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ANTIMALARIAL, FERRIC REDUCING ANTIOXIDANT POWER AND ELEMENTAL ANALYSIS OF *Caesalpinia pulcherrima* LEAF EXTRACT

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Abstract

The increasing rise of resistance of malaria parasites to established antimalarial drugs have necessitated the continued search for new drug entities especially those with fresh modes of action. Caesalpinia pulcherrima is a plant with many pharmacological and medicinal properties. The leaf extract and fractions were studied for their antimalarial, ferric reducing antioxidant power and elemental composition.Various fractions of the leaf extracts were obtained using vacuum liquid chromatography (VLC). The in vivo antimalarial activity was evaluated against plasmodium berghei parasites. The required dose was given according to the weight of the animal two hours after inoculation of parasites on D_1, then once daily for three more days (D_2-D_4). The antioxidant activity was carried out using UV-Visible spectrometer at 593 nm. The elemental analysis was done using Atomic Absorption Spectrophotometer. There was a dose dependent increase in percentage chemo-suppression of the parasites by the different groups with maximum effect at 800 mg/kg (61.57-34.86 % from day five (5)to day eight (8) respectively). The highest FRAP activity was observed in 100% ethyl acetate with 314.90 ± 3.94 mmol, while the lowest antioxidant power was observed in 50% ethyl acetate:50% n-hexane fraction with 48.50 ± 1.10. 90%methanol:10% distilled water fraction did not indicate any FRAP activity. The results obtained revealed that the phytochemicals with very potent antioxidant power are more present in the ethyl acetate fraction. Elemental compositions (ppm) in the powdered leaf of C. pulcherrima were, sodium (Na); 20.27 ± 0.12, iron (Fe); 13.50 ± 0.08, calcium (Ca); 9.0 ± 0.14, and copper (Cu); 0.30 ± 0.08. Lead (Pb) was not detected. This study has shown that Lead concentration was below detectable limit, while the concentrations of Fe and Cu were within established permissible limits. Appreciable amount of Na and Ca were also indicated. C. pulcherrima leaf extract and fractions contain biologically active principles that are relevant antimalarial and antioxidant agents with acceptable mineral compositions.

Key words: *Caesalpinia pulcherrima*, antimalarial, antioxidant power, elemental composition

Introduction

Malaria is one of the most life-threatening infectious diseases worldwide, claiming the lives of millions of people each year [1]. It is an acute and chronic tropical infection caused by a parasite of the genus *Plasmodium* [2]. There are five species that can cause human malaria: *P. falciparum*, *P. vivax*, *P. malariae*, *and P. ovale and P knowlesi* [2]. The female mosquitoes of the genus *Anopheles* transmit the protozoa to humans. Some signs and symptoms of the illness are high fever, chills, headache, anemia, and splenomegaly [2]. Most serious and fatal complications are caused by *P. falciparum* [2].

Majority of the human race, especially from developing countries depend on the traditional system of medicine for the management of various diseases [3]. Medicinal plants, herbs and algae are naturally provided remedies to cure disease with negligible side effects [4]. They are also a pool of new and fresh antioxidants principally those with high phenolic contents [5]. Hence there is an ever-increasing concern in natural antioxidants from food, predominantly vegetables and fruits. Several public health reports support their protecting effects against many diseases [6–8]. Oxidative stress is implicated in the pathophysiology of malaria. Plasmodium parasites digest haemoglobin leading to the production of heme. Heme triggers the production of ROS which are involved in the pathophysiology of malaria [9, 10] and can lead to the development of anemia [11, 12] and apoptosis [13]. All these are implicated in the pathogenic mechanisms triggered by the parasite [14] as well as free radical production [15] and antioxidant defenses [16] in host cells to prevent or stop an infection.

A free radical can be defined as any molecular species capable of independent existence that contains an unpaired electron in an atomic orbital; some free radicals arise normally during metabolism [17]. Other sources are pollution, radiation, herbicides, cigarette smoke, stress, lack of exercise and lack of sleep leading to oxidative stress. Oxidative stress is a term used to explain the oxidative damage (chain reaction) resulting when a free radical attacks a stable molecule. An antioxidant is defined as molecules stable enough to donate an electron to free radicals and terminate the chain reaction before vital molecules are damaged [17].

