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INHIBITION OF B-HAEMATIN SYNTHESIS IS NOT ASSOCIATED WITH ANTIPLASMODIAL ACTIVITY OF HYDROMETHANOLIC STEM EXTRACT OF *COSTUS AFER* KER GAWL. (COSTACEAE)

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ABSTRACT

Introduction: *Costus afer* Ker Gawl (Costaceae) is commonly used as a medicinal plant throughout tropical Africa. It is useful in traditional medicine for the management of diabetes, veneral diseases, malaria, epilepsy, skin eruptions, arthritis and inflammation.

Aim: The study aimed at evaluating the haemin polymerization inhibition activity of hydromethanolic stem extract of *Costus afer* Ker Gawl (HMECA) and its residual aqueous fraction (RAFCA).

Methodology: The method described by Huy *et al* was used in investigating the haemin polymerization inhibition activity of the hydromethanolic stem extract of *Costus afer* and its residual aqueous fraction. Qualitative phytochemical screening and estimation of total phenolic content of the extracts were also carried out using standard procedures.

Results: The hydromethanolic stem extract of *Costus afer* and its residual aqueous fraction produced non-dose dependent inhibitions of β-haematin formation with 1 mg/ml and 2 mg/ml of the extract and its residual aqueous fraction producing 39.6% and 18.0% inhibitions of β-haematin formation respectively as compared to 96.9% produced by chloroquine phosphate, the reference standard. Qualitative phytochemical screening of the extracts revealed the presence of alkaloids, flavonoids, tannins, saponins, hydroxyanthraquinones and carbohydrates. Cardiac glycosides, Cyanogenic glycosides, anthraquinone glycosides and steroids/triterpenes were absent. The total phenolic contents of HMECA was higher than those of RAFCA (129.8 mg TAE/g of extract and 70.2 mg TAE of fraction respectively) and 254.9 mg QE/g of extract and 152.9 mg QE/g of fraction for the flavonoid content respectively).

Conclusion: The results of this study have shown that the antiplasmodial activity of hydromethanolic stem extract of *Costus afer* and its residual aqueous fraction may be due to other mechanism (s) of action other than inhibition of β-haematin formation.

Keywords: Antiplasmodial activity, β-haematin formation, *Costus afer*, hydromethanolic, residual aqueous fraction

INTRODUCTION

In spite of great local and global efforts for the prevention, control and treatment of malaria, the disease remain a major public health challenge worldwide and especially in the developing countries and tropical region of the world. A large number of antimalarial drugs are available for the prevention and treatment of malaria. There are also a number of insecticides for the elimination of the malaria parasite vector, the Anopheles mosquito. However, the rapid development of resistance of malaria parasites and Anopheles mosquitoes to the use of various antimalarial drugs and insecticides respectively has made the search for newer

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and more effective drugs not only a necessity but a matter of urgency as well if we are to overcome the huge burden of malaria. During the intraerythrocytic stages of malaria parasite, the parasite depends on the RBC haemoglobin as main source of food for growth and multiplication[3]. Plasmodium takes in haemoglobin into its acidic food vacuole where it breaks it down by the activity of various proteases[4] into amino acids and haem[5]. The amino acids are used for protein synthesis by the parasites[6] and accumulation of free haem (Fe^{+3}) induces oxidative stress by generating reactive oxygen species, subvert and lyse membranes as well as inhibit the action of a number of enzymes, thus leading to parasite death[6]. To overcome the toxicity of free haem and avoid death, the malaria parasite has several detoxification mechanisms such as polymerization of haem to a non-toxic haemozoin (haemin)[6,7], haem-binding proteins and degradation of free haem by hydrogen peroxide [8,9] or by glutathione-dependent degradation in the parasite cytoplasm[10]. Some antimalarial drugs act via the inhibition of haemozoin formation which is the most important mechanism for detoxification of toxic free haem by the parasite[6]. Considering the paramount importance of haem polymerization in parasite life cycle, a number of antimalarial drugs target haem polymerization for their activity[11]. While many malaria parasites have developed resistance to antimalarial drugs as a means of survival, the parasite cannot develop resistance against haem polymerization and as such this haem polymerization pathway is a good axis for the development of new and effective antimalarial drugs[12]. The antiplasmodial activities of the methanol stem extract of *Costus afer* Ker Gawl and its residual aqueous fraction have been reported in *Plasmodium berghei* infected mice[13,14] as well as their *in vitro* antimalarial activities against chloroquine-sensitive, chloroquine-resistant and artemether-resistant strains of *Plasmodium falciparum*[15]. Literature search did not reveal the mechanism of antiplasmodial action of *Costus afer*. This study therefore investigated the haemin polymerization inhibition activity of this plant as a possible mechanism of antimalarial action. β-haematin, which is identical to haemozoin, is a synthetic polymer that is used for malaria *in vitro* assessments[16] and a simple colorimetic inhibition assay of haem crystallization was used in this study[6].

