Nutritional Intervention and Therapeutic Use of Azadirachta Indica (Neem) Fruit Juice as It Clears Malaria Parasites and Replenishes Depleted Blood Levels in Plasmodium Berghei Infected Swiss Albino Mice

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Abstract

One of the major pathogeneses of malaria parasite infection is the invasion and destruction of the Red Blood Cells, which requires the synergistic administration of antimalarial and most times essential nutrients for effective treatment. Previous studies have shown that various non-edible parts of Azadirachta indica tree may have anti-malarial potentials, hence the investigation on the effects of edible fresh juice of Azadirachta indica fruit on various hematological parameters in addition to its potential to clear Plasmodium berghei in infected mice. The phytochemical constituents of A. indica fruit juice were carried out by Gas Chromatography-Mass Spectroscopic (GC-MS) method. Determinations of creatinine, urea, and lipid profile were carried out using Auto-analyzer. The chromatogram of GC-MS analysis of A. indica fruit juice showed four (4) peaks as follows dodecanoic acid (4.37%), oleic acid (14.19%), 13-octadecyl (17.05%) and 15-tetracosanoic acid methyl ester (47.13%) as the major constituent. The unique pathway for plasmodial fatty acid synthesis has become a possible target of drug action. Adult male mice were placed in six groups (n = 6). Group A mice were fed a normal diet and water ad libitum only while groups B to E were fed a normal diet and water ad libitum and further infected with Plasmodium berghei. The parasitemia was confirmed on the third day of infection. Groups C, D, E, and F were further given 5mg/kg body weight of Artesunate, 4.3ml/kg, 8.6 ml/kg, and 12.9 ml/kg of fruit juice respectively for fourteen days and then sacrificed. The percentage of parasitemia in infected groups was very high but treatment with Artesunate and various doses of fruit juice significantly reduced the percentage of parasitemia. Interestingly, 0.86mg/kg dose of the fruit juice caused a high significant reduction in the percentage of parasitemia comparable to the potency of Artesunate. Additionally, infection with Plasmodium berghei yielded a significant decrease in the levels of various hematological indices in the infected mice which were normalized with the administration of Artesunate and the fruit juice of Azadirachta indica. Fruit juice of Azadirachta indica has been shown to have novel dual benefits of clearing malaria parasites, serving as a blood tonic and nutritional supplement.

INTRODUCTION

Malaria is one of the most common chronic diseases that affect mainly people in the tropical regions of the world. It is about the most important tropical parasitic diseases in Sub-saharan Africa. World Health Organization (WHO) estimated around 300–500 million acute clinical malarial cases every year and around 1 million deaths every year due to malaria parasitemia [1]. It is currently a major global concern particularly in malaria pandemic regions due to the morbidity rate and economic burden. In 2016, WHO reported about 216 million cases of malaria with about 445, 000 deaths due to malaria worldwide [2]. More so, within the pandemic regions of the world, the greatest prevalence of malaria is in India and Africa. According to World Health Organization Regional Office for Africa in 2015, about 88% of global cases of malaria and 90% of global deaths due to malaria occurred in the African region [3].

Furthermore, in Africa particularly Nigeria, the prevalence is relatively high perhaps due to the humid environments, poor drainage system, poor attitude towards environmental cleanliness, ignorance, lifestyle, and sleeping habit. In general, malaria is associated with lots of resistance to various
antimalarial drugs due to abuse, fever, and other chronic conditions such as migraine headache, psychiatric conditions, and destruction of RBC leading to anemia and cerebrovascular diseases [4, 5]. Despite recent pharmaceutical development of several drugs for effective treatment and management of malaria, this infection is yet to be eradicated in many countries of the world particularly in the malaria pandemic regions [6]. Presently available drugs respond to three classes of compounds: (a) aryl amino alcohol compounds such as quinine, (b) antifolates-dihydrofolate reductase inhibitors such as pyrimethamine, and (c) artemisinin derivatives. Artemisinin was first isolated in 1970 by Chinese scientists from *Artemisia annua* [7, 8].

