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Phytochemical and Antimalarial Screening of Extracts of Leaves of *Morinda Lucida* (Rubiaceae)

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Abstract: ***Morinda lucida is a medicinal plant popular for its traditional uses in the treatment of several illnesses such as malaria, inflammation, diabetes, jaundice, hypertension, and dysentery. In this study, the powdered dried leaves of Morinda lucida were successively extracted with petroleum ether, ethyl acetate and methanol to obtain the extracts of the various organic solvents respectively. The extracts obtained were investigated for the presence of phytochemical and further screened for in vivo antimalarial activities. The results of the preliminary phytochemical screening of the extracts of leaves of Morinda lucida revealed the presence of alkaloids, terpenoids, phenols, reducing sugar, steroids, saponins, flavonoids, tannins, carbohydrates, lignans, quinines, xanthones and peptides. Quinones were significantly absent in all the extracts. The Rane's curative antimalarial test showed that the extracts at all the doses (250, 500, and 1000 mg/kg, per body weight) administered possess dose dependent antiplasmodial activity against Plasmodium berghei as evident from the parasitaemia suppression. The highest percentage suppression was recorded against the methanol extract at the concentration of 1000 mg/kg which was similar to that of the standard drug, chloroquine (5 mg/kg per body weight) used in the study. The results of this study demonstrated that the various extracts of Morinda lucida have promising antimalarial activity which can be attributed to the phytochemicals present in the plant.***

Keywords: *Morinda lucida*, Leaves extract, Phytochemicals, Antimalarial activity

1. Introduction

Malaria is today a global mosquito - borne infectious disease that adversely affects humans and other animals. It is a disease caused by single - celled micro - organisms of the *Plasmodium* group and most commonly spread by an infected female *Anopheles* mosquito. When a mosquito bites, it introduces the parasites from the mosquito's saliva into a person's blood [1]. It can also be transmitted through an organ transplant, a transfusion and the use of shared needles or syringe. Once the parasites are inside the body, they travel to the liver, where they mature after several days. The mature parasites enter the bloodstream and begin to infect the red blood cells [2]. Within 48 to 72 hours, the parasites inside the red blood cells multiply, causing the infected cells to burst open. They continue to infect the red blood cells, resulting in symptoms that occur in cycles that last two to three days at a time [3]. The most vulnerable persons are those with no or little immunity against the disease

The common symptoms of malaria which typically develop within 2 - 4 weeks following the infection include shaking chills that can range from moderate to severe, high fever, profuse sweating, headache, nausea, vomiting, abdominal pain, diarrhea, and anemia [2]. In severe cases it can cause yellow skin, seizures, coma or death [2].

Plasmodium falciparum is the most lethal species [4] [5] [6] and is quite problematic in the tropics [7]. *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae* generally cause a milder form of malaria [3] [2]. The species *Plasmodium knowlesi* rarely causes disease in humans [2]. Malaria endemic regions are now faced with an unprecedented situation in which the only affordable treatment options are rapidly losing therapeutic efficacy. This is because, *Plasmodium* species keeps on changing its genetic make up to adapt to some malarial drugs [5] resulting in drug resistance which is now common against all classes of antimalarial drugs apart from artemisinin. Treatment of resistant strains becomes increasingly dependent on this class of drugs. The high cost of artemisinin limits its use in the developing world [5] [6].

Plants were once a primary source of almost all the medicines in the world [8]. They still continue to provide humankind with new phytochemicals that are used as medicines for different sicknesses [8] [9]. Several secondary metabolites are responsible for the biological activity such as antimalarial of plants. These natural products are synthesized in plants for their survival. Most of these metabolites are synthesized from building blocks derived from acetyl coenzyme A (acetyl - CoA) (**1**), mevalonic acid (**2**) as well as shikimic acid (**3**) and are used respectively in the acetate, mevalonate and shikimate biosynthetic pathways.