MATERIALS AND METHODS

Collection and Identification of Plant Material

Costus afer (whole plant), weighing about 5 kg, was collected from Kagoro Hills in Kagoro, Kaura Local Government Area of Kaduna State, Nigeria in September, 2017 and it was identified and authenticated by Mallam Shehu Umar Gallah, a Taxonomist in the Department of Biological Sciences, Kaduna State University, Kaduna. A voucher specimen (Voucher number 01/1087) was prepared and deposited at the Herbarium unit of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria for future references.

Extraction and Fractionation

Preparation and Extraction of Hydromethanolic Stem Extract of *Costus afer*

The leaves were plucked off from the stem and stem was then debarked by removing the leafy covering. The pitch was cut into small

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pieces, air-dried in the laboratory at ambient temperature and humidity until constant weight was achieved. The dried stem was size-reduced using a wooden mortar and pestle. The pulverized stem was then ground into a fine powder using an electric grinder (Binatone). The resulting powder was stored in a dry air-tight container until it was required for use.

Three hundred grams (300g) of the powdered stem was macerated with 1.8L 70% v/v methanol (JHD, China) for 72 hours at room temperature with occasional shaking. The mixture was filtered using muslin cloth, followed by Whatman No 1 filter paper to ensure all debris were filtered out. The filtrate obtained was then concentrated at 45^{o} C using a Rotary evaporator under reduced pressure. The residue was dried in an oven at 45^{o}C. The dried extract was weighed and the percentage yield was calculated as follows:

$$\% \text{ Yield} = \frac{\text{Weight of dried extract (g)}}{\text{Weight of powdered plant material (g)}} \times 100$$

One gramme (1 g) of the dried extract was weighed and dissolved in 10 ml distilled water to get a stock solution of 100 mg/ml and other concentrations were prepared by serial dilution of the stock solution on each day of the experiment.

Fractionation of Hydromethanolic Stem Extract of *Costus afer*

Ten (10) grammes of the hydromethanolic extract was dissolved in 150 ml distilled water and filtered. The filtrate was successively partitioned with equal volumes of dichloromethane, ethylacetate and n-butanol in a separating funnel in increasing order of polarity. The mixture was gently shaken and allowed to separate. The required layer was then decanted into a measuring cylinder. This process was repeated 4 times.

The dichloromethane and ethylacetate fractions were concentrated at 50^{o} C using a rotary evaporator under reduced pressure. The concentrates were then dried in an oven at 50^{o} C.

The n-butanol fraction and the residual aqueous fraction were concentrated to dryness in a water bath at 50^{o} C.

The various concentrates were weighed and percentage yields were calculated as follows:

$$\% \text{ Yield of fraction} = \frac{\text{Weight of concentrate}}{\text{Weight of extract}} \times 100$$

Chemicals and Reagents

Bovine haemin chloride (Sigma-Aldrich, Germany), Chloroquine phosphate powder (Sigma-Aldrich, Germany), Dichloromethane A.R (JHD, Guangdong Guanghua Sci-Tech Co., Ltd, China), Dimethyl sulfoxide (DMSO) (JHD, Guangdong Guanghua Sci-Tech Co., Ltd. China), Ethylacetate A.R (JHD, Guangdong Guanghua Sci-Tech Co., Ltd, China), N-Butanol A.R (JHD, Guangdong Guanghua Sci-Tech Co., Ltd, China), Methanol A.R (JHD, Guangdong Guanghua Sci-Tech Co., Ltd, China), Sodium acteate (JHD, Guangdong Guanghua Sci-Tech Co., Ltd. China), Tween 20 (Kermel chemical co., China), Tween 80

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Equipment

Avery balance (W and T, Avery Ltd, Birmingham, England, Dry Oven (DHG-9030), Electric suction pump (Searchtech Instruments, England. C7A-23D), Dry Oven (DHG-9030), Electronic pipettes (Microlux), Flat-bottom 96-Well microtiter plate (Star Laboratory, England), Incubator (National Appliance Company, U.S.A Model 630, Serial no 1-81-1550-1), Microtiter plate reader (Molecular Devices co., Manlo Park, CA. USA), Rotary Evaporator (Searchtech Instruments, England. RE 52-3), Water Bath (Model DK-420, No L-606382).

