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INVITRO ANTIOXIDANT AND HEMIN BIOMINERALIZATION INHIBITORY PROPERTIES OF *Cassia sieberiana DC.* AQUEOUS STEM BARK EXTRACT

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ABSTRACT

Medicinal plants are the most affordable source of disease treatment in Africa. Different parts of plants are screened for their curative and disease management of various illnesses. *Cassia sieberiana* (D.C) is a medicinal plant widely distributed in the Southern Sahel, Sudan Savanna and distributed in the semi-arid north eastern part of Nigeria. It is a source of some bioactive compounds and has an antioxidant activity. This study was aimed to determine *in-vitro* antioxidant and hemin biomineralization inhibitory properties of *Cassia sieberiana (*D.C). Aqueous stem bark extract. Phytochemicals screening and *in vitro* free radical scavenging activity of aqueous stem bark extract of *Cassia sieberiana* D. C was conducted using 3 – 5 weeks old albino rats .Results showed the presence of carbohydrates, alkaloids, tannins, flavonoids, saponins and steroids. The extract displayed significant scavenging activity of DPPH at higher concentration doses (9mg/ml). The extract displayed non-hemolytic activity at high concentrations, with the maximum inhibition at 8mg/ml, while the inhibition of hemolysis induced by PHZ (phenylhydrazine) was dose dependent, with a significant increase in erythrocyte membrane protection at 10mg/ml, with IC^{50} at 1.729 mg/ml. The extract displayed potential antimalarial activity against the bio-mineralization of heme, increased inhibition was exhibited with increased concentration (IC^{50} =5.808). These findings suggest that the aqueous extract of *Cassia sieberiana* possesses antimalarial properties likely mediated due to the presence of secondary metabolites and its ability to scavenge free radicals prevents the bio-mineralization of heme. Thus, it can be further explored for the effective management of malaria.

Keywords: *Cassia sieberiana,* DPPH, Phytochemical, Hemolysis

INTRODUCTION

Malaria is one of the major public health problems in the world which are transmitted by the bite of female anopheles mosquito and caused by plasmodium species (Autino *et al*., 2012) .About 1.2 billion populations are at risk of transmission(Odikamnoro *et al*.,2018). Cases of malaria in Africa is presented in Nigeria, where disease transmission occurs all year (Ali *et al.*, 2015).

Cassia sieberiana (D.C.) commonly known as Marga in Hausa belongs to the Family: Caesalpiniaceae or Leguminosae. It is found in the forest of dry areas (Obidah *et al*., 2009*).* It is distributed in Saharan and sub-Saharan Africa. It is used for the treatment of many diseases in Nigeria particularly in north eastern and north western Nigeria, where it is used for treatment of malaria, inflammatory conditions, rheumatism, jaundice, diarrhea, deworming and as aphrodisiacs. The plant is usually available and acceptable to most of the consumers (Abdulrazak *et al.*, 2015).

Herbal medicines used in the treatment of diseases were known to have a natural antioxidants property (Mary *et al*., 2018) .These plants have the ability to neutralize free radicals by blocking the process of oxidation (Sharareh *et al.*, 2015). Most of these medicinal plants are accepted and are safer than their synthetic counterparts (Karimi *et al*., 2015). Most medicinal plants toxicity however, most medicinal plants have not been comprehensively assessed (Sharareh *et al.*, 2015). However, most of the medicinal plants have not yet been evaluated for antioxidant activity due to having a large variety of the species.

Free radicals over production causes oxidative stress which leads to DNA, lipid and protein damages that are associated with the following chronic diseases: cancer, coronary artery diseases, hypertension and diabetes (Pizzino *et al*.,2017) . Naturally occurring antioxidants in plants, such as ascorbic acid, vitamin E and phenolic compounds have the ability to reduce the oxidative damage associated with many diseases (Altemimi *et al*., 2017. Pure natural compounds have been reported to have antioxidant properties (Kasote *et al*., 2017) .Natural antioxidants are considered bioactive and safe. Phenolic compounds are natural antioxidants that have the ability of donating an electron or hydrogen atom to the free radical in order to quench oxygen-derived free radicals (Sadeghi *et al.*, 2015). Therefore, the search for new anti-malarial and antioxidants drugs is very important.This study will serve as an insight into the potential antioxidant and antimalarial properties of the plant extract and for exploration as effective therapeutic agent for the treatment of malaria and oxidative cell damages