The quinoline-containing antimalarial drugs, chloroquine, quinine, and mefloquine, are important components of chemotherapy against malaria. These drugs act by accumulating and interfering with the digestion of hemoglobin in the blood stages of the malaria life cycle. Chloroquine is a dibasic drug that diffuses down the pH gradient to accumulate about 1000-fold in the acidic vacuole of the parasite. The high intravacuolar concentration of chloroquine inhibits the polymerization of haem and during hemoglobin breakdown, it builds up to poisonous levels, thereby killing the parasite with its toxic waste [9]. The mechanism behind the antimalarial activity of antifolates is their inhibition of the enzymes dihydrofolate reductase (PfDHFR) and dihydropteroate synthase (PfDHPS), both of which are involved in the folate metabolic pathway. PfDHPS is present in Plasmodium cells, and there is no equivalent enzyme in human cells. Doxycycline (DOX) is a key antimalarial drug thought to kill Plasmodium parasites by blocking protein translation in the essential apicoplast organelle [10]. Artemisinin is believed to act via a two-step mechanism. Artemisinin is first activated by antiparasitic haem-iron which catalyzes the cleavage of this endoperoxide. A resulting free radical intermediate may then kill the parasite by alkylating and poisoning one or more essential malarial protein(s) [11]. Additionally, most drugs currently used in the clinical treatment of malaria usually present patients with various side effects in addition to the current issue of drug resistance and associated hidden hunger conditions [12–15]. The drug metabolism and clearance from the body further increases metabolic burden on the body systems, especially the liver and kidneys leading sometimes to toxicity with consequent damage to important tissues and organs [16, 17]. More so, due to the destruction of the RBC – a characteristic feature of the pathogenesis of malaria [5, 18], most clinical prescriptions of antimalarial drugs are often accompanied with supplements to cushion their side effects and boast blood levels [19]. This, however, increases the cost of medication with a consequent economic burden [20]. Hence, an urgent need for concerted efforts into identifying novel treatment options perhaps using nutraceuticals (natural products), which could have both therapeutic and nutritional potentials, thus helping in the alleviation of this global menace.

Treatment of malaria should be of high priority to endemic areas like Africa due to the magnitude of the problem associated with the infection, side effects, loss of man-hours and resources, high cost of medicine, problems of resistance, and death. These call for multiple approaches to tackle this worldwide problem. One of the recent considerations in nutritional intervention in health and diseases is to identify unique parasite biochemical pathways that may serve as targets for new drugs and seek possible nutritional intervention with therapeutic potentials that may inhibit unique parasite biochemical pathways. These nutrients will not only provide supplementations, and reduce the high cost of drugs but
as organic materials, would not add to the metabolic burden on the liver in the xenobiotic transformation and side-effects of chemically synthesized drugs that generate reactive oxygen species in their metabolism, which have been implicated in hepatotoxicity, neurotoxicity, genotoxicity and DNA damage, deleterious oxidative processes, inflammation, aging, and other diseases.

In traditional African society like in Igboland, people that manifest symptoms of malaria and fever are advised to take red palm oil and lipid chemistry indicates that fats and oil are fatty acid esters of alcohols. Consequently, there are new advances in Fatty Acids as antimalarial, antimycobacterial, and antifungal agents [21]. The new developments concerning fatty acids as antimalarial agents and the importance of enzyme inhibition, in particular, the inhibition of the enoyl-ACP-reductase (Fab I) of *P. falciparum*, the principal agent responsible for malaria have attracted fresh attention from scientists. *Plasmodium falciparum*, the most deadly malaria parasite of the phylum Apicomplexa, has been found to contain an apicoplast, an organelle that originally arose from a cyanobacterium through a secondary endosymbiotic process and thus possesses two membranes that may imply its resistance to certain drugs [22].

*Azadirachta indica* is locally known as neem. It is a tree in the mahogany family of Meliaceae and native to India, Bangladesh, Thailand, and Nepal. It also grows well in tropical and sub-tropical regions of the world. Neem is one of the most important medicinal plants that have been declared worldwide as the “tree of the 21st century” by the United Nations. The tender shoots and flowers of the neem tree are eaten as vegetables in India. The neem tree plant has several medicinal and therapeutic applications [23]. Extracts from *Azadirachta indica* leaf have been reported to have antimalaria activities [24]. The medicinal and therapeutic potentials may be attributed to the nutrients and phytochemical constituents. These phytochemicals are secondary metabolites produced by plants to enable them to adapt to harsh climatic conditions, attract pollinating insects, or prevent them from being eaten up.

