each were treated with a placebo (vehicle; 3% dimethyl sulfoxide, 10% tween 80 in PBS) and 5 mg/kg of CQ for negative and positive controls respectively. The mice were consecutively treated orally for 3 days (D1, D2, and D3) with equivalent doses. Blood films were taken on the fifth day (D4) from tail snips, fixed in methanol and stained with 10% Giemsa stain (Abdela et al., 2014). Microscopic examination of thin blood film provided parasitemia counts in 4 fields of ~100 erythrocytes per view. The difference between mean numbers of parasites per view in the negative control group and those of the test groups were calculated and expressed as percentage suppression (chemo suppression) according to the formula (Tona et al., 2001):

\[
\text{PS} = \frac{A-B}{A} \times 100
\]

Where; A=Mean parasitemia in the negative control group on day 4, B=Corresponding parasitemia in the test group.

The mean survival time (days) for each group was determined over a period of 30 days post infection.

**Toxicity evaluation**

**Cytotoxicity evaluation**

The MTT assay that is based on the principle of conversion of yellow tetrazolium MTT to purple formazan dye by mitochondrial dehydrogenases of living cells was used for cytotoxicity determination (Van Meerloo et al., 2011). Vero cells obtained from kidney epithelial cells of the African green monkey, stored in liquid nitrogen in KEMRI; Centre for Traditional Medicine and Drug Research were used. The Vero cells were grown in Eagle’s minimum essential medium, supplemented with 5% foetal bovine serum (FBS) in 25 ml cell culture flasks. They were incubated at 37°C in 5% CO2 incubator (Kurokawa et al., 1995). The cells were sub-cultured three times a week to achieve 80% confluence. Afterwards, the cells were seeded with 5 × 10⁶ cells per well in 96-well plates. They were then incubated at 37°C for the next 2 days. Fresh MEM (GIBCO, Grand Island, NY) containing test extracts at different concentrations was added to replace the culture medium and later incubated for another 2 days. Triplication of the cells for each sample was done in triplicate wells to allow detachment. A hemocytometer was used to count viable cells. Data from inhibition was then plotted as dose-response curves and CC50 (concentration able to cause visible alterations in 50% of intact cells) was determined. Selectivity index (SI) was used as parameter of clinical significance of the test samples by comparing general toxins and
Table 1. In vivo antiplasmodial activity of five extracts.

| Extract/drug | Extract yield (%) | W2 IC_{50} (Mean±SD) (µg/ml) | D6 IC_{50} (Mean±SD) (µg/ml) |
|--------------|-------------------|-------------------------------|-----------------------------|
| H_{2}O       | 7.50              | >200                          | >200                        |
| Hexane       | 6.00              | >200                          | 151.25±22.96                |
| DCM          | 3.00              | 138.25±14.31                  | 141.34±10.23                |
| MeOH         | 2.75              | 80.48±12.29                   | 31.93±4.31                  |
| EtOAc        | 3.00              | 69.81±5.24                    | 53.79±6.02                  |
| Control (CQ) |                   | 65.13±17.17 ng/ml             | 13.54±1.20 ng/ml            |

The results are expressed as mean ± SD of mean of IC_{50}s of the extracts and control (CQ).

**RESULTS**

**In vitro assay**

The results from the in vitro antiplasmodial assay of five different extracts of *N. sativa* against the two strains of *P. falciparum* and respective extract yields are listed in Table 1. The ethyl acetate and methanolic extracts showed good activity against D6 and W2 strains. However, best activity was exhibited in methanolic extract followed by ethyl acetate extract against D6 strain. Notably, ethyl acetate extract demonstrated better activity against W2 strain when compared to methanolic extract. Water extract had IC_{50} >200 µg/ml thus no activity against the two strains of *P. falciparum*. Hexane and DCM extracts proved inactive against D6 and W2 strains with IC_{50} >130 µg/ml. Adopted categorization of antiplasmodial activity was as follows; high (IC_{50} ≤ 10 µg/ml), moderate (IC_{50} 10-50 µg/ml), low (IC_{50} 50-100 µg/ml) and inactive (IC_{50} >100 µg/ml) (Gathirwa et al., 2008).

