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# Antiplasmodial and Acute Toxicity Studies of Fractions and Cassane-Type Diterpenoids from the Stem Bark of *Caesalpinia pulcherrima* (L.) Sw.

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ABSTRACT

Malaria is a major public health disease affecting millions of people worldwide especially in sub-Saharan Africa, with annual deaths of over 4 million. The emergence of resistant strains of Plasmodium parasite to currently used drugs necessitates the search for newer and affordable cure for malaria from medicinal plants sources. *Caesalpinia pulcherrima* (L) Sw. is used in traditional medicine for the treatment of various diseases including malaria. Phytochemical, acute toxicity studies and antiplasmodial activities were carried out on the stem bark extracts of the plant. Fraction (HEEA) was fractionated over silica gel column to obtain pure compounds (characterized by IR, UV, 1D and 2D spectroscopy) which were subjected to antiplasmodial investigations. Phytochemical studies revealed the presence of saponins, flavonoids, phenols, terpenoids, tannins, and alkaloids. The $LD_{50}$ was established at 5656.85 mg/kg body weight in Swiss albino mice. Of all the fractions, HEEA exhibited the highest antiplasmodial activities against both the D6 and W2 *Plasmodium falciparum* clones at $IC_{50}$ 3.7 and 5.3µg /mL, respectively. Two known compounds; Pulcherrin J (**1**) and 6*β*-cinnamoyloxy-7*β*-hydroxyvouacapen-5*α*-ol **(2)** were isolated from HEEA and investigated for antiplasmodial activities. They showed significant inhibition of parasites growth in the D6 and W2 clones with $IC_{50}$ values 10.25- >10.62 µM and 10.25- >10.62 µM, for compound **1** and **2**, respectively, as against those of the standard antimalarial drugs (Chloroquine and Artemisinin) with $IC_{50}$ values <0.0937 and <0.1062, respectively. These findings revealed that *C. pulcherrima* stem bark possess significant antiplasmodial activities and could be a promising source of newer antiplasmodial agents.

***Keywords***: *Caesalpinia pulcherrima*, phytochemical studies, acute toxicity, antipasmodial activity, Pulcherrin J.

## Introduction

Majority of the human race, especially from developing countries depend on the traditional system of medicine for the management of various diseases.[1] Thousands of plant species are used medicinally, chiefly as herbal preparations in the indigenous systems of medicine in different countries and are sources of safe, cheap and potent drugs which have continued to be effective, successful, popular and modern chemistry has not been able to substitute a good number of them[1] .Plants are natural reservoirs of medicinal agents which are widely assumed to be safe, but many are potentially toxic.[2] In spite of the millions of chemical compounds currently synthesized in the laboratory, and available for screening for the action of therapeutic value, natural products, particularly of plants origin remain the most important sources of new drugs.[3] In recent years, a significant renewal of interest in natural products as potential sources for new medicines has been observed by scientists as well as pharmaceutical companies[4]. Thus, searches for new drugs with better and cheaper substitutes from plant origin are a natural choice. The medicinal value of these plants lies in some chemical substances that produce a definite physiological action on the human body.[5]

In the face of considerable progress made in the treatment of parasitic diseases, malaria remains a major public health challenge in prevalent areas of the world, predominantly because of the widespread resistance of malaria parasites to currently existing anti-malarial agents, the resistance of the mosquito vectors to currently obtainable insecticides, the limited success in the development of malarial vaccines and the devastating and undesirable reactions of conventional anti-malarial drugs.[1] These developments and the intricacy of producing efficient vaccines underscore the imperative need for new antimalarials. In developing countries, especially in Africa, accessible treatments against malaria are mainly based on the use of traditional herbal medicine. Indeed, local plants play a significant role in treatment of many infectious diseases and a good number of them rely on herbal remedies[1]. *C. pulcherrima* (CP) is a flowering plant in the legume family fabaceae. They are normally grown as ornamental flowers in tropical gardens[6]. The plant is known to be a rich source of cassane-type diterpenoids, lupeol, lupeol acetate, carotenoids, quercetin, rutin, beta sitosterol, glycosides, phenols and steroids[7,8-10]. Different parts of the plant have