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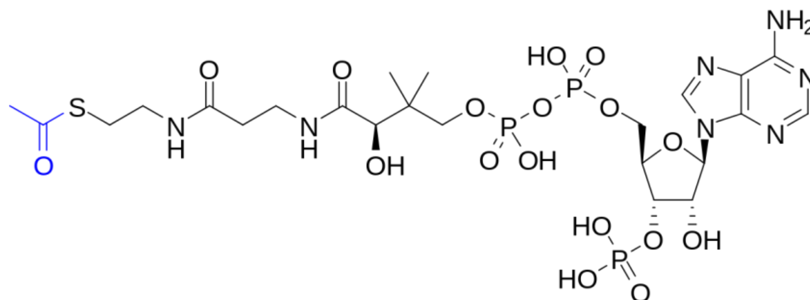
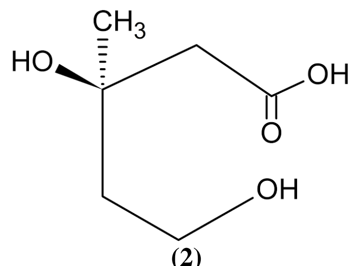


(1)



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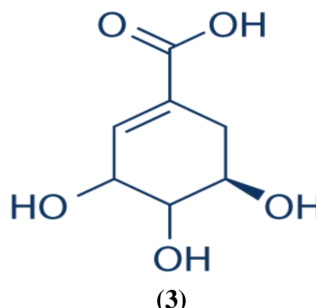

(3)

Through these biosynthetic pathways, phytochemicals like alkaloids, phenols, and terpenoids are produced. Most antimalarial and anti - inflammatory compounds are derived from alkaloids, terpenes and some phenolic compounds.

Morinda lucida belongs to the family *Rubiaceae.* It is widely used in rural areas in West and Central Africa for the treatment of many diseases [10]. In Central and West Africa, infusions and decoctions of root, bark and leaves are used as treatment against different fevers, including yellow fever, malaria, trypanosomiasis and feverish conditions during childbirth [11] [12]. Among the Yorubas in West Africa, the fresh leaves of the plant are often macerated in palm wine and the tincture which is bitter is taken orally for the treatment of diabetes [10]. The bark of *M. lucida* is widely used in Cameroon as a decoction for treating Diabetes mellitus [13]. The plant is also used in cases of insomnia, hypertension, cerebral congestion, dysentery, stomach - ache, ulcers, jaundice, leprosy and gonorrhea [11] [12]. The powdered root bark as well as plasters made from stem or root bark is used as a dressing against itch and ringworm as well as wound infections and abscesses [11]. Leaves and twigs are sold in markets as a medicinal tonic for young children [11]. Apart from oral teas from the leaves being used as a general febrifuge, they are also used as analgesics and laxatives [11]. The leaves are also used to treat infertility and irregular menstruation in women of some parts of West Africa [11].

2. Materials and Methods

Collection of Plant Material

Fresh leaves of *Morinda lucida* were collected from Ikachi Ukpa, Oju Local Government Area, Benue State, Nigeria, on Monday 15th January, 2018, by 6:13 PM. They were authenticated in the Herbarium of the National Institute for Pharmaceutical Research and Development (NIPRD), Idu, Abuja by Mr. Akeem A. Lateef, where a Voucher Specimen with the Herbarium Number: NIPRD/H/6801 was deposited. The leaves of *Morinda lucida* collected were gently rinsed with water from a running tap to remove all the adhering dirt and air - dried at room temperature to constant weight for four (4) weeks and then pulverized using a mortar and pestle. The powdered plant material was collected, the weight was taken and it was stored in a tightly closed polythene bag from where a given quantity was taken for extraction.

Extraction of Plant Material

About 2kg of the air - dried pulverized leaves of *Morinda lucida* was macerated in 2.5 L X 4 of Petroleum ether (40-60^{0}C) in an aspirator bottle with continuous swirling for seven (7) days [14]. The extract obtained was filtered using sterile Whatman No.1 filter paper and left on the bench at room temperature to dryness to obtain the crude extract. The weight of the crude extract obtained was taken and recorded. The left over marc from the petroleum ether extraction was air - dried at room temperature for a day and was successively extracted with equal volume of ethyl acetate and methanol respectively for seven (7) days each, following the same procedure for petroleum ether extraction. All extracts were stored in a refrigerator for chemical and biological study.

Preliminary Phytochemical Screening

The crude extracts of *M. lucida* obtained were subjected to preliminary qualitative phytochemical screening for secondary metabolites such as saponins (Frothing test), tannins, reducing sugars, flavonoids (alkaline reagent test), terpenoids (Salkowski's test), alkaloids (Wagner's reagent), steroids (Liebermann - Burchard test), carbohydrates (Molisch's test), phenols, lignans, xanthones, peptides and quinones. This screening was based on standard procedures and protocol [15] [16] [17] [18] [19].