**Cytotoxic activities**

The two active extracts (methanolic and ethyl acetate) were tested for cytotoxicity and exhibited relatively weak cytotoxicity against Vero cells with CC_{50}s of 164.13 and 73.66 µg/ml respectively with low selectivity index indicating mild cytotoxicity as shown in Table 2.

**In vivo antimalarial activity of methanolic and ethyl acetate extracts of *N. sativa* against *P. berghei* ANKA infected Swiss albino mice.**

The antimalarial activity of the two potent extracts (methanolic and ethyl acetate) against *P. berghei* ANKA infected Swiss albino mice by the 4-day suppressive method is presented in Table 3. Chemosuppression was observed in a dose dependent manner after four days of antimalarial screening of *N. sativa* extracts (125-500 mg/Kg). The mean parasitemia in the groups treated with methanolic extract ranged from 4.03±0.67 to 7.18±0.73 while that of animals treated with ethyl acetate extract
Table 2. Cytotoxicity (CC50) of methanolic and ethyl acetate extracts to Vero cells.

| Extract | CC50 (µg/ml) | SIa (W2) | SIa (D6) |
|---------|-------------|----------|----------|
| MeOH    | 164.13      | 2.04     | 5.14     |
| EtOAc   | 73.66       | 1.06     | 1.37     |

a SI-selectivity index calculated as CC50/IC50.

Table 3. Mean of parasitemia, % parasitemia suppression and survival time after treatment termination.

| Drug/ extract | Dose (mg/Kg) | Mean ± SD parasitemia (%) | % suppression of parasite | Mean survival time (days) |
|---------------|-------------|---------------------------|--------------------------|--------------------------|
| MeOH          | 125         | 7.18±0.73                 | 56.29                    | 10.50±0.58               |
|               | 250         | 5.58±0.39                 | 66.05                    | 11.25±0.96               |
|               | 500         | 4.03±0.67                 | 75.52                    | 16.25±0.96               |
| EtOAc         | 125         | 6.75±0.31                 | 59.02                    | 11.00±0.82               |
|               | 250         | 4.72±0.19                 | 71.27                    | 12.40±1.14               |
|               | 500         | 4.04±0.87                 | 75.30                    | 15.60±1.52               |
| CQ            | 1.20±0.23   | 92.59                     |                          | 28.25±1.50               |
| Vehicle (3% dimethyl sulfoxide, 10% tween 80 in PBS) | 16.45±3.08 | 5.00±0.82 |

The results are expressed as mean ± SD.

varied from 4.04±0.87 to 6.75±0.31. The mean parasitemia in the negative control group was 16.45±3.08. There was a significant percentage parasitemia difference between the test groups when compared with the untreated control group (p< 0.001). The extracts demonstrated highest percentage parasitemia reduction at 500 mg/Kg. However, there was a slight difference in percentage parasitemia reduction between the positive control and the two extracts. The extracts were able to prolong survival of the animals after treatment termination (p < 0.001) in comparison to the negative control.

Acute toxicity study

The acute toxicity study results indicated that the extract caused no mortality at a dose of 1000-2000 mg/Kg within the first 24 h as well as subsequent 14 days. There were no physical and behavioral signs of over-toxicity like; loss of appetite, inability of movement, diarrhea amongst others. This suggests that LD50 of the extracts is greater than 2000 mg/Kg.