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been known to possess great medicinal value globally. The aerial parts are used as an abortifacient, emmenagogue, purgative, stimulant and emollient among the Nigerian communities.[3] The fruits, flowers, leaves, and stem barks are used as regular remedies for the treatment of a number of disorders including pyrexia, menoxenia, wheezing, bronchitis and malarial infection among the Chinese communities [11]. The sap from the leaves is also said to be antipyretic, the sap from the flower is known to treat sores and the seeds are said to heal a bad cough, breathing difficulty and chest ache. Extracts from the root are also said to be abortifacient.[6,12] Different workers, have reported the antiplasmodial activities of the leaves and stem bark of CP,[13,14] 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 with $P<0.005$ and 14.6 ± 1.3 µg/mL[15] against PB. In spite of these recognized arrays of reports on the therapeutic and pharmacological potentials of this plant, there is yet a dearth of information on the *in vitro* antiplasmodial activity on the fractions and isolated compounds from the stem bark of *C. pulcherrima*. Therefore, this study aims to investigate the *in vivo* acute toxicity, cell cytotoxicity, and *in vitro* antiplasmodial potentials of the fractions and isolated compounds from the stem bark of *C. pulcherrima*, in order to validate its ethnomedicinal use in the treatment of human malaria infection in the tropics.

## Materials and Methods

*General experimental*
The NMR spectra were determined on Bruker Avance AV-300 and AV-400 spectrometers in $C_3D_6O$. Chemical shifts are expressed in parts per million (ppm) using TMS as the internal standard. EI-MS was recorded at an ionizing voltage of 70 eV (direct probe) on a double focusing magnetic sector mass analyzer (JEOL JMS-600H).

*Collection and preparation of C. pulcherrima stem bark*
The fresh stem bark of *C. pulcherrima* was collected in June 2014 from Ugbowo Campus, University of Benin, Nigeria. The plant sample was identified by Mr. Ugbogu O.A. and Mr. Shasanya O.S. of the Forestry Research Institute of Nigeria, (FRIN) Nigeria, where a voucher specimen with number FHI 109969 was deposited. The stem bark was air dried at 28°C and ground to fine powder. The powdered stem bark was weighed and stored in an air-tight container.

*Extraction of Crude powdered sample*
The powdered stem bark of *C. pulcherrima* (2.5 kg) was extracted with 7 L of methanol by maceration at room temperature for 96 hours. The extract was concentrated to dryness using a rotary evaporator at reduced pressure. The concentrated extract was weighed and the percentage yield calculated based on the initial weight of the crude powdered sample. The extract was stored in an air-tight container and kept in a refrigerator at 4ºC until further analysis.

*Phytochemical Screening*
Simple chemical tests to detect the presence of alkaloids, tannins, saponins, flavonoids, terpenoids and phenols were carried out using standard methods [16-18].

*Acute Toxicity Studies*
*Animals*
Twenty-Five Swiss albino mice of either sex with average weight of 24.5 g obtained from the Nigerian Institute of Medical Research (NIMR), Lagos, Nigeria, were used for this investigation. The mice were housed in standard environmental condition of ambient temperature and relative humidity, and 12 h light and 12 h dark cycles. They had free access to standard pellet diet and water according to the National Institutes of Health (NIH) Guide for the care and use of laboratory Animals.[19]

*Experimental*
The acute toxicity study was performed by a method described by Igbe *et al*,[20] with modifications. The mice were divided into five separate groups of five mice each labeled 1-5. Groups 2-5 were the test groups, while group 1 was the control. Groups 2-5 received 1000, 2000, 4000, 8000 mg/kg of the methanol crude extract suspended in gum acacia respectively by oro-gastric syringe, while group 1 (control) received 10 % gum acacia solution by oral route. The animals were observed for common symptoms of toxicity and mortality within 24 hours, and the animals that survived after 24 hours were observed for any signs of delayed toxicity for 14 days. The median lethal dose ($LD_{50}$) was calculated using Equation 1:

$$LD50 = \sqrt{a * b}$$

Where
a = lowest lethal dose (where death of mice occurred)
b = highest non-lethal dose (where no death of mice occurred)[20, 21]

*Antiplasmodial/Parasite lactate dehydrogenase assay*
The *in vitro* antiplasmodial assay procedure employed was an adaptation of the parasite lactate dehydrogenase (pLDH) assay developed by Makler *et al*.[22] The assay was performed in a 96-well micro plate and included two *P. falciparum* clones. [Sierra Leone D6 (chloroquine-sensitive) and Indochina W2 (chloroquine resistant)]. In primary screening, the different fractions were tested in duplicate only on the chloroquine-sensitive (D6) strain of *P. falciparum*. The fractions and compounds showing >50% growth inhibition of the parasite were subjected to screening. The standard antimalarial agents chloroquine and artemisinin were used as positive controls, with DMSO (0.25 %) as negative control. The selectivity indexes were determined by measuring the cytotoxicity of samples on mammalian cells (VERO, monkey kidney fibroblast). All experiments were carried out in duplicate.