Caesalpinia pulcherrima (CP) is a flowering plant in the legume family fabaceae. They are normally grown as ornamental flowers in tropical gardens [18, 19]. The plant is known to be a rich source of cassane-type diterpenoids, lupeol, lupeol acetate, carotenoids, quercetin, rutin, beta sitosterol, glycosides, phenols, steroids, flavonoids and alkaloids [19, 20-23]. Different researchers have reported the antiplasmodial activities of the leaves and stem bark of CP [19, 21, 22]. In these studies, the leaves of CP were shown to exhibit moderate antiplasmodial activity against *Plasmodium berghei* (PB), while the stem bark showed significant antiplasmodial effects [19, 21]. In spite of these recognized arrays of reports on the therapeutic and pharmacological potentials of this plant, there is yet a scarcity of information on the *in vivo* antiplasmodial activity on the extracts from the leaves of *C. pulcherrima.* Therefore, this study aims to investigate the *in vivo* antiplasmodial potentials, ferric reducing antioxidant power and elemental content of *C. pulcherrima* leaf extract, in order to validate its ethnomedicinal use in the treatment of human malaria infection in the tropics.

Materials and Methods

Sample collection and preparation

Fresh leaves of *C. pulcherrima* were collected on April, 2016 at the University of Benin. The plant was identified in the Department of Pharmacognosy, Faculty of Pharmacy, University of Benin. The leaves were dried at ambient temperature and were ground to powder by a mechanical grinder. The powdered leaves were stored in an air-tight container and kept for further analysis.

Determination of some metals in *c. Pulcherrima*

The Sample (1 g) was digested with concentrated nitric acid and hydrochloric acid in a ratio of 3:1 on a hot plate. At the end of complete digestion it was filtered using a Whatman filter paper No. 1 into a 50mL volumetric flask and made up to mark with distilled water. Metals concentrations were determined by Atomic Absorption Spectrophotometer.

Preparation of extracts

Exactly five Hundred gramms (500 g) of the leaves of *C. pulcherrima* were exhaustively macerated separately in 3 litres of methanol for 98 hours. The resultant mixture was filtered using cheese cloth and then Whatman's (No. 1) filter paper and the filtrate concentrated to dryness in vacuum at 40°C using rotary evaporator.

Experimental animals

Swiss mice (18 –24 g) of either sex obtained from the University of Benin, Edo state, Nigeria were used for the study. The animals were kept in plastic cages at room temperature and moisture, under naturally illuminated environment of 12:12 hour dark/light cycle. They were fed on standard

diet and had water *ad libitum* according to the NIH Guide for the care and use of laboratory Animals.

Parasite inoculation

Plasmodium berghei was obtained from the National Institute of Medical Research (NIMR), Lagos, Nigeria. Each mouse used in the experiment was infected intraperitoneally with 0.1 mL of infected blood containing about 1 x 10^7 *P. berghei* – parasitized erythrocytes. The inoculum consisted of 5x10^7 *P. berghei* – parasitized erythrocytes per mL. This was prepared by determining both the percentage parasitaemia and the erythrocytes count of the donor mouse and diluting the blood with isotonic saline in proportions indicated by both determinations.

Preparation of giemsa solution

Giemsa powder (3.5 g) was dissolved in a mixture of 250 mL of glycerol and 250 mL of methanol. The procedure was done in a dark room. The solution was poured in a dark reagent bottle and kept in a dark cupboard for a week.

Preparation of phosphate buffer saline (pbs)

Sodium dihydrogen phosphate (10.9 g) and 3.2 g of disodium hydrogen phosphate were dissolved in distilled water and the solution was made up to 1000 mL using distilled water. The pH of the solution was adjusted to 7.2 using dilute solution of NaOH.