In Africa, leaves, seeds, roots, bark, and the flowers of the neem tree are used in the treatment of different diseases such as jaundice, stomach ulcers, and several parasitic diseases [25]. A large amount of scientific evidence is available on the potential of various parts of *Azadirachta indica* tree due to their endowments as a source for the development of human and animal healthcare products. For example, neem seed oil and essential oils from leaves and bark have been shown to inhibit the growth of various genera of pathogenic bacteria such as Mycobacterium and several parasitic protozoa including Plasmodium [26].

Farahna [27] reported the anti-plasmodia effects of *Azadirachta indica* in experimental cerebral malaria in mice. Isah [28] and Tepongning [29] reported the antimalarial properties and standardization of tablets of *Azadirachta indica* (Meliaceae) in mice. *Azadirachta indica* ethanol extract protects neurons from apoptosis, and inflammation and mitigates brain swelling in experimental cerebral malaria [30–32].

Furthermore, the fruit juice of *Azadirachta indica* has been shown to contain large amounts of nutritional constituents [33]. However, its medicinal potentials are yet to be investigated and further exploited for possible therapeutic uses. Doves and bats are frequently seen feasting on the fruit juice and discarding
the seeds after sucking the succulent juice from the fruit. It was suspected that animals use instinct to source cures for diseases and as such, it could be a result of Avian malaria that directs birds' instincts to fruit juice of *Azadirachta indica* both for cure and nutrient source. Following this animal instinct, we hence investigated whether the fruit juice extract of *Azadirachta indica* could have an anti-plasmodial effect on Swiss albino mice infected with *Plasmodium berhei* (NK 65) and if it could as well enhance the depleted levels of selected hematological parameters in the mice as a source of nutraceuticals.

**MATERIALS AND EXPERIMENTAL DESIGN**

The large amount of fresh ripe fruits of *Azadirachta indica* was harvested from the 2nd Baze Bar at Abakaliki, Ebonyi State, Nigeria where the trees were planted as shade trees for relaxation. They were washed with distilled water and the fresh juice was extracted using a juice extractor.

**GC-MS Analysis of Fruit Juice of A. indica:** The fruit juice of *A. indica* was transferred into a 2ml vial. GC-MS analysis on the fruit juice was carried out using an Agilent 7890A-5975C GC-MS system employing the following conditions: HP5-column (30m x 0.25mm x 0.25µm), operating in electron impact mode at 70eV; carrier gas flow (a constant) = 1ml/min. Injection volume = 0.5µl, split ratio = 10:1, injector temperature = 250°C, ion source temperature = 280°C, oven temperature, Initial = 110°C (hold 2mins), 110°C to 200°C at 10°C/min, 200°C to 280°C at 5°C/min (hold 9mins) and mass spectra were taken at 70eV. Interpretation of mass spectrum GC-MS was conducted using the National Institute of Standards and Technology (NIST) Database. The spectrums of the unknown components were compared with the spectrum of known components stored in the NIST library. The name, molecular weight, formula, retention time, and structure of the components of the test materials were ascertained.

**Experimental Animal and Extract Administration**

Adult, male Swiss albino mice were obtained from the Animal House of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka, Nigeria. They were acclimatized for 7 days in aluminum cages in the Animal House of the Department of Biochemistry, Ebonyi State University, Abakaliki, before treatment. During this period, they were fed with commercial growers' mash (pellets). Clean water was provided daily and animals were allowed free access to food and water *ad libitum*. At the end of the seven (7) days acclimatization period, the animals were weighed and randomly placed into six different groups (*n* = 6). Each group was kept in a separate cage while the weights were used to determine the equivalent dose of 300, 600, and 900 ml/kg body weight respectively on the basis that a 70 kg body weight man could take a single glass of juice (300 ml) daily either to facilitate digestion or as a beverage.

**Parasitemia**

A quantity (1ml) of plasmodium-infected blood was dissolved in normal saline. Using a syringe, the dissolved plasmodium was injected via the intra-peritoneal route into the mice. The mice were allowed for 72 hours before blood samples were collected from the tail for staining, microscopic examination, and confirmation of parasitemia. Only mice that had the plasmodium parasite were further used for the study.
Grouping

Mice in group A were fed a normal diet and water *ad libitum* and served as normal control. Groups B to E mice were fed a normal diet and water *ad libitum* and further infected with *Plasmodium berghei*. Group B mice were left untreated. Group C animals were further treated with 5mg/kg body weight Artesunate as the standard drug treatment group. Groups D and E were also treated with the fruit juice of *Azadirachta indica* fruit twice daily through oral intubation according to their body weight at doses of 4.3 ml/kg, 8.6 ml/kg, and 12.9 ml/kg body weight respectively, and served as test groups. Artesunate is a semi-synthetic derivative of artemisinin used primarily as a treatment for malaria and as a standard drug in the laboratory for malaria studies. The animals were treated as described in the grouping for seven (7) days.