MATERIALS AND METHODS

Collection and Identification of plant Material

The fresh stem bark of *Cassia sieberiana* DC. was collected from the National Institute for Pharmaceutical Research and Development, (NIPRD), Idu Abuja. The identification and authentication was done by Dr Jamila Aliyu Ibrahim from Department of Medicinal Plant Research and Traditional Medicine, National Institute for Pharmaceutical Research and Development, (NIPRD), Idu Abuja and a voucher specimen

(NIPRD/H/6827) was deposited at NIPRD Herbarium for future reference. The stem bark of *Cassia sieberiana* DC was cleaned and shade-dried at room temperature for a period of two weeks. The dried stem was ground into coarse powder using a mortar and pestle and it was then pulverized using an electric blender. The powder obtained was then used to prepare the plant extract.

Extraction of Plant Material

The dried and pulverized stem bark was extracted by weighing out and soaking 50g of the powder in 500ml of the solvent (water) using the solute-solvent proportion of 1:10 in a conical flask. The mouth of the conical flask was covered with aluminum foil and stirred vigorously then left to stand for 48 hours. Thereafter, extract was filtered using Whatman no 1 filter paper to obtain the filtrate which was then concentrated to dryness over a water bath temperature of 60°C to obtain a solid crude extract. The extract was maintained in the refrigerator at a temperature of 20℃ until use for the study.

Experimental Animals

Albino rats of 3 – 5 weeks old were used for the study. They were maintained at the Animal Facility Centre (AFC) of the Department of Pharmacology and Toxicology, NIPRD, Abuja. The animals were acclimatized to the working environment for a week before the commencement of the experiment. The animals were housed in standard cages with sawdust shavings as beddings. Dark and light cycles were maintained at 12 hours each. The animals were fed *ad libitum* and had free access to water.

Phytochemical Screening

The extracts were subjected to qualitative phytochemical screening according to standard methods described by Trease and Evans (1989) sofowora, (1998).

Preparation of 30mM of EDTA

EDTA (1.17g) was dissolved in 100ml of distilled water.

Preparation of 0.05M phosphate buffer at pH 7.4

Potassium dihydrogen phosphate KH_2PO_4 (0.68g) was dissolved in 100ml distilled water and 0.87g of dipotassium hydrogen phosphate K_2HPO_4 was dissolved in 100ml distilled water, the salts were mixed together at the proportion of 39ml of KH_2PO_4 and 61ml of K_2HPO_4 to attain the right pH.

DPPH radical scavenging activity

The free radical scavenging activity was determined using a DPPH assay. DPPH was freshly prepared in methanol and wrapped in aluminum foil and kept in the dark to prevent autoxidation. A 100 µL volume of the DPPH solution was added to 100 µl of various concentrations (3µg/ml-10µg/ml) of test compounds prepared in phosphate buffer (pH 7.4).After 20 min of incubation in the dark, absorbance was taken spectrophotometrically at 492 nm. The experiment was carried out in triplicates and a blank solution containing only the solvent without the plant extract was subjected to the same DPPH treatment and this was used as a standard control.

Free radical scavenging activity was calculated using:

100-[{(As-Ab)/Ac} × 100]

Where: As - Ab = Net absorbance of sample; Ac = Absorbance of control. The effective concentration of extract necessary to decrease the initial DPPH absorbance by 50% (EC_{50}) was calculated by linear regression where the abscissa (x) represents extract concentrations and the ordinate (y) represents the average percentage (%) scavenging capacity.

Haemolysis assay

Freshly obtained non-infected RBCs were washed thrice with three volumes of phosphate buffered saline (PBS, pH 7.4). The haemolysis assay was performed at 5% haematocrit with different concentrations (3µg/ml-10µg/ml) of compounds in a final volume of 1.4ml, followed by incubation at 37°C for 40minutes. Saponin (0.05% in PBS) was used as a positive control, whereas 0.2% DMSO served as the vehicle control. After incubation, the tubes were centrifuged at 400*g* for 7 minutes to obtain sedimented erythrocytes. The supernatants were diluted in the ratio 1:4 in distilled water in separate test tubes. The absorbance of the supernatants was measured at 538nm to determine the amount of haemoglobin released upon RBC . The experiment was performed in triplicates.