Evaluation of suppressive activity of extract on early infection (4 day test)

This test was used to evaluate the antiplasmodial activity of the leave extract and chloroquine against early *P. bergheiberghei* infection in mice. On the first day (D1), twenty-five Swiss albino mice were inoculated (intraperitoneally) with *P. berghei* parasitized erythrocyte (0.1 mL of infected blood containing about 1 x 10^7*P. bergheiberghei* – parasitized erythrocytes) and randomly divided into five groups (Group A, B, C, D and E) of five animals each and treated for the next four consecutive days (D1 – D4). Groups A, B and C received daily doses of the extract (200, 400 and 800 mg of extract/kg body weight of animal respectively) by oral route, group D received no treatment, while group E received 5 mg/kg of chloroquine daily by oral route. The required dose was given according to the weight of the animal two hours after inoculation of parasites on D1, then once daily for three more days (D2 – D5) [24].

Evaluation of parasitaemia

On day five (D5) of the study, thick and thin films were prepared with blood collected from the tail of each mouse. The thin film was fixed with methanol and both films were stained with Giemsa and the thin blood films of infected and treated mice were examined for parasitaemia level under the microscope from day five through day eight (D5 – D8). The stained slides were mounted in oil immersion. Red blood cells were counted in 10 fields and the parasitized also noted. The percentage parasitaemia (Pp) in a group was calculated as:

$$\text{Pp}=\frac{\text{number of parasitised red blood cells}}{\text{total number of red blood cells}}\times 100$$

The average percentage suppression for each dose of each extract was calculated in comparison to controls as follows:

$$\text{Average \% suppression} = \frac{X-Y}{X}\times 100$$

Where, X= Average % parasitaemia negative control

Y= Aaverage % parasitaemia treated groups.

Ferric reducing antioxidant power (FRAP) analysis

Prepared FRAP solution (3.6 mL) was added to distilled water (0.4 mL) and incubated at ambient temperature about 37^{o}C for 5 minutes. Then this solution mixed with unknown concentrations of the plant extract (80 µL) and incubated for 10mins at about 37^{o}C. The absorbance of the reaction mixture was measured at 593 nm using the UV-Visible spectrometer. Ferrous sulphate standard was prepared which was used for the construction of calibration curve of Absorbance against concentrations (0.1,0.2,0.4,0.6,0.8,1.0) from which the various concentrations of the fractions of the leaf extract were determined at a known absorbance [25].

Results and Discussion

The results obtained from the *in vivo* antimalarial studies, ferric reducing antioxidant power and elemental analysis of the extract from the leaves of *C. pulcherrima* are shown in Table 1, 2, 3 and 4

below. The calibration curve and experimental plot are also shown in Figure 1and 2 below.

Percentage yield of the extract

The percentage yield of the leaf extract of *C. pulcherrima* obtained was 15.83%.

Elemental analysis of leaves of *C. pulcherrima*

Some heavy metals in the leaves of *C. pulcherrima* were determined using Atomic Absorption spectrophotometer (AAS).

Table 1-Elemental analysis of the Leaves of *C. pulcherrima*

Elements	Wavelenght (nm)	Amount (ppm)
Sodium (Na)	589.3	20.27 ± 0.12
Iron (Fe)	248.3	13.50 ± 0.08
Calcium (Ca)	422.7	9.0 ± 0.14
Copper (Cu)	324.7	0.30 ± 0.08
Lead (Pb)	283.2	-

The concentration (ppm) of lead was below detection limit

Comparison of %parasitaemia of the leaves extract of *C. pulcherrima* and standard drug (chloroquine)

Table 2-Effect of the extracts and standard drug (chloroquine) on *Plasmodium berghei NK65*

		% Parasitaemia			
Treatment	Dosage (mg/kg)	D_5 (% ± SD)	D_6 (% ± SD)	D_7 (% ± SD)	D_8 (% ± SD)
No treatment		5.23 ± 0.59	7.86 ± 0.44	10.25 ± 0.40	13.28 ± 0.25
Extract	200	2.90 ± 0.22	4.02 ± 0.13	6.21 ± 0.14	10.78 ± 0.23
Extract	400	2.53 ± 0.16	3.98 ± 0.21	6.03 ± 0.16	9.82 ± 0.31
Extract	800	2.01 ± 0.15	3.21 ± 0.17	5.61 ± 0.27	8.65 ± 0.22
Chloroquine	5	0.25 ± 0.06	0.65 ± 0.14	1.14 ± 0.18	1.83 ± 0.22

Not treated group = Negative control, Extract = n-Hexane - ethyl acetate (50-50) VLC fraction of the leaves of *C. pulcherrima*,

Chloroquine = positive control, SD=Standard deviation.