Blood Sample Collection and Analysis

After 7 days, the rats were fasted overnight, sacrificed in anesthetized with halothane, and the blood was collected by cardiac puncture, using 5ml syringes into EDTA vacutainers for determination of packed cell volume (PCV) which is the percent of the packed red cells in a volume of whole blood was determined using the standard method. The PCV, hemoglobin count, differential count, erythrocyte, and white blood cell counts were determined using a hemocytometer. The parasitemia was determined using the microscopic method, which is a gold standard for laboratory confirmation of parasitemia.

Statistical Analysis

Data were expressed as mean ± SEM and all statistical analyses were performed using Graph Pad Prism 5.04 (GraphPad, La Jolla, CA). The statistical test was done using one-way ANOVA and then the means were compared with Dunnett’s test. In some cases, the Student’s t-test was subsequently used after one-way ANOVA to obtain the statistical trend between any two groups of interest. In general, p < 0.05 was considered as the statistical significance level.

Result

The chromatogram of GC-MS analysis of *A. indica* fruit juice showed four (4) peaks with dodecanoic acid (4.37%), oleic acid (14.19%), 13-octadecyl (17.05%) and 15-tetracosanoic acid methyl ester (47.13%) as the major constituent (Table 1).
Table 1
Gas Chromatography-Mass Spectroscopy (GC-MS) Constituents of *A. indica* Fruit juice

<table>
<thead>
<tr>
<th>Peak</th>
<th>Compound</th>
<th>Retention time</th>
<th>% Total Area</th>
<th>Bioactivity/ Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dodecanoic acid</td>
<td>41.83</td>
<td>4.37</td>
<td>Dodecanoic acid is a straight-chain, twelve-carbon medium-chain saturated fatty acid with strong bactericidal properties; the main fatty acid in coconut oil and palm kernel oil. It has a role as a plant metabolite, an antibacterial agent, and an algal metabolite.</td>
</tr>
<tr>
<td>2</td>
<td>Oleic acid</td>
<td>45.44</td>
<td>14.19</td>
<td>Oleic acid is a fatty acid that occurs naturally in various animal and vegetable fats and oils. Oleic acid is found to have antibacterial activity, particularly in inhibiting the growth of several Gram-positive bacterial species.</td>
</tr>
<tr>
<td>3</td>
<td>15-tetracosanoic acid methyl ester</td>
<td>46.55</td>
<td>47.13</td>
<td>Tetracosanoic acid is a C24 straight-chain saturated fatty acid. It has a role as a volatile oil component, a plant metabolite, a human metabolite, and a <em>Daphnia tenebrosa</em> metabolite. It is a very long-chain fatty acid and a straight-chain saturated fatty acid. It is a conjugate acid of a tetracosanoate.</td>
</tr>
<tr>
<td>4</td>
<td>13-Octadecenal</td>
<td>61.00</td>
<td>17.05</td>
<td>(Z)-13-Octadecenal is an organic compound that belongs to the group of alpha, beta-unsaturated aldehydes. It has been found to have antimicrobial activity against <em>Pseudomonas aeruginosa</em> and may be useful as a potential antibacterial agent. The compound has been shown to inhibit bacterial growth by binding to the pyruvate form of the enzyme pyruvate: ferredoxin oxidoreductase. This binding prevents the formation of an enzyme-substrate complex and inhibits pyrite oxidation in bacterial cells, which is essential for energy production. (Z)-13-Octadecenal also has insecticidal properties and can be used as a pheromone or as a constant flow system for gas chromatography.</td>
</tr>
</tbody>
</table>