DISCUSSION

There is need to seek improved antimalarial drug substitutes in order to fight resistance against P. falciparum (Mueller et al., 2000). The most dependable source of remedy is possibly medicinal plants (Gasquet et al., 1993; Wright and Phillipson, 1990). The world is a rich source of medicinal plants and more research needs to be conducted to exploit their uses. Quinolones and artemisinin derivatives, the most widely effective antimalarials were principally obtained from traditional plants (Waako et al., 2005). N. sativa, having been used traditionally to heal many ailments, it has been mentioned to contain antiparasitic activity although underutilized in antimalarial studies (Kooti et al., 2016). This study employed P. falciparum in vitro culture and P. berghei ANKA; in vivo model which takes into account the prodrug effect and involvement of the immune system in infection eradication for prediction of treatment outcomes. Many conventional antimalarial agents such as chloroquine, mefloquine and lately artemisinin derivatives have been established using rodent malaria model (Waako et al., 2005). The four day suppressive test mostly assesses the antimalarial activity of candidates on early periods of infection (Verma et al., 2011). The best antimalarial drug should be safe without adverse effects (Saito et al., 2018). As a result, cytotoxicity and toxicity studies were appropriate to identify potential application of N. sativa in malaria management. The results indicate that the concentrations of the extracts used to carry out the in vivo experiments were nontoxic with no mortality recorded during the first 24 h and subsequent 14 days at 1000-2000 mg/Kg dosage. Experimental animals survived
for the entire 4 days of the 4 day suppressive test without mortality. Usually, when the mice die in the course of the 4’ Day suppressive test or before the fifth day, the death is attributed to the safety of the test drug rather than the animal parasitemia levels (Satayavivad et al., 1998). Therefore, cytotoxicity and toxicity studies of the plant were important to determine selective indexes and lethal doses correspondingly. However, a weak cytotoxicity of IC_{50}s of 164.13 and 73.66 µg/ml with low selectivity index for methanolic and ethyl acetate extracts respectively was recorded. Al-Sheddi et al. (2014) noted earlier that N. sativa is weakly cytotoxic at higher concentrations. Additionally, our findings agree with those of Zaoui et al. (2002) that demonstrated relatively low toxicity levels and organ stability upon use of N. sativa extracts. This suggests that at lower concentrations, N. sativa extracts can be used safely to manage malaria. Therefore, the cytotoxicity and toxicity study justifies the safety of the plant in treating malaria.

The extracts demonstrated significant (p<0.05) parasitemia reduction activity in all the antimalarial evaluations. The in vitro study showed antiplasmodial activity of the five different extracts enabling the selection of the most effective extracts. Methanolic and ethyl acetate extracts showed the best activity with IC_{50}s of 80.48±12.29 and 69.81±5.24 against W2 strain and 31.93±4.31 and 53.79±6.02 against D6 strain respectively. These two active extracts also demonstrated good activity in vivo. The in vivo assay showed significant reduction in percentage parasitemia on the test groups compared to negative control group (p<0.001). These indicates the need for more studies on the use of the two solvents for extraction of active antimalarial constituents from N. sativa as shown by Mzena et al. (2018) in a different antimalarial plant study. The presence of single or diverse bioactive compounds possibly present in the seed extracts might have played a greater role in the antimalarial activity portrayed by the plant. Our results concurred with the findings of Ashcroft et al. (2018), who recorded much higher parasitemia suppression (>90%) upon use of methanolic extracts of N. sativa seeds. However, their results upon using aqueous extracts differed significantly with our findings as they reported up to 88.18% parasitemia reduction. Nonetheless, their seeds origin was different from ours. As a result, this may influence the bioactive compounds present in the seeds thus the activity. Mahmood et al. (2003) explained that the possible constituents of N. sativa have the ability to obstruct production of nitric oxide (NO) in macrophages. This in turn leads to escalation of tryptophan degradation via indolamine deoxygenase stimulation in human peripheral macrophages. Consequently, the parasite dies as it is deprived of a vital amino acid. The immunomodulatory properties of N. sativa seeds that have been exhibited in past work done by Haq et al. (1999), might largely contribute to host-parasite interaction as noted earlier on by Anthony et al. (2005). Consistent with this model, Salem (2005) attributed anti-microbial effects in his study to the immunomodulatory properties of N. sativa seeds constituents. The abovementioned factors might have singly or together achievable supported the antimalarial effects of the seed extracts exhibited in this study. The limitation of the study is that identification of specific compounds responsible for antimalarial activity was not attained as this was beyond the scope of the current study. We therefore recommend that further analysis should be done on N. sativa seeds to identify the specific antimalarial compounds present.

Conclusion

The results in this study provide room for future comprehensive investigations on the plant through bioassay-guided fractionation, isolation and characterization of the bioactive compounds leading to establishment of novel antimalarial compounds fighting against drug resistant malaria. This study explains the use of N. sativa plant traditionally in the Middle East folk medicine and in Mombasa County.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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