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(RESEARCH ARTICLE)



Isolation, characterisation and *in vivo* anti-malarial investigation of pulcherrimin A from *Caesalpinia pulcherrima* stem bark

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Abstract

Malaria is responsible for about a million deaths yearly. The fight against malaria is faced with the occurrence of widespread resistance of the malaria parasite, *Plasmodium spp.* The search for plant derived anti-malarial drugs has become greatly imperative. This study was aimed to isolate and investigate the *In vivo* anti-malarial activity of pulcherrimin A isolated from the stem bark of *Caesalpinia pulcherrima*. Ethyl acetate fraction of the stem bark extract was subjected to fractionation over silica gel column to obtain pure compound which was characterized as 3,5,6,7-tetrahydroxy-19-vouacanoic acid;(3 β ,5 α ,6 β ,7 β)-form,6,7-dibenzoyl (pulcherrimin A) a known compound using various spectroscopic techniques. Pulcherrimin A was evaluated for *In vivo* anti-malarial activity against *P. berghei* infected mice using the 4-day suppressive test. Different doses (50, 200 and 400 mg/kg/day) of pulcherrimin A were administered to the mice after parasite inoculation. A maximum parasitaemia suppression of 68.18% was observed for the middle dose (200 mg/kg/day) in contrast with 40.91% for the highest dose (400 mg/kg/day). The study therefore revealed that pulcherrimin A isolated from the stem bark of *C. pulcherrima* exhibited moderate-dose significant ($p < 0.05$) inhibition of *P. berghei* parasite, thus authenticating the local usage of different parts of the plants in the treatment of malaria and other pyrexia-related infections.

Keywords: Anti-malarial; *P. berghei* parasite; Pulcherrimin A; *Caesalpinia pulcherrima*

1. Introduction

Malaria is a disease responsible for deaths' of over one million people yearly; approximately three billion people in the world are at risk of malaria caused by the *Plasmodium* parasite. In 2018, the World Health Organization (WHO) reported 85% of the estimated 405,000 deaths in Africa and India (with Nigeria and Democratic Republic of Congo taking the lead at 24 and 11%, respectively), with the remaining from America, Middle East and Europe [1]. To date, parasite resistance to anti-malarial medicines has been documented in 3 of 5 *plasmodium sp.* known to affect humans; *P. falciparum*, *P. vivax* and *P. malariae*, and this is a concern in the fight against malaria [2]. For thousands of years, all over the world, people have used plant derived medicines for the treatment of malaria and other diseases. These medicinal plants have active compounds, some of which have been isolated and their mechanisms of action evaluated [3,4]. In the past, compounds from natural origin represent a main source for novel drug discovery for treatment of many diseases, some of which includes; plant and marine extracts, alkaloids, terpenes, flavonoids, quassinoids, limonoids, chalcones and coumarines [5-7]. These natural products have shown positive treatment responses, which can be improved upon

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via synthetic strategies to obtain anti-malarial active components against not only drug sensitive, but also drug resistant strains of *plasmodium spp* [8].

Caesalpinia pulcherrima commonly known as 'Bride of Barbados' belongs to the family Fabaceae (pea family), is used traditionally as an abortifacient and in the treatment of various ailments including; ulcer, hepatitis, diarrhoea, dysentery, malaria and injury infections [9,10]. Different parts of *C. pulcherrima* have been reported to exhibit medicinal properties such as anti-inflammatory, antimicrobial, antioxidant, anticancer, and antimalarial activities. A phytochemical examination of *C. pulcherrima* has led to the isolation and characterisation of many secondary metabolites, typically the cassane-type diterpenoids, some of which are Pulcherrins A-R and Pulcherrimins A-F [11-24].

Therefore, this study, to the best of our knowledge, aims to investigate (for the first time) the *In vivo* anti-malarial potential of the isolated compound (pulcherrimin A) from the stem bark of *C. pulcherrima*, in order to validate its ethnomedicinal use in the management of human malaria.

2. Materials and methods

2.1. Extraction of crude powdered sample

The ground sample of *C. pulcherrima* stem bark (2.5 kg) was extracted with methanol (7.5 L) by maceration at room temperature for 96 hrs (3x). The crude extract (220 g) was obtained after concentration, using a rotary evaporator under reduced pressure at 45°C. The extract was weighed and the percentage yield was calculated. The extract was kept in a refrigerator at 4°C for further analysis [25].