Test for saponins

To 2 mL of each of the extracts was added 6 mL of distilled water in a test tube. The mixture was shaken vigorously, allowed to stand. There was a formation of persistent foam

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on the surface of the mixture lasting for more than 10 minutes.

Test for tannins
About 2 mL of each of the extracts was treated with 4 mL of 10% alcoholic $FeCl_3$ solution in a test tube and observed. There was a formation of dark green colour solution.

Test for reducing sugar
About 2 mL of each of the extracts was mixed with Fehling A and Fehling B separately and observed. There was a formation of a green colour with Fehling A and a brown colour with Fehling B.

Test for flavonoids
About 2 mL of each of the extracts was mixed with 2 mL of dilute NaOH in a test tube; the mixture formed a golden yellow colour.

Test for quinones
About 2 mL of each of the extracts was treated with conc. HCl in a test tube. No colour change was observed in the mixture.

Test for terpenoids
About 3 mL of concentrated H_2SO_4 and 2 mL of chloroform were added to 5 mL of each of the extracts in a test tube. A monolayer of reddish brown colouration of the interface was observed.

Test for alkaloids
The extracts 2 mL each were treated with 5 drops of Wagner's reagent in a test tube. A reddish brown precipitate was formed by the methanol extract solution.

Test for steroids
About 2 mL of each of the extracts was treated with 2 mL of chloroform, 10 drops of acetic anhydride and 2 drops of conc. H_2SO_4. The solutions were observed for the formation of red colouration.

Test for carbohydrates
To about 2 mL each of the extracts, 2 drops of Molisch's reagent was added and shaken. This was followed by the addition of 2 mL of conc. H_2SO_4. A reddish violet ring at the junction of two layers was immediately formed in the ethyl acetate and methanol extract solution.

Test for phenols
To about 2 mL of each of the extracts was added 1% ferric (III) chloride in methanol/water (1: 1). A dirty green precipitates were formed in the ethyl acetate and methanol extract solution.

Test for lignans
About 0.5 mL of aqueous solution of each of the extracts was added to 2 mL of 2% (v/v) furfuraldehyde in a test tube. The formation of red colour was observed in each of the solutions.

Test for xanthones
About 3 drops of 10% sodium hydroxide solution was added to about 1 mL of each diluted extract in isopropyl alcohol. A yellow - red colouration was recorded.

Test for peptides
An aqueous sample of each of the extracts was treated with an equal volume of about 1% sodium hydroxide solution followed by a few drops of aqueous copper (II) sulphate. The methanol extract solution turned purple.

Test for quinones
About 2 mL of each of the extracts was treated with conc. HCl in a test tube. No colour change was observed in the mixture.

Antimalarial Screening

Parasite Inoculation
All animals were quarantined for 7 days prior to infection. Blood from mice infected with *P. berghei* NK - 65 was used to infect the animals used for the screening. Standard inoculums of $1x10^7$*P. berghei* infected erythrocytes in 0.2 mL were prepared by diluting infected blood with 0.9% normal saline. Each mouse was inoculated by intra - peritoneal injection with a blood suspension (0.2 mL) containing $1x10^7$ parasitized erythrocytes [20]. The parasite was maintained by serial passage of blood from infected to non - infected mice on a weekly basis.

Curative Test
The curative potential of each of the extracts and standard drug was carried out [21] according to established protocol. The mice were injected intra - peritoneally with standard inoculums of $1x10^7$*Plasmodium berghei* NK - 65 infected erythrocytes on the first day (day 0). Seventy two hours later, the mice were divided into 9 groups of five mice each. The groups were orally treated with each of the extracts (250, 500 and 1000 mg/kg/b. w. /day). Two control groups *(n=5)* were used namely, negative control (infected and treated with 5 mL/kg/b. w. /day normal saline) and positive control (infected and treated with 5 mg/kg/b. w. /day of Chloroquine). The treatment was carried out for 4 consecutive days. Blood samples were collected from the tip of the tails of the animals on day 5 (24 hours after the treatment).

Parasitaemia monitoring
Parasitaemia was monitored using an established method [22]. Briefly, blood samples were collected from the tip of the tails of the animals on day 5. Thin blood smears slides were dried, fixed for 15 minutes using methanol, and subsequently stained with 10% Giemsa for 25 minutes. Stained slides were washed off using phosphate buffer, pH 7.2 and allowed to dry. The slides were immersed in oil and viewed at x100 magnification. Each slide was observed at three different fields and the parasitized red blood cells (RBCs) and total number of RBCs for each field was recorded [23].