Phytochemical Composition

The fruit juice of *Azadirachta indica* was found to contain some active phytochemicals such as terpenoids, tannins, alkaloids, glycosides, flavonoids, total phenols, saponins, and carotenoids in concentrations of 38.74 ± 2.78, 34.34 ± 3.84, 16.25 ± 1.30, 4.00 ± 0.35, 3.91 ± 0.06, 3.10 ± 0.26, 1.07 ± 0.35 and 0.07 ± 0.01 mg/100ml respectively (Fig. 1). Resistance of the plasmodium parasite to the existing drugs, including chloroquine, mefloquine, and artemisinin-based combination therapy (ACT), is a serious global issue in malaria treatment and control. This warrants an urgent quest for novel compounds with antiplasmodial activity, particularly from natural sources such as medicinal plants. Alkaloids have over the years been recognized as important phytoconstituents with interesting biological properties. The first successful antimalarial drug was quinine, an alkaloid, which was extracted from the Cinchona tree [34].
Several classes of phytoconstituents are responsible for the antimalarial activity of plants including alkaloids, terpenes, steroids, and flavonoids. Alkaloids are considered an important group exhibiting diverse biological activities, particularly antimalarial activity. They constitute an important class of structurally diversified compounds that are having the nitrogen atom in the heterocyclic ring and are derived from amino acids. Large numbers of alkaloids have been isolated from different plant sources and reported for their potent antimalaria activity [35].

Several alkaloids having varying terpenoid backbones exhibit antiplasmodial activity. Terpenes and terpenoids also possess a wide range of biological activities including anticancer, antimicrobial, anti-inflammatory, antioxidant, and antiallergic. Common dietary flavonoids, including quercetin and kaempferol, have been reported to inhibit the in vitro viability of 3D7 *P. falciparum* [36, 37]. Tannins have been shown to exhibit antiplasmodial activity against *Plasmodium falciparum* [36].

**Effect of Fruit Juice on Percentage Parastemia**

Administration of specific doses of *Azadirachta indica* fruit juice significantly (p < 0.05) cleared plasmodium parasites in male albino mice infected with *Plasmodium berhei* (NK 65). *Plasmodium berhei* (NK 65) parasite was found to be high in mice infected with the parasite and left untreated. Artesunate which is a standard drug in the family of Artemesinin-based drugs currently used clinically for the treatment of malaria significantly reduced the levels of *Plasmodium berhei* (NK 65) in the infected mice. Interestingly, it was observed that treatment with various doses of *Azadirachta indica* fruit juice also reduced the levels of *Plasmodium berhei* (NK 65) in the infected mice. More interestingly, treatment with the fruit juice dose of 200 mg/kg body weight of mice reduced the levels of *Plasmodium berhei* (NK 65) to a level comparable to the effect of the standard drug. This suggests that the fruit juice of *Azadirachta indica* may have antiplasmodial potential comparable to a standard antimalarial drug, Artesunate.

Administration of specific doses of *Azadirachta indica* increased both red blood cells and haemoglobin levels in male albino mice infected with *Plasmodium berhei* (NK 65).

Red blood cells and haemoglobin were significantly decreased in male albino mice infected with *Plasmodium berhei* (NK 65). As expected, after seven days of administration of various doses of *Azadirachta indica* fruit juice, both levels of red blood cells and haemoglobin were significantly increased.

**Administration of specific doses of Azadirachta indica normalizes packed cell volume in male albino mice infected with Plasmodium berhei (NK 65).**

Infection of male albino mice with *Plasmodium berhei* (NK 65) resulted in a significant decrease in its packed cell volume. Conversely, after seven days of administration of various doses of *Azadirachta indica* fruit juice the packed cell volume of the male albino mice was significantly normalized.

**Administration of specific doses of Azadirachta indica normalizes White Blood Count in male albino mice infected with Plasmodium berhei (NK 65).**
As expected, the levels of White Blood Cells were significantly decreased in male albino mice infected with *Plasmodium berhei* (NK 65). However, administration of various doses of *Azadirachta indica* fruit juice in male albino mice infected with *Plasmodium berhei* (NK 65) significantly normalized the decreased levels of White Blood Cells in the mice.