2.2. Chromatographic fractionation

The crude extract (200 g) was fractionated by silica gel chromatography (SiO₂ 13 x 150 cm) using n-hexane (9.4 L), n-hexane:ethyl acetate (1:1) (12.5 L), ethyl acetate (8.2 L), ethyl acetate:methanol (1:1) (13 L) and methanol (7 L), in increasing proportions of polarity to give five different fractions for n-hexane (100%) (HE), n-hexane:ethyl acetate (50%) (HEEA), ethyl acetate (100%) (EA), ethyl acetate:methanol (50%) (EAME) and methanol (100%) (ME). The various fractions were afterwards concentrated using the rotary evaporator at reduced pressure and their percentage yields calculated. The fractions together with the crude extract were kept in sample bottles and stored in the refrigerator at 4°C for further analysis [25].

2.3. Isolation

2.3.1. Ethyl acetate fraction

Ethyl acetate fraction (EA) (20 g) was chromatographed on column chromatography (SiO₂, 5 x 135cm) eluting with DCM:MeOH 100:0 (3 L), 99:1 (10 L), 98:2 (10 L), 96:4 (6 L), 94:6 (6.25 L), 92:8 (5.75 L), 90:10 (4.25 L), 88:12 (5.25 L) and 0:100 (3 L) in a stepwise gradient. Fractions (250 mL each) were collected and monitored with TLC to give five major fractions (CP200-216, CP217-238, CP239-268, CP269-289, and CP290-301).

2.3.2. Fraction CP239-268

Fraction CP239-268 (3.2 g) was chromatographed on LH-20 Sephadex column chromatography (4 X 125 cm) with methanol (4 L) and eluates (5 mL each) were collected and monitored with TLC to give one major subfraction CP252. Subfraction CP252 (1.82 g) was rechromatographed on normal silica gel chromatography (2x120 cm) with 5% MeOH in DCM (2 L). The precipitate formed was suspended in n-hex:DCM (1:1), filtered, dried and weighed to give a pure compound (CP252).

2.4. Experimental animals

Healthy Swiss mice of both sexes (8–12 weeks, weighing 20–25 g) bred and maintained at the Nigerian Institute of Medical research (NIMR), Lagos, Nigeria were used. The animals were kept in cages and housed in a standard animal house under environmental conditions (23-25°C, 12 h/12h light/dark cycle). They had access to standard pelleted diet and water *ad libitum*. All mice were acclimatized for one week prior to the study. This study was approved by the ethical review board of Faculty of Life Sciences of University of Benin with a reference number HRPGC/578/2015. All experiments were conducted in accordance with the National Institute of Health Guide for care and use of laboratory animals [26].

2.4.1. Parasite inoculation

The NK65 strain of *Plasmodium berghei* (which is chloroquine sensitive) was used for this study. The parasite was obtained from the Nigerian Institute of Medical Research, Yaba, Lagos (NIMR). An infected donor mouse with *P. berghei* strain of the rodent malaria *Plasmodium spp* was used for parasite inoculum preparation. Each mouse was injected intraperitoneally with 0.1 mL of the infected blood containing about $1 \times 10^6 P. berghei$ parasitized red blood cells.

2.5. In vivo anti-malarial activity study

A four (4) day suppressive test was adopted and used for this study [27]. The Swiss mice were randomly divided into groups of five (5) with each group having five (5) animals in a cage. Groups A, B and C were administered pulcherrimin A at doses of 50, 200 and 400 mg/kg, respectively, Group D served as a negative control and was given distilled water while the last group (E) was administered with 5 mg/kg of chloroquine phosphate which served as the positive control. The animals were administered with pulcherrimin A and chloroquine phosphate solution, 2 hours after the inoculation of the parasite on day 0 (D0) and everyday till day 3 (D3) using an oro-gastric syringe. On day 4, a thin blood film was made from the tail of each mouse. The smear was prepared by spreading the blood on a clean slide over an area of 1.5 cm × 2.5 cm, allowed to dry and fixed with methanol, stained with 3% Giemsa for 45 minutes and examined with microscope under the oil immersion (Olympus CX, Japan). The percentage parasitaemia and percentage suppression were calculated using the modified formula [28].

$$\text{Percentage parasitemia} = \frac{\text{number of parasitised red blood cells}}{\text{total number of red blood cells}} \times 100 \quad (1)$$

$$\text{Average \% suppression} = \frac{X - Y}{X} \times 100 \quad (2)$$

Where, X = Average % parasitaemia negative control

Y = Average % parasitemia treated groups.