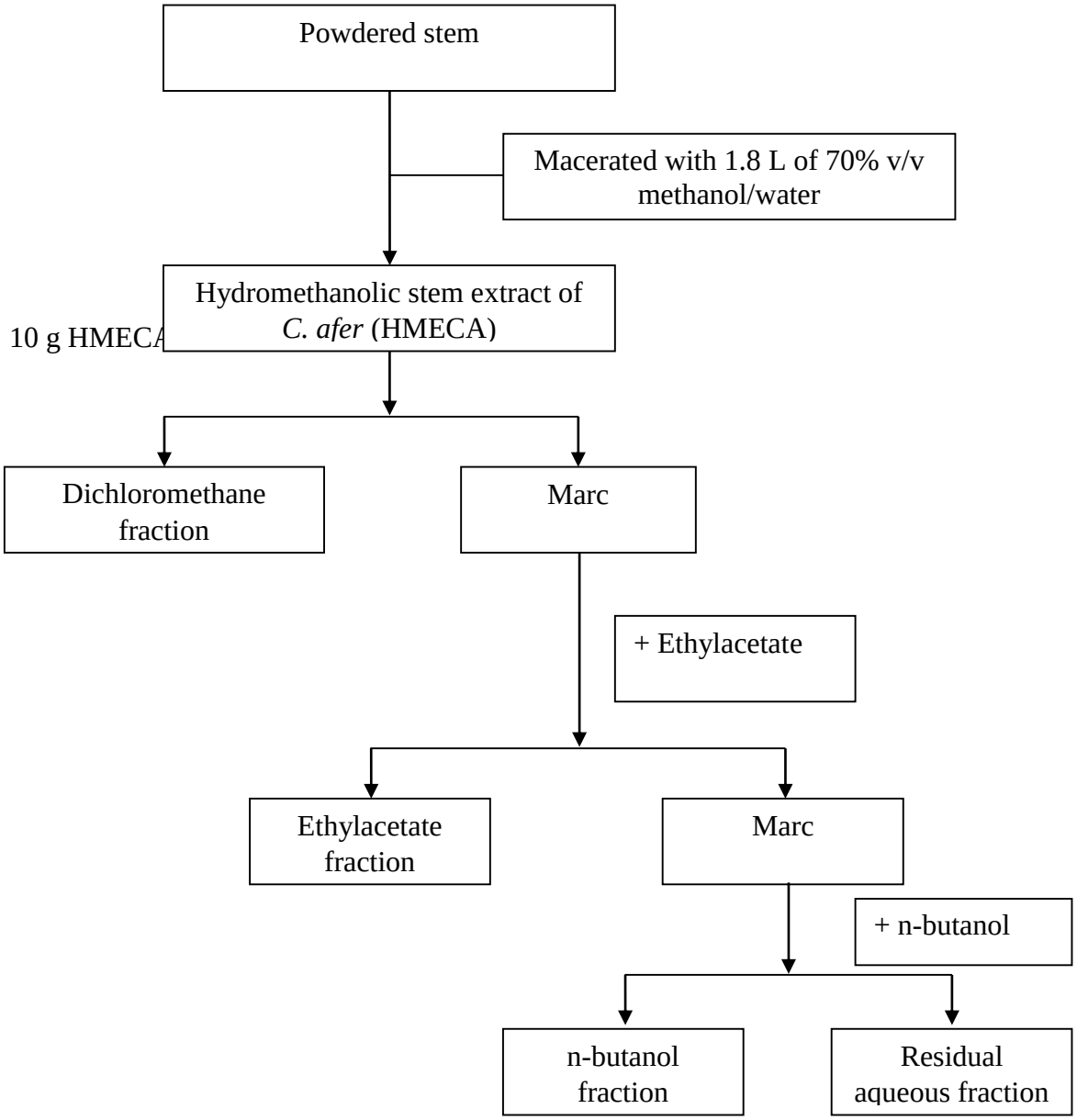

Figure 1: Solvent Partitioning of Hydromethanolic Stem Extract of *Costus afer*

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Inhibition Assay of β-haematin Formation