**DISCUSSION**

A large amount of mature and fresh ripe fruits of *A. indica* was harvested from Abakaliki, Ebonyi State, Nigeria. Fresh ripe fruits of *A. indica* were collected from the Abakaliki area of Nigeria. The analysis was carried out by GC-MS and 4 compounds were identified. Most of the compounds that were identified are presented in Table 1. Different fatty acid methyl esters of the *A. indica* fruit juice were identified using the National Institute of Standards and Technology (NIST) Database. The major fatty acid identified were dodecanoic acid (4.37%), oleic acid (14.19%), 13-octadecenal (17.05%) and 15-tetracosanoic acid methyl ester (47.13%). Fatty acids such as oleic (18:1), palmitic (16:0), linoleic (18:2), and stearic (18:0), were reported by Castro [38] and Duarte [39] in propolis samples. A total of 10 compounds were identified by [40] from the propolis collected from the Tamilnadu region of India, using GC-MS to show the presence of fatty acids. Among 10 compounds the major fatty acid present were as 9-octadecenoic acid (3.2%), decanoic acid (2.12%), 9,12-hexadecanoic acid (1.29%), octadecadienoic acid methyl ester (0.49%) and alcohols such as 1-tetradecanol (0.89%), octadeanol (0.69%), 1-dotricontanol (0.48%) and 2,3-epoxy-5,8-hexadecadien-1-ol (0.6%) [40, 41]. The synthetic mixes recognized in the ethanol concentration of the bark of *Azadirachta indica* were introduced [42]. GC-MS investigation uncovered the presence of Decosanoic corrosive, 22-trimethyl siloxy)- methyl ester, (1-Benzenesulfonyl-1H–pyrrol–3-yl) acidic corrosive, methyl ester, 1-H-pyrrole-3-propanoic corrosive, 2-(methoxycarbonyl-4-(2-ethoxy-2-oxoethyl)- methyl ester, Propanoic acid,2 hydroxy-2-methyl ethyl ester, and 3,5-pyridine dicarboxylic corrosive, 2-4-6-trimethyl-diethyl ester. The antimicrobial action of leaves from *Psidium guajava* and *A. indica* was broken down by GCMS. The outcome demonstrated that 47 bioactive phytochemical mixes were recognized in the ethanol part of *A. indica* [42]. Biney revealed that the presence of methyl or ethyl esters of unsaturated fats can likewise be considered as qualities of the *A. indica* plant [43]. From this outcome, it very well may be reasoned that every one of these constituents is of pharmacological significance.

Vital metabolic activities take place in the apicoplast of the plasmodium parasite. Some of the processes include the biosynthesis of isoprene, haem, and fatty acids. Meanwhile, the pathway for fatty acid biosynthesis in the apicoplast of Plasmodium is different from the fatty acid biosynthesis pathway in humans and higher eukaryotes. Humans and eukaryotes normally use a Type I fatty acid synthase (FAS I) system, where each fatty acid biosynthetic step is catalyzed by a single protein with multiple domains. On the contrary, the apicoplast has a Type II fatty acid synthase (FAS II) system with each fatty acid biosynthetic pathway carried out by a discrete enzyme encoded by a different gene [44]. This type II FAS system is absent in humans but is common in plasmodium, bacteria, and algae [45]. Interference with the plasmodial type II FAS system can serve as a target of drug action to destroy the parasite without harming the human host. In the parasite, fatty acid biosynthesis is critical for cell membrane formation, an important source of energy, essential in signal transduction, protein acylation, growth, differentiation,
and homeostasis in *P. falciparum*. Lipid biosynthesis is elevated during the erythrocytic phases of the parasite [45] because when the parasite is invading a host, it needs to protect itself by creating a so-called parasitophorous vacuole, in part as a protection from the immune system of the host. In this process, the parasite needs to make its fatty acids *de novo* to form and expand its membrane. In *P. falciparum* the principal membrane fatty acids are decanoic acid (10:0), lauric acid (12:0), and myristic acid (14:0).

Earlier work in 1992 by Kumaratilake and collaborators reports on the antimalarial properties of *n*-3 and *n*-6 polyunsaturated fatty acids, where acids such as 22:6 (*n*-3) and 20:5 (*n*-3) were the best in the studied series for *in vitro* killing of intra-erythrocytic forms of *P. falciparum* [46]. It was also reported that docosahexaenoic acid [22:6 (*n*-3)] was the best in the studied series of fatty acids in killing *P. falciparum* (> 90% death) at concentrations of 20–40 µg/ml [46]. The methyl esters of the fatty acids were reported to be as potent as the free acids in killing the parasite. Later work in 1995 by Krugliak and collaborators reported on the antiplasmodial effect of a series of C18 fatty acids against the FCR3 strain of *P. falciparum*, and these fatty acids displayed some inhibitory activity (≤ 200 µg/ml) against both the intact infected cells and the free parasites [47]. In this particular work, oleic acid (9–18:1) was the most inhibitory fatty acid with an IC₅₀ of 23 µg/ml, and linoleic acid (9,12–18:2) displayed an IC₅₀ of 76 µg/ml.