3. Results

3.1. Physical constants and spectral data of compound 1 (CP252)

Compound 1 (CP252): pulcherrimin A

IUPAC name: 3,5,6,7-tetrahydroxy-19-vouacanoic acid; (3 β , 5 α , 6 β , 7 β)-form, 6,7-dibenzoyl

State: white solid

% yield: 0.64% (1.279 g)

$[\alpha]_D^{21.94} +81$ (c = 1 mg/mL CHCl₃)

R_f: 0.55; Hexane:Acetone (5.5:4.5)

Melting point: 190-191.5°C

IR (KBr): 3449.0(O—H), 2932.8(C—H), 1714.3 (C=O), 1629.2, 1456.2, 1285.0, 1114.5, 1024.8, 712.7 cm⁻¹

UV (MeOH) λ_{max} 273 nm

HR EI-MS m/z: 588.2384 [M⁺]: C₃₄H₃₆O₉

Degree of unsaturation (DoU): 17

EI-MS (rel. int %) (M⁺) m/z: 588.2(2.4), 344.1(4.9), 311.1(3.9), 300.1(9.8), 282.1(37.3), 267.1(15.0), 249.1(5.8), 175.1(30.4), 145.0(9.5), 122.0(71.8), 105.0(100.0), 91.0(7.8), 77.0(45.3),

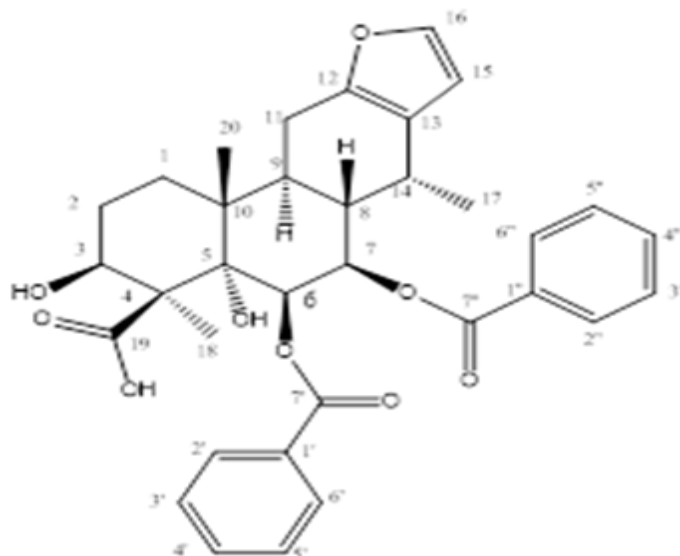


Figure 1 Chemical structure of compound 1

Table 1 $^1\text{H-NMR}$ (400 MHz, CD_3COCD_3) Spectroscopic data of compound 1 (CP252) (δ in ppm, multiplicities, J in Hz)

Position	Compound 1		Multiplicity
	δ_c	δ_H	
1a	34.24	1.68 (m)	CH_2
1b		1.96 (dd, $J=13.8, 3.8$)	
2a	29.02	1.76 (m)	CH_2
2b		2.47 (dd, 14.0, 3.6)	
3	74.82	3.80 (d, $J=8.0$)	CH
4	55.81	-	C
5	80.03	-	C
6	70.16	6.18 (d, $J=4.0$)	CH
7	72.59	5.85 (dd, $J=11.6, 4.0$)	CH
8	36.58	2.42 (m)	CH
9	37.82	2.60 (m)	CH
10	42.05	-	C
11a/b	28.12	2.64 (m)	CH_2
12	149.67	-	C
13	122.09	-	C
14	28.87	2.82 (m)	CH
15	110.21	6.20 (d, $J=2.0$)	CH
16	141.41	7.29 (d, $J=1.6$)	CH
17	17.34	0.98 (d, $J=7.2$)	CH_3
18	20.62	1.63 (s)	CH_3
19	176.85	-	C

20	17.78	1.64 (s)	CH ₃
1'	165.79	-	C
2'	131.08	-	C
3'/7'	130.03	7.8 (m)	CH/CH
4'/6'	128.88	7.35 (m)	CH/CH
5'	133.36	7.55 (m)	CH
1''	166.30	-	C
2''	131.77	-	C
3''/7''	130.25	7.83 (m)	CH/CH
4''/6''	128.99	7.45 (m)	CH/CH
5''	133.36	7.55 (m)	CH
3-OH	-	3.09 (s)	OH
5-OH	-	4.62 (s)	OH

Table 2 Chemo suppressive effect of pulcherrimin A against *P. berghei* infected mice

Groups	Average %Parasitaemia
50 mg/kg	0.95±1.24
200 mg/kg	0.70±0.38
400 mg/kg	1.28±0.86
CQ	0.00
Negative control	2.22±0.45