Phenylhydrazine – induced erythrocyte haemolytic assay

A 20% erythrocyte suspension was prepared using blood obtained from healthy rats prior to blood withdrawal, the rats were anaesthetized with ketamine (60 mg/kg body weight). Blood was collected using a syringe into EDTA tubes. The non-pre incubation method was done as follows: the incubation mixture comprises of 1 ml of phenylhydrazine hydrochloride (0.5 mM) prepared in phosphate buffered saline (PBS), 1.9 ml of different concentrations of *C. siberiana* (3µg/ml-10 µg/ml) prepared in PBS and 0.1 ml of 20% erythrocyte suspension made to a total volume of 3 ml with PBS solution. The mixture (RBC, extract and phenylhdrazine) was incubated at 37ºC for 1 h then centrifuged at 1000 g for 10 min. The extent of haemolysis was measured by recording the absorbance of the supernatant at 540 nm. Suitable blank controls were kept to nullify the effect of solvents and inherent hemolysis.

Hemebiomineralization inhibition test

Chloroquine was included in each series of experiments as positive control. Freshly prepared 6.5 mM solution of heminchloride of 100 µL in 0.2M NaOH was mixed with 200 µL of 3 M sodium acetate, 25 µL of 17.4 M acetic acid and 25 µL of compound dissolved in dimethyl sulfoxide (DMSO). DMSO was used as a negative control. After 24-hour incubation at 37ºC and centrifugation for 15 min at 8000g, the supernatant was discarded. The pellet obtained was washed twice with 200 µL DMSO and once with 200 µL of distilled water. The pellet was dissolved in 200 µL 0.1M NaOH and after a further sevenfold dilution with the same solvent, the absorbance was measured at 405 nm using a multiplate reader. Results were expressed as percentage of inhibition of beta-hematin formation in test samples relative to that of the negative control.

RESULTS AND DISCUSSION

Table 1: Phytochemical analysis of aqueous stem bark extract of *C. sieberiana*

Phytochemical Tests	Test Performed	Results
Alkaloids	Mayer's test	+
Carbohydrates	Molisch's test	+
Flavonoids	Ferric Chloride test	+
Phlobatannins	Hydrochloride test	-
Saponins	Froth test	+
Steroids	Salkowski test	+
Sugars	Fehling's test	-
Tannins	Ferric Chloride test	+

+: Indicates the presence of chemical constituents;
-: Indicates the absence of chemical constituents

Table 2: DPPH Radical Scavenging Activity of aqueous stem bark extract of *C. sieberiana*

Concentration (mg/ml)	Mean absorbance	Net absorbance	Antioxidant activity
Control	0.24±0.07***	0.0000	0
3	0.81±0.01***	0.2357	3.55
4	0.86±0.11***	0.1159	52.59
6	1.17±0.01***	0.2007	17.86
8	1.21±0.02***	0.1762	27.88
9	1.28±0.039***	0.0957	60.83

One way ANOVA, *** $p<0.001$ compared to control

Table 3: Anti-hemolytic activity of aqueous stem bark extract of *C. siberiana*

Concentration (mg/ml)	Mean Absorbance	Percentage Inhibition (%)
10	0.65±0.077	17.277710
9	0.63±0.03	20.185420
8	0.61±0.02*	22.840290
7	0.67±0.00	15.718500
6	0.75±0.02	5.267594
5	0.70±0.01	11.040880
4	0.68±0.01	14.454280
3	0.71±0.03	10.071640
Control	0.00±0.00	-

Values represent mean ± SEM of 3 replicates ($p<0.05$), significantly different from the control groups. *$P<0.05$.

Table 4: Erythrocyte membrane stabilizing activity of aqueous stem bark extract of *C. sieberiana* measured in terms of % inhibition of hemolysis induced by phenylhydrazine

Concentrations (mg/ml)	Absorbance of erythrocyte haemolysis	Percentage Inhibition (%)
10	0.50±0.07***	69.656290
9	0.69±0.15***	57.738460
8	0.89±0.06**	45.759610
7	0.99±0.01**	39.332930
6	1.41±0.06	14.114300
5	0.92±0.06**	44.071590
4	1.48±0.24	9.721376
3	1.47±0.10	10.290830
Control	1.64±0.10	0.000000

LC_{50}=1.729. Values represent mean ± SEM of 3 replicates. **(p<0.01), ***(p<0.001) (Data analysis and post hoc test) significantly different from control groups.