Haemin polymerization inhibition test was conducted according to the method described by Huy *et al*.[1]. Haemin chloride (163 mg; Sigma) was dissolved in 10 ml Dimethyl Sulfoxide (DMSO). The solution was passed through a 0.2 µm pore membrane filter to remove insoluble particles. From the molar mass of haemin chloride being 120.576 g/L (equivalent to 120.576 mg/ml), the concentration of the haemin chloride prepared is equal to 135 µM. The stock solution was kept at 4° C until it was required to be used and according to Trang *et al*.[17], this stock solution can remain stable for up to one month. 8.23 ml of this haemin stock solution was diluted to 10 ml with 1M acetate buffer (pH 4.8) just before being used to yield 111.1 µM haemin. Chloroquine phosphate (Sigma-Aldrich), hydromethanolic extract and residual aqueous fraction of *Costus afer* were dissolved in distilled water to obtain various concentrations (0.5, 1.0, 2.0, 4.0 and 8.0 mg/ml). The density of tween 20 is 1.1 g/ml or 1100 g/L and from the method of Huy *et al*.[6], a concentration of 0.012 g/L tween 20 which yieded the highest fraction of haeme (80%) converted to β-haematin was used to initiate β-haematin formation. 1.1 µl of tween 20 was added to 100 ml distilled water to get a concentration of 0.012 g/L tween 20. 100 µl of haemin chloride (111.1 µM) in sodium acetate buffer (pH 4.8) was added into each of 51 wells of a microtiter plate. The first three wells (A1- C1) had only haemin chloride and served as the negative control ($A_{control}$). Wells D1 to F1 received 100 µl of tween 20 (0.012 g/L) in addition to the 100 µl of haemin chloride ($A_{min.}$). The remaining 45 wells received 100 µl each of various concentrations (0.5 to 8.0 mg/ml) of chloroquine, hydromethanolic extract and residual aqueous fraction of *Costus afer* and the initial 100 µl of haemin chloride and these wells served as standard drug/sample wells ($A_{standard/sample}$). The samples were incubated at 37 °C for 250 minutes. The samples were mixed by being pipetted three times and then the plate was read at 405/630 nm using a microtiter plate reader (Molecular Devices co., Manlo Park, CA. USA)

The fraction (f) of haemin converted to β-haematin was calculated as follows ;

$$f = \frac{(\text{A control} - \text{A sample})}{(\text{A control} - \text{A min})} \quad [6]$$

Where, $A_{control}$ = absorbance of the haemin without Tween 20 or standard drug/extract at 405/630 nm, A_{sample} = absorbance of haemin in the presence of both Tween 20 and drug/extract and A_{min} = absorbance of haemin with Tween 20 in the absence of drug/extract at 405/630 nm.

Percentage of inhibition of β-haematin formation was calculated using the following equation;

$$\% \text{ inhibition} = (1\text{-}f) \times 100$$

$$\text{or } \frac{(\text{A sample} - \text{A min})}{(\text{A control} - \text{A min})} \times 100$$

The average values obtained from the triplicate assays were plotted against concentrations and the IC_{50} and IC_{90} (ie molar equivalents of chlroquine, extract and fraction relative to haematin required to inhibit haem polymerization by 50% and 90% respectively) values were calculated graphically.

STATISTICAL ANALYSIS

All tests were performed in triplicates and the data were analysed by One-way Analysis of

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Variance (ANOVA) using SPSS version 20 (2011) software package.

RESULTS

The hydromethanolic stem extract of *Costus afer* and its residual aqueous fraction produced non-dose dependent inhibitions of β-haematin formation with the 1.0 mg/ml concentration of the hydromethanolic extract producing the highest inhibition of 39.6% and 2.0 mg/mlconcentration of the residual aqueous fraction producing a maximum inhibition of β-haematin formation of 18.0%. IC_{50} and IC_{90} values of 38.52 ± 0.84 and 78.52 ± 1.00 mg/ml and 54.50 ± 0.50 and 99.90 ±1.05 mg/ml were obtained for the aqueous and residual fractions of *Costus afer* respectively (Table 1).

The standard drug, chloroquine phosphate produced dose-dependent inhibitions of β-haematin formation and at 8.0 mg/ml concentration, it produced 96.9% inhibition of β-haematin formation with IC_{50} and IC_{90} values of 21.15 ± 1.11 mg/ml and 38.05 ± 0.78 mg/ml respectively (Table 1).