Antimalarial activity of leaves extract of *C. pulcherrima*

Table 3- Mean survival time and %Chemo-suppression of *Plasmodium berghei berghei*by*the Leave of C. pulcherrima*and Standard drug (chloroquine)

		Average %chemo-suppression				
Treatment	Dosage (mg/kg)	D_5 (%)	D_6 (%)	D_7 (%)	D_8 (%)	Mean Survival time (M_t ± SD) days
No treatment		-	-	-	-	14 ± 3.22
Extract	200	44.55	48.85	39.41	18.83	27 ± 2.49
Extract	400	51.63	49.36	41.17	26.05	24 ± 2.72
Extract	800	61.57	59.16	45.27	34.86	22 ± 2.49
Chloroquine	5	95.22	91.73	88.97	86.22	28 ± 0.45

Not treated group = Negative control, Extract = n-Hexane - ethyl acetate (50-50) fraction of the leave of *C. pulcherrima*, Chloroquine = Positive control.

Table 4.FRAP analysis of various fractions of the leaf of *C. pulcherrima*

Fractions	Absorbance	Concentration
100% ethyl-acetate	1.049	314.90±3.94[a]
50% ethyl-acetate:50% n-hexane	0.163	48.50±1.10[b]
50% ethyl-acetate:50% Methanol	0.568	170.21±3.10[c]
100% methanol	0.211	63.10±1.02[d]
Crude	0.493	147.932.59[e]

Concentrations were reported in percentage mmole± SD of the various leaves fractions. Values with different alphabets indicate significant difference at $p<0.05$

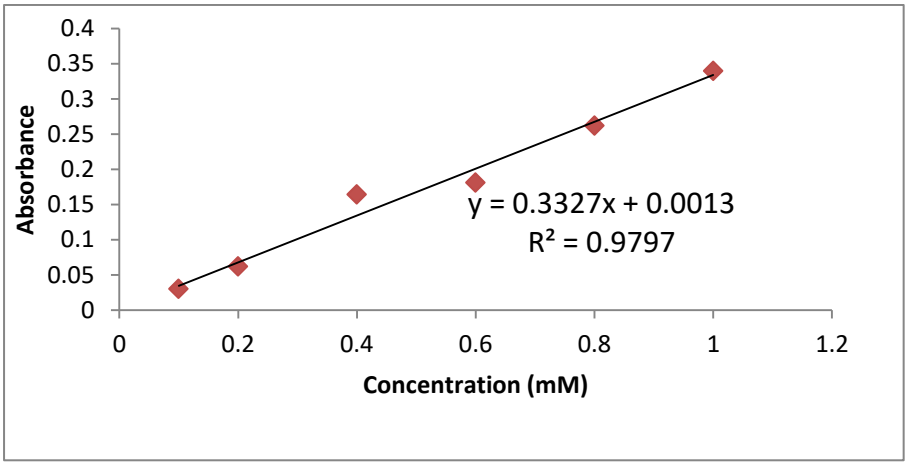

Figure 1Calibration curve for FRAP assay ($FeSO_4.7H_2O$)