Average percentage parasitaemia was calculated using the formula:

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$$\% \, Parasitaemia = \frac{Total\ number\ of\ parasitized\ erythrocytes}{Total\ number\ of\ erythrocytes\ counted} \times 100$$

Average percentage suppression was calculated using the formula:

$$\% \text{ Suppression} = \frac{P_{nc} - P_{tg}}{P_{nc}} \times 100$$

Where:
P_{nc} = Parasitaemia in negative control
P_{tg} = %
Parasitaemia in treated group with plant extracts and standard drug.

Statistical evaluation of data
The results were presented as the mean % + Standard Error of Mean (SEM) for each group of experiments. The test groups were compared with the negative control group using one way ANOVA by SPSS version 9.05 software. All data were analyzed at a 95% confidence interval. P<0.05 were considered statistically significant.

3. Results and Discussion

Preliminary Phytochemical Screening
The results of the preliminary phytochemical screening of the successive extracts of the dried powdered leaves of *M. lucida* are shown in **Table 1** below.

Table 1: Results of Preliminary Phytochemical Screening of Extracts of *M. lucida* Leaves

Phytochemicals	Extracts		
	Petroleum ether	Ethyl acetate	Methanol
Alkaloids	-	-	+
Terpenoids	+	+	+
Phenols	-	+	+
Reducing Sugar	-	+	+
Steroids	+	+	+
Saponins	-	-	+
Flavonoids	-	-	+
Tannins	-	+	+
Carbohydrates	-	+	+
Lignans	+	+	+
Quinones	-	-	-
Xanthones	-	+	+
Peptides	-	-	+

Key: (+) = Present, (-) = Absent

Antimalarial Screening (Curative Test)
The results of the antimalarial screening of the extracts of the dried powdered leaves of *M. lucida* are shown in **Tables 2a – 2c**.

Table 2a: Results of Antimalarial Screening of Petroleum Ether Extract of *M. lucida* Leaves (Curative Test)

Treatment	Percentage Parasitaemia	Percentage Suppression
Normal Saline	0.7 + 50	0
PE (250 mg/kg)	0.3 + 09	58.80
PE (500 mg/kg)	0.5 + 22	30.40
PE (1000 mg/kg)	0.3 + 86	48.53
Chloroquine	0.2 + 21	70.53

Key: PE = Petroleum ether extract, Normal saline = Negative control, Chloroquine = Positive control

Table 2b: Results of Antimalarial Screening of Ethyl Acetate Extract of *M. lucida* Leaves (Curative Test)

Treatment	Percentage Parasitaemia	Percentage Suppression
Normal Saline	0.7 + 50	0
EtOAc (250 mg/kg)	0.5 + 37	28.40
EtOAc (500 mg/kg)	0.2 + 19	70.80
EtOAc (1000 mg/kg)	0.3 + 87	48.40
Chloroquine	0.2 + 21	70.53

Key: EtOAc = Ethyl acetate extract, Normal saline = Negative control, Chloroquine = Positive control

Table 2c: Results of Antimalarial Screening of Methanol Extract of *M. lucida* Leaves (Curative Test)

Treatment	Percentage Parasitaemia	Percentage Suppression
Normal Saline	0.7 + 50	0
MeOH (250 mg/kg)	0.4 + 97	33.73
MeOH (500 mg/kg)	0.6 + 01	19.87
MeOH (1000 mg/kg)	0.2 + 07	72.40
Chloroquine	0.2 + 21	70.53

Key: MeOH = Methanol extract, Normal saline = Negative control, Chloroquine = Positive control

4. Discussion

The results of the preliminary phytochemical screening of the petroleum ether extract revealed the presence of terpenoids, steroids and lignans. Terpenoids, phenols, reducing sugar, steroids, tannins, carbohydrates, lignans and xanthones were positive to the test carried out on the ethyl acetate extract. The screening revealed the presence of twelve (12) phytochemicals found to be present in the methanol extract. They include alkaloids, terpenoids, phenols, reducing sugar, steroids, saponins, flavonoids, tannins, carbohydrates, lignans, xanthones and peptides. The phytochemicals common to the three extracts were terpenoids, steroids and lignans. Quinones were significantly absent in all the extracts. The results are presented in **Table 1.** These secondary metabolites reported from this investigation are known for their broad spectrum of pharmacological and physiological properties in medicinal applications [24]. The results agree with some previous studies on the phytochemical screening of *M. lucida* extracts [25].