*Isolation and crystallization of compounds* **1** and **2**
Ground *C. pulcherrima* (L.) Swartz stem bark (2.5 kg) was soaked in methanol (7.5 L) at ambient temperature. 220 g of the crude extract was obtained after filtration and concentration, by using a rotary evaporator at 45ºC. 200 g of the crude extract was fractionated over silica gel column(230 - 400 mesh) with different solvents in order of increasing polarities, n-hexane (9.4 L); n-hexane:ethylacetate (1:1) (12.5 L), ethyl acetate (8.2 L), ethyl acetate:methanol (1:1) (13 L) and methanol (7 L). The different fractions obtained were concentrated *in vacuo* to give 0.45 g (0.23%), 38.81 g (19.41%), 25.75 g (12.75%), 127.73 g (63.87%) and 4.18 g (2.09%) for n-hexane, n-hexane:ethylacetate (1:1), ethyl acetate, ethyl acetate:methanol (1:1) and methanol, respectively. The fraction obtained on elution with n-hexane:ethyl acetate (1:1) (HEEA) gave the highest antiplasmodial activity and as a result, was re-chromatographed over silica gel ($SiO_2$, 6.5x135 cm column) with increasing proportions of n-hexane with ethyl acetate [100:0 (7.5 l), 95:5 (10 l), 90:10 (24.5 l), 85:15 (7.5 l), 80:20 (6 l), and 0:100 (4.5 l)]. Each obtained fraction (250 mL of each) was monitored with TLC and combined into 12 main fractions coded as CP4–9, CP10–17, CP18–33, CP34–48, CP49–61, CP63–76, CP77–92, CP93–123, CP124–135, CP136–139, CP140–152 and CP153–162. The fraction obtained from the above column gave crystalline precipitates, which were suspended in n-hexane, filtered and dried to obtain compound 1(130.4 mg) named pulcherrin **J**.[23]
Sub-fraction CP93-123 (6 g) was re-chromatographed with n-hexane:EtOAc eluting in step-wise gradient from 100:0 to 80:20. Fractions (100 mL each) were collected and monitored with TLC to give 3 sub fractions (CP93-123A, B and C). Sub fraction CP93-123A was re-chromatographed using same solvent system as above. A crystalline precipitate was formed which was suspended in n-hexane:EtOAc (97:3) and filtered, and the residue dried to give compound **2** (6$\beta$-cinnamoyl-7 $\beta$-hydroxy-vouacapen-5$\alpha$-ol)[24]. The spectral data, crystal data, data collection and structure refinement details of Compounds **1** and **2** can be obtained from [23, 24], respectively.

## Results and Discussion

The percentage yield from crude extract and fractions are as shown in table 1 below:

The phytochemical analysis of the methanol and aqueous crude extracts of the stem bark of *C. pulcherrima* revealed the presence of saponins, phenols, flavonoids terpenoids and tannins. These findings are in agreement with those of Sivasankari[25] and Sharma and Rajani[26]. Phytochemicals are compounds that act as free radical scavengers to help eradicate the highly charged oxygen molecules that are by-products of metabolized oxygen[27] and are believed to provide several

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health benefits.[28] Saponins are known to exhibit anti-inflammatory activity and erythrocyte haemolysis.[29, 30] Flavonoids commonly found in fruits and vegetable have been linked to decreased risk of mortality from coronary heart diseases and many more.[31] Alkaloids, Phenols and Tannins are also known for their antimicrobial, antidiarrhoeal and anthelmintic properties.[32]

In screening drugs, determination of $LD_{50}$ is usually an initial step in the assessment and evaluation of the toxic properties of a substance.[33] In this study, there was no sign of toxicity and change in behavioral pattern observed in the experimental animals treated with crude extract for doses up to 2000mg/kg. However, at doses 4000 to 8000 mg/kg body weight, there were obvious signs of toxicity: lethargic, anorexia, sleepiness and deaths. The $LD_{50}$ was established at 5656.85 mg/kg. These results supported the findings of Sharma and Rajani on the aerial parts of *C. pulcherrima*[17] and the findings of Ogu *et al*.,[34] on the aqueous extract of the stem bark of *C.* pulcherrima.[28] 'Based on Hodge and Sterner scale', a test drug administered orally is considered extremely toxic at $\leq 1$ mg $kg^{-1}$, highly toxic at 1-50 mg $kg^{-1}$, moderately toxic at 50-