Values are mean ± SEM (n=5). CQ= Chloroquine

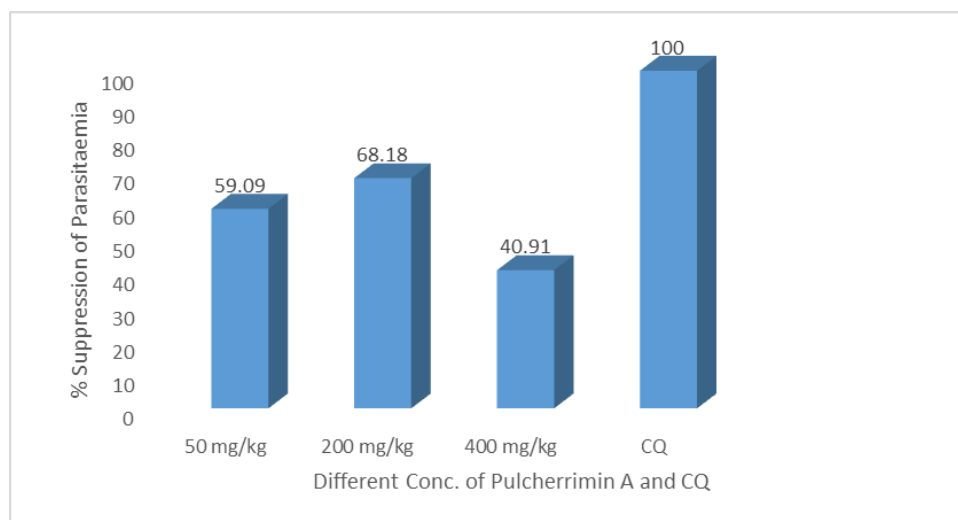


Figure 2 Average % Suppression of parasitaemia

4. Discussion

Compound 1 (CP252) was isolated as a white solid. The EI-MS of compound 1 has a molecular ion peak (M^+) at 588.2. The molecular formula was deduced to be $C_{34}H_{36}O_9$, from High Resolution EI-MS m/z : 588.2384 [M^+] which requires 17 degrees of unsaturation (DoU). The 1H - and ^{13}C -NMR spectral data of compound 1 confirmed the presence of a beta hydroxyl group at C-3, resulting in an increase of the chemical shift of the alpha hydrogen (δ 3.8, d, $J=8.0$). The primary methyl group of C-18 (s, δ 1.51, ^{13}C ; δ 20.62) has carboxyl functionality (^{13}C δ 177.7) attached to a neighbouring carbon. The ^{13}C -NMR, 1H -NMR and MS also revealed the presence of benzyloxy groups at C-6 and C-7. The ^{13}C - and 1H -NMR spectroscopic data were characteristics of the cassane-type diterpenoids. On the basis of the spectroscopic data (MS, IR, UV, 1 and 2D NMR) and comparison of data with literature, the isolated compound was established to be pulcherrimin A and was assigned 3,5,6,7-tetrahydroxy-19-vouacanoic acid;(3 β , 5 α ,6 β ,7 β)-form,6,7-dibenzoyl [11].

Pulcherrimin A exhibited an anti-malarial activity against *P. berghei* in mice. This is observed by a corresponding increase in suppression as the dose increased from 50 to 200 mg/kg, an indication of anti-malarial activity [29]. However, a further increase of the dose to 400 mg/kg resulted in an increase in percentage parasitaemia with a corresponding decrease in suppression, indicating a decrease in antimalarial activity. Similar results were obtained by Tekleab et al [30], whereby the middle dose of ethanolic extract of *Paulinacipinnata* showed a higher chemosuppressive effect against *P. berghei* than the upper and lower doses.

In vivo antiplasmodial activity can be classified as moderate, good, and very good if an extract displayed percentage parasitaemia suppression equal to or greater than 50% at a dose of 500, 250, and 100 mg/kg body weight per day, respectively [31, 32]. Based on this classification, pulcherrimin A could be said to have exhibited a good (68%) anti-malarial activity at 200 mg/kg. The fact that pulcherrimin A showed a good anti-plasmodial activity at 200 mg/kg and not 400 mg/kg, indicates that pulcherrimin A could be recommended for use at moderate doses for optimal result. However, the *in vitro* antimalarial investigation of Compounds 1 against the fundamental parasite of human malaria; *P. falciparum* [D6; chloroquine sensitive and W2; chloroquine-resistant strains] should be carried out to further substantiate the findings of this study.

5. Conclusion

The study revealed that pulcherrimin A isolated from the stem bark of *C. pulcherrima* exhibited moderate-dose significant inhibition of *Plasmodium berghei*, thus authenticating the local usage of different parts of the plants in managing malaria and pyrexia-related diseases. However, it is therefore recommended that the acute, sub-acute and chronic toxicity level of pulcherrimin A be examined for the purpose of medication. This study, to the best of our knowledge, is the first report of the *in vivo* anti-malarial evaluation of pulcherrimin A isolated from *C. pulcherrima* stem bark.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

We declare that we have no conflict of interest.

Statement of ethical approval

This study was approved by the ethical review board of Faculty of Life Sciences of University of Benin, Nigeria, with a reference number HRPGC/578/2015. All experiments were conducted in accordance with the National Institute of Health Guide for care and use of laboratory animals.

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