Alkaloids mainly derived from plant sources, are a large group of secondary metabolites containing usually basic nitrogen in a heterocycle, which are broadly in chemical structure and in pharmacological action [26]. Despite the toxicity of some alkaloids which is widely recognized, they are a source of many biologically active phytochemicals with great potential for medicinal and agricultural uses. Many alkaloids have attractive pharmacological effects and are used as medications, such as recreational drugs, or in entheogenic rituals [40].

Terpenoids are modified class of terpenes with different functional groups and oxidized methyl group moved or removed at various positions. Most of the terpenoids with the variation in their structures are biologically active and are used worldwide for the treatment of many diseases. Many terpenoids inhibited different human cancer cells and are used as anti - cancer drugs such as taxol and its

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derivatives. Terpenes and its derivatives are used as antimalarial drugs such as artemisinin and related compounds. Meanwhile, terpenoids play a diverse role in the field of foods, drugs, cosmetics, hormones, vitamins, and so on [27]. Terpenoid - derived drugs have contributed significantly to human disease therapy and prevention. Some terpenoids drugs have provided tremendous benefits for patients and for the pharmaceutical industry. Artemisinin and its derivatives comprise a multi - million - dollar market worldwide. Terpenoids indisputably continue to be important compounds for drug discovery. In the last two decades in particular, many terpenoids with promising biological activities have been isolated from diverse marine environments. These marine terpenoids exhibit an impressive array of novel structural motifs, many of which are considered to be derived from biosynthetic pathways that are exclusive to marine organisms. Moreover, most of these marine terpenoids possess remarkable biological activities whose potential benefits extend beyond the marine ecosystem and embody the development of new antifungal, anticancer, anti - inflammatory, and antiviral drugs [28].

Epidemiological evidence has demonstrated the remarkable health - promoting effects of phenolics on chronical ailments, including anti - carcinogenic, anti - inflammatory, and antioxidant activities. Phenolics have also been shown to be potent antibacterial and antiviral agents. For instance, phenolics constrained the growth and proliferation of hepatitis C virus (HCV); this virus is a primary blood - borne pathogen causing liver cirrhosis and hepatocellular carcinoma (HCC), thus inhibiting infection in primary human hepatocytes [29]. The anti - carcinogenic capacity is a primary disease - preventive effect of phenolics; they retard the initiation and progression of cancers by constraining the transformation of normal cells, the growing tumours, angiogenesis, and metastasis. Moreover, phenolics stimulate the expression of tumor - suppressing proteins such as p53, Phosphatase and tension homolog (PTEN), p21, and p27 [30].

The level of reducing sugars in wine, juice, and sugarcane are indicative of the quality of these food products, and monitoring the levels of reducing sugars during food production has improved market quality [31]. The carbonyl groups of reducing sugars react with the amino groups of amino acids in the Maillard reaction, a complex series of reactions that occurs when cooking food. Maillard reaction products are diverse; some are beneficial to human health, while others are not [32].

Steroids are perhaps one of the most widely used groups of drugs in present day. Beside the established utilization as immunosuppressive, anti - inflammatory, anti - rheumatic, diuretic, sedative, anabolic and contraceptive agents, recent applications of steroid compounds include the treatment of some forms of cancer, osteoporosis, HIV infections and treatment of declared AIDS. The microbial biotransformation of steroids, yielded several novel metabolites, exhibiting different activities [33].

Saponins have considerable potential as pharmaceutical and/or nutraceuticals agents in natural or synthetic form. Saponins, from a variety of sources have been shown to have hypocholesterolemic, anti - coagulant, anti - carcinogenic, hepatoprotective, hypoglycemic, immunomodulatory, neuroprotective, anti - inflammatory and antioxidant activity [34].

Flavonoids are known to exhibit many biological properties which are beneficial for human health. They are rich sources of natural antioxidants in human diets. Flavonoids neutralize the harmful effects of free radicals in the best of ways and thus help in the prevention of many diseases. They interact with a great number of cellular targets such as antioxidant free radical scavenger activities and also the anti - inflammatory, antibacterial, antiviral, anti - aging and especially anti - cancer properties [35].