In 2005, a naturally occurring C₁₈ fatty acid, named scleropyric acid, was isolated from the twigs of *Scleropyrum wallichianum*, and also shown to display good antiplasmodial activity (IC₅₀ = 7.2 µg/mL) against a K1 multidrug-resistant strain of *P. falciparum* [48].

Despite all current measures to curtail the spread of malaria infection, the disease is still ravaging the human population, particularly in malaria-endemic regions of the world. The economic burden continues to soar in addition to its comorbidities [49–51]. Current efforts in malaria research are towards the development of medications with less economic cost and more efficacy in combating this menace [52, 53]. Over the years, studies on the medicinal potentials of *Azadirachta indica* have predominantly focused on some parts of the plant such as the seeds, leaves, bark, roots etc. Some studies have developed extracts from the leaves, stem bark, and roots to treat several human diseases and ailments [54–57]. However, fruit juice has since been neglected and has never been exploited as a useful source of nutraceuticals that can be used in the treatment of diseases. Igwenyi revealed the biochemical composition and nutritional potentials of the fruit juice of *Azadirachta indica* and suggested that more investigations should be done to explore possible therapeutic potentials of the fruit juice [33]. Interestingly, this present study evaluated the potential of the fruit juice of *Azadirachta indica* in clearing malaria parasites in addition to replenishing blood levels usually depleted due to malaria pathogenesis.

As expected, our result revealed a high level of parasitemia in animals infected with *Plasmodium berhei* (NK 65). Treatment with Artesunate which is a standard drug for malaria studies and treatment led to a significant reduction in the levels of the parasites in the treated animals. Both World Health Organization and Malaria Consortium have since recommended the use of Artemisinin Combination Therapy as a standard drug for clinical treatment of malaria infection particularly in the malaria endemic regions of the world [6, 58]. Interestingly, treatment of the infected animals with fruit juice of *Azadirachta indica* as shown in Fig. 2 led to a significant reduction (p < 0.05) in the levels of *Plasmodium berhei* (NK 65). More
interestingly, there were no significant differences (p > 0.05) between the anti-parasitic effect of the standard drug treatment and the treatment with fruit juice of *Azadirachta indica* on all the days of treatment. Fruit juice of *Azadirachta indica* is yet to be reported to have anti-plasmodial activity, hence this study has shown a novel therapeutic potential of *Azadirachta indica* fruit juice. Additionally, the use of the fruit juice of *Azadirachta indica* as medication for malaria infection offers some metabolic advantage over conventional drugs particularly as it relates to drug metabolism in the liver. Drug as xenobiotic increases metabolic burden on the liver [59, 60] whereas juice is barely metabolized in the liver due to its organic nature and high nutritional content.

Furthermore, one of the hallmarks of the pathogenesis of malaria infection is the destruction of the structural integrity of the Red Blood Cells (RBC) leading to the rupture of the membrane and further release of haemoglobin in the blood [61–63]. The haemoglobin is further broken down to its primary structure of the amino-acid sequence. This is incorporated by the parasite for the building of its proteins while the heam is processed to bilirubin for excretion [64, 65]. We confirmed this hypothesis in Fig. 3 of our result. *Plasmodium berhei* (NK 65) infection led to a significant reduction in the levels of haemoglobin in the infected and untreated group. Conversely, treatment with Artesunate led to a significant elevation (p < 0.05) in the mean values of haemoglobin in the animals. Moreover, treatment with fruit juice elevated the mean values of haemoglobin in the animals to values comparable to that of the standard malaria drug. Perhaps, this may partly be due reduction in parasitic load in the animals and partly due to the repair of the cell membrane of the damaged RBC. The fruit juice may contain bioactive components that could be helpful in membrane repair and resistance to oxidative processes as previously suggested by Igwenyi [33].