Table 1: Percentage inhibition of β-haematin synthesis and IC_{50} and IC_{90} values of the hydromethanolic stem extract of *Costus afer* and its residual aqueous fraction

Drug/Sample	Concentration(mg/ml)	%inhibition of β-haematin synthesis	IC_{50} (mg/ml)	IC_{90} (mg/ml)
Chloroquine	0.5	35.9	21.15±1.11	38.05±0.78
	1.0	52.0		
	2.0	50.6		
	4.0	77.2		
	8.0	96.9		
Hydromethanolic extract of *Costus afer*	0.5	26.9	38.52±0.84*	78.52±1.00*
	1.0	39.6		
	2.0	17.1		
	4.0	28.5		
	8.0	20.1		
Residual aqueous fraction of *Costus afer*	0.5	12.3	54.50±0.50*	99.90±1.05*
	1.0	6.4		
	2.0	18.0		
	4.0	13.0		
	8.0	10.1		

Values are means ± S.E.M, n = 3, One Way ANOVA, *$p \leq 0.05$ = significant difference between the IC_{50} and IC_{90} values of Hydromethanolic extract and Residual aqueous fraction compared with the standard drug, chloroquine.

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DISCUSSION

During the intraerythrocytic stage of the malaria parasite, haemoglobin is ingested by the parasite and used as its major protein source. As the parasite develops further, approximately 80% of haemoglobin in RBCs are degraded by the developing trophozoites into haem (iron rich molecule) and globin molecules. While globins are converted to amino acids for use by the parasite, the haem is toxic and when it is allowed to accumulate, it results in membrane lysis that eventually lead to parasite death. To overcome the toxicity of free haem, malaria parasites convert the toxic haem to a non-toxic haemozoin, otherwise called the malaria pigment[18]. Various drugs are used in the treatment of malaria, each acting on a specific pathway. The quinolines act on the haem polymerization pathway and antifolates act by inhibiting the synthesis of folic acid.

Chloroquine, a weak base, undergoes protonation to CQ^{2+} in the highly acidic digestive vacuoles (pH 4.7) of susceptible *Plasmodium* which prevents chloroquine from leaving the food vacuole leading to accumulation of chloroquine and its binding to haem to form a highly toxic haem-chloroquine complex which is thought to kill the parasites via oxidative damage to membranes, digestive proteases, or other critical molecules and ultimately death of the parasites[19,20]. Lumefantrine also acts probably by preventing parasite detoxification of haem[21]. Artemisinins may act by formation of potentially toxic haem-adducts and it may also generate free radicals that alkylate and oxidize proteins and possibly lipids in parasitized erythrocytes[22]. Other antimalarial drugs such as primaquine are thought to act as oxidation-reduction mediators and such activities contribute to antimalarial effects by generating reactive oxygen species or by interfering with mitochondrial electron transport in the parasite[23].

The result of the haemin polymerization inhibition activity (HPIA) assay showed that both the hydromethanolic stem extract of *Costus afer* and its residual aqueous fraction were not active in inhibiting β-haematin formation as they produced maximum inhibitions of only 39.6% and 18.0 % at doses of 1 mg/ml and 2 mg/ml respectively, as compared to 96.9% haemin polymerization inhibition by the standard drug, chloroquine phosphate at a dose of 8.0 mg/ml. There are two prominent mechanisms known with antimalarial compounds inhibiting haemin (haem) polymerization; alteration of polymerization conditions or sequestration of haemin to form toxic drug-haemin complexes as in the case of artemisinin [24]. The mechanism of the antimalarial activity of chloroquine is believed to relate to haemin (haem) crystallization; it binds haem and inhibit β-haematin (haemozoin) formation in the lysosomal digestive vacuole of the parasite[8]. It is likely that phosphate is responsible for the inhibition effect of β-haematin formation caused by chloroquine phosphate as it has been reported that phosphate and chloride salts inhibited haemin polymerization by 85% and 97% respectively[25], but no inhibition was observed with chloroquine sulphate. According to Mosaddegh *et al*.[26] ,if the percentage of haemin detoxification inhibition is greater than 90%, the assay is considered as positive, whereas values less than 90% indicate a negative result.

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CONCLUSION

The result of this study showed that the antiplasmodial activity of *Costus afer* may not be due to the inhibition of β-haematin formation in the lysosomal digestive vacuole of the parasite. Other mechanisms of antimalarial action may explain the antimalarial activity of the hydromethanolic stem extract of *Costus afer* and its residual aqueous fraction. Bioactive compounds responsible for antiplasmodial activity of this plant should be isolated and characterized and further studies should be conducted to determine the possible mechanism(s) of antiplasmodial action of this plant.

Ethical Approval: Not applicable

Competing Interests: The authors have declared that no conflicting interests exist

Authors' Contributions

JAA conceived the research idea, designed and carried out the experiments under the supervision of MBB, CBA and EJI. JAA wrote the draft manuscript. All the authors corrected and approved the final manuscript.

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