Table 5: Heme biomineralization inhibition activity of aqueous stem bark extract of *C. Siberiana*

Concentrations (mg/ml)	Mean Absorbance	Percentage Inhibition (%)
10	0.043±0.002***	47.29451
9	0.042±0.001***	48.16192
8	0.040±0.003***	50.06196
7	0.031±0.002***	61.46221
6	0.050±0.002***	37.71169
5	0.047±0.003***	41.88352
4	0.038±0.001***	52.91202
3	0.043±0.004***	46.50971
Chloroquine	0.081±0.001***	52.49897

LC_{50} =5.808. Values represent mean ± SEM of 3 replicates. *** (p<0.001) significantly different from control group.

DISCUSSION

The results of the phytochemical screening of the aqueous stem bark extract of *C. siberiana* revealed the presence of tannins, steroids, alkaloids, flavonoids, saponins and carbohydrates which is similar to the findings of Shittu *et al.,* (2010) and Donkor *et al.,* (2013).

Diverse assortments of plant secondary metabolites are known to possess biologically active compounds that are responsible for tremendous pharmacological activities which may benefit in protection against diseases (Atanasov *et al*.,2015) .They act as anti-cancer agents, anti-inflammatory agents, antioxidant agents, anti-ulcer agents, as well as preventing various diseases(Sahoo *et al*.,2013). The observed antioxidant activity of extracts may be due to the neutralization of free radicals (DPPH), either the transfer of hydrogen atoms or by transfer of an electron. The scavenging effect can be attributed to the presence of active phytochemicals in them such as saponins, flavonoids and tannins. This is supported by the studies of David *et al*., (2004) and Okokon *et al*., (2008), who reported that Saponins, flavonoids and tannins act as primary antioxidants or free radical scavengers that can counteract the oxidative damage induced by the malaria parasite. Hemolytic assay was performed because compounds possessing potent biological activity may not be useful in pharmacological preparations if they possess hemolytic effect. Saponins used in this study as positive control for evaluation of hemolytic action, produced changes in the erythrocyte membrane, causing rupture and release of characteristic hemoglobin pigments hence showing no hemolytic inhibition. Spectrophotometry analysis revealed that the extract did not cause significant hemolysis compared to saponin. The quantitative screening of *C. siberiana* shows that the plant is rich in tannins and this supports findings of Gurib-

Fakim, (2006) which reports that plants rich in tannins are employed in traditional medicine in the treatment of microbial infections and as hemostatic agents. In this erythrocyte membrane stabilization study, *C. sieberiana* inhibited the haemolysis of erythrocytes induced by phenylhydrazine in a dose dependent manner. This reveals the ability of the extracts to scavenge most of the free radicals generated, since the mechanism for hemolysis of erythrocytes in this model was due to the generation of different free radicals. This could be attributed to the presence of antioxidants and this is supported by Claro *et al*., (2006) who stated that Vitamins C and E which are high in antioxidants contributes to the decrease in oxidative stress caused by Phenyl hydrazine *in vitro*. In this erythrocyte membrane stabilization study, *C. sieberiana* inhibited the haemolysis of erythrocytes induced by phenylhydrazine in a dose dependent manner. This reveals the ability of the extracts to scavenge most of the free radicals generated, since the mechanism for hemolysis of erythrocytes in this model was due to the generation of different free radicals. This could be attributed to the presence of antioxidants and this is supported by Claro *et al*., (2006) who stated that Vitamins C and E which are high in antioxidants contributes to the decrease in oxidative stress caused by PHZ *in vitro.*

CONCLUSION

This study shows that the aqueous stem bark extract of *Cassia Siebreiana* DC.shows potential for development as an antimalarial agent. Antioxidant, free radical scavenging, anti-hemolytic and hemebiomineralization inhibitory activities are likely mechanisms through which its antimalarial effects are mediated. Thus, it can be further explored as effective therapy against malaria infection.

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