We further examined the effects of the juice on Packed Cell Volume (PCV) (Fig. 4). PCV indicates the percentage of RBC in circulation, thus the volume occupied by the RBCs in a particular volume of blood. A decrease in PCV indicates RBC loss perhaps due to cell destruction, general blood loss, and/or failure of hemopoietic cells in the bone marrow [66–69]. As expected, infection with *Plasmodium berhei* (NK 65) caused a significant decrease in the mean value of PCV in infected and untreated animals. It is a known fact that *Plasmodium berhei* (NK 65) thrives in the blood stage of malaria pathogenesis by invading and destroying the RBC [5, 18]. Hence, the PCV in the infected and untreated animals may have decreased due to RBC destruction which is consistent with studies of others. Interestingly, treatment with the fruit juice significantly increased (p < 0.05) the PCV to a mean value comparable to the standard drug group. In clinical treatment of malaria infection particularly in pregnant women, blood tonics are usually prescribed in addition to the antimalarial drug [19, 70–72]. This is to replenish the lost RBC due to malaria infection whereas the critical antimalarial component clears the parasites. Thus, we may have identified yet another novel blood-replenishing potential of *Azadirachta indica* fruit juice, which would need to be explored and exploited in further investigation. Fruit juice of *Azadirachta indica* thus may have dual potentials of clearing malaria parasites and replenishing depleted RBC due to malaria pathogenesis. Fruit juice of *Azadirachta indica* may also have an additional economic advantage over conventional malaria drugs by eliminating prescriptions for malaria patients and alleviating the metabolic burden on the liver and kidneys.
To confirm whether the novel blood-replenishing potential of *Azadirachta indica* fruit juice is a holistic one (i.e. affecting other blood components), we evaluated the levels of the White Blood Cells (WBC). As shown in Fig. 5, infection with *Plasmodium beighei* (NK 65) parasites significantly decreased (p < 0.05) the levels of WBC in the infected animals which are consistent with the results of other investigators, particularly in humans [61, 63, 73, 74]. WBCs are necessary for combating infectious agents with the potential of causing diseases [75, 76]. Their decrease in blood circulation leads to a lowering of the body's immune system and further increases one's susceptibility to various diseases [77]. Perhaps, malaria infection lowers body immunity through depletion in the WBCs circulation as previously shown by other studies [63, 78, 79]. Interestingly, we further observed that treatment with *Azadirachta indica* fruit juice led to a significant increase (p < 0.05) in the mean values of WBC in the treated animals. Fruit juice of *Azadirachta indica* may be useful in boosting the immune system through elevation of WBCs. Perhaps, it could further be recommended for people in low-income regions who are not able to afford costly multivitamins to enhance their protection against various diseases, which usually result due to lowered immune system.

In conclusion, this present study has identified novel therapeutic potentials of *Azadirachta indica* fruit juice which would require further investigations for full deployment into pharmaceutical development of natural products and new therapies for malaria infection. The antiplasmodial activity of the juice extract can be attributed to the fatty acid compositions and phytochemical constituents. The yellow colour will appeal to children and the sweet taste of the juice will find a very useful application in pediatrics and antimalarial syrup production with additional nutritional and energy value. This is necessary as the high cost and toxic side effects of synthetic and orthodox medicine have created the challenge for the development of strategies and novel organic candidates for nutritional intervention in health and diseases.

**Declarations**

**Ethical Consideration**

Ethical Permission and approval were sought from the Research Ethics Committee (REC) of University of Nigeria, Nsukka, Nigeria before the commencement of this research study. The care and handling of the animals was done in accordance to the guidelines of National Institute of Health Guide for the Care and Use of Laboratory Animals, 8th edition (2011). In compliance with the internally accepted 3Rs (Replacement, Reduction, and Refinement) principles of animal experimentation, the appropriate guidelines were applied.

**Consent for publication**

The authors hereby grant permission and consent for the publication of the manuscript in BMC Complementary Medicine and Therapies and its associated platforms. We understand that this publication may include, but is not limited to, text, images, figures, tables, and any other content related to the work.
Availability of data and materials

Data available on request from the author:

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References


**Figures**
Figure 1

Phytochemical composition of Fruit Juice of *Azadirachta indica*

Figure 2

Administration of specific doses of *Azadirachta indica* fruit juice cleared plasmodium parasites in male albino mice infected with *Plasmodium berhei* (NK 65).
Figure 3

Administration of specific doses of *Azadirachta indica* increase both red blood cells and haemoglobin levels in male albino mice infected with *Plasmodium berhei* (NK 65).

Figure 4

Administration of specific doses of *Azadirachta indica* normalize Packed Cell Volume in male albino mice infected with *Plasmodium berhei* (NK 65). Treatment groups with similar alphabets are not statistically significant whereas there is statistical significance between treatment groups with dissimilar alphabets.
Figure 5

Administration of specific doses of *Azadirachta indica* normalizes White Blood Count in male albino mice infected with *Plasmodium berhei* (NK 65). Treatment groups with similar alphabets are not statistically significant whereas there is statistical significance between treatment groups with dissimilar alphabets.