Tannins, including gallo and ellargic acid (epigallitannins), are inhibitors of HIV replication.1, 3, 4 - Tri - O - galloylquinic acid, 3, 5 - di - O - galloyl - shikimic acid, 3, 4, 5 - tri - O - galloylshikimic acid, punicatin, punicalagin inhibited HIV replication in infected H9 lymphocytes with little cytotoxicity. Two compounds, punicalin and punicacortein C, inhibited purified HIV reverse transcriptase [36]. Hydrolysable tannins have also shown potential antibacterial effects against Helicobacter pylori [37].

The bioactivity of most carbohydrates is mostly dependent on resistance to digestion in the upper gastrointestinal tract (GIT), the stomach, and small intestine. Trehalose can reduce insulin resistance and improve glucose management in addition to having anti - inflammatory properties. Soluble polysaccharides increase viscosity of the upper GIT content, which enhances trapping of bile acids, preventing cholesterol reabsorption and promoting their removal through the feces [38].

The breadth of the biological activities of lignans has been appreciated relatively recently, especially their anticancer potency, anti - inflammatory, anti - nociceptive, anti - ulcerogenic and antifungal potency. Various lignans have exhibited antiviral and antibacterial activity, e. g., against Gram - positive bacteria through alteration of biofilm formation, bacteria metabolites, membrane receptors and ion channels [39]. For instance, pinoresinol has demonstrated activity against some virus [40]. Lignans also have the capacity to inhibit NF - kB activity on human mast cells (HMC - 1). Thus, reduces pro - inflammatory cytokines production. Furthermore, lignans are able to suppress nitric oxide (NO) generation and decrease inflammatory cell infiltration [41] [42] [43]. Many studies have demonstrated the strong antioxidant activity of plant extracts, attributed to several highly - effective antioxidants, including lignans (e. g., lariciresinol, matairesinol, secoisolariciresinol, pinoresinol, and nortrachelogenin) [44]. Among the natural antioxidants, lignans exhibit particular high antioxidant activity and thus have potential as preventive and/or therapeutic clinical tools [45].

Xanthones are reported to give CNS stimulation and have anti - inflammatory activity. Naturally occurring xanthones have emerged as an important class of organic compounds in view of their remarkable pharmacological and other biological activities which include stimulation and anti - inflammatory activity. It has now been observed that a number of plant products which are in regular use as

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chemotherapeutic agents contain xanthones as active constituents [46].

Bioactive peptides are known for exerting health beneficial properties and thus are considered as a lead compound for the development of nutraceuticals or functional foods. In the past few decades, a wide range of food - derived bioactive peptide sequences have been identified, with multiple health beneficial activities. However, the commercial application of these bioactive peptides has been delayed because of the absence of appropriate and scalable production methods, proper exploration of the mechanisms of action, high gastro - intestinal digestibility, variable absorption rate, and the lack of well - designed clinical trials to provide the substantial evidence for potential health claims.

The results of this study showed that the petroleum ether, ethyl acetate and methanol extracts possess dose dependent antiplasmodial activity as evident from the parasitaemia suppression with the methanol extract having the highest activity. The result of the curative test at 1000 mg/kg is similar to that of the standard drug, chloroquine (5 mg/kg/day) which was used in the study.

The pharmacological activities of medicinal plants are believed to arise from its constituent phytochemicals. A number of phytochemical groups may be responsible for the observed antimalarial activity. Alkaloids are popular for their toxicity against cells of foreign organisms such as bacteria, viruses and protozoans (to which malaria parasites belong). Quinine (an alkaloid) is a popular antimalarial alkaloid in current clinical use. Saponins are another group associated with antiprotozoa activity [20]. The mechanism of action by which saponins work might be through their detergent effect on cell membranes [47]. Another mechanism of action could be elevation of red blood cell oxidation or by inhibiting protein synthesis in parasite [47].

5. Conclusion

The results of the findings carried out during this study have provided scientific base that support the traditional uses of leaves extracts of *Morinda lucida* in the treatment of malaria and many other diseases. The methanol extract with the highest percentage yield has the highest number of phytochemicals as proved by the result of the preliminary phytochemical screening and demonstrated the highest activity in the curative antimalarial screening compared to the petroleum ether and ethyl acetate extracts. The results of this study demonstrated that the various extracts of *M. lucida* have promising antimalarial activity which can be attributed to the phytochemicals present in the plant.

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