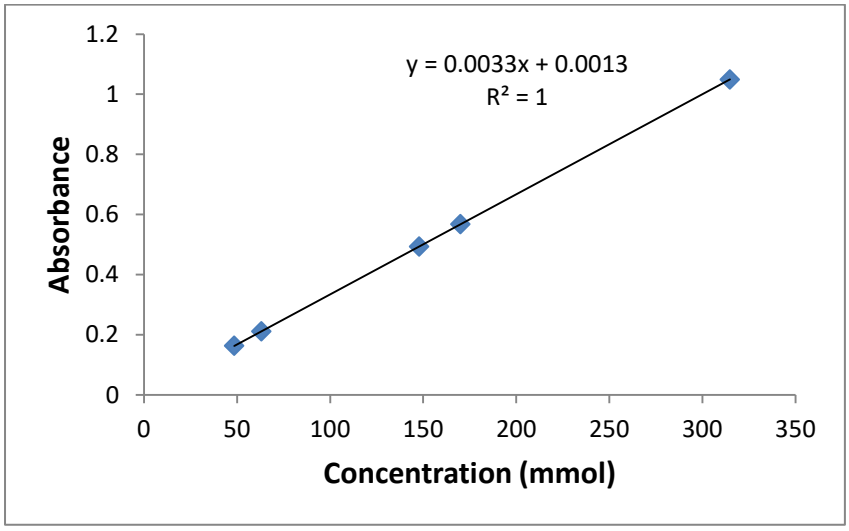

Figure 2: Absorbance against concentration for the various fractions of the extracts of leaf of C. *pulcherrima*

The chemo- suppressive activity of the extract (Table 3) shows that, the extract may have attacked the young *Sporozoites* cells, thereby inhibiting their growth or killing them like the

standard drug that was used and/or the extract helped in boosting the immune system of the mice, re-enforcing it against microbial attack. The suppressive activity of the extract increased with increase in the dosage of the extract administered through D_5 to D_8 suggesting that the extract was more at an "advantage" over the parasite with increase in dosage, that is, an increase in dosage lead to a greater amount of bioactive component. However, there was a reduction in the activity from D_5 to D_8 for both the extract, suggestively this may be because some of the young parasite which escaped the period of treatment and grew to maturity and were subsequently released into the blood stream [19, 26, 27].

The antioxidant properties of the extract were evaluated. As mentioned earlier, anti-oxidants are vital in the mopping up of free radicals from the body**.** The extracts showed a considerable antioxidant effect from absorbance of 0.163nm to 1.047nm with 100% ethyl-acetate having the highest value. The concentration of the extracts are reported as mean ± standard deviation as shown in (Table 4) from the plot of Y=0.0033X + 0.0013, thus the more intense the colour of the reagent, the higher the absorbance which shows a higher power of antioxidant capacity. The antioxidant activity of the plant extract could be as a result of phenolics and other phytochemicals present in the plant, majorly flavonoid [28]. From the analysis done, it shows that the leaves possess an antioxidant property capable of quenching free radicals. These antioxidants delay or inhibit cellular damage mainly through their free radical scavenging property [29].

The relative abundance of elements in the *C. pulcherrima* leaves was in the order of Na>Fe>Ca>Cu. There are several factors that affect the concentration of heavy metals and it includes bioaccumulation capacity, season, pH, geographic factors, time of harvesting, age and salinity [30].According to the WHO, the permissible level of Fe, Pb, Cd, Cr, Cu, Ni, and Zn in medicinal plant and food is 20, 10, 1, 1.5, 10, 15, and 50 ppm, respectively [31].This study has shown that Lead concentration was below detectable limit, while the concentrations of Fe and Cu were within the limit of the WHO.In humans, Fe plays an important role in red blood cell production and is used against anemia, tuberculosis, and growth disorder [32].Cu helps in absorption of iron. It is also used for cellular defense, protecting mucus membrane and preventing anemia, osteoporosis, delayed wound healing and the development of aortic aneurysms, and loss of hair colour [33]. Pb is a non-essential trace element and has a detrimental effect on the human body. Pb poisoning causes convulsions, chronic hepatitis, Central Nervous System disorder, anemia, and kidney damage and reduces fertility and delayed puberty [34].

Conclusion

It has been shown from this study that *C. pulcherrima* extract and fractions contain bioactive components that could be used for the treatment of malaria. There was a dose dependent increase in percentage chemo-suppression of the parasites by the different groups with maximum effect at 800 mg/kg. It was also revealed from the study that the 100 % ethyl acetate extract had the highest concentration and thus having the highest antioxidant power as compared to other fractions. The leaf extract also exhibited concentrations of elements and heavy metals within the limits given by the WHO with no trace of Pb which is highly desirable. Further studies to isolate the various bioactive components and identify the compounds are being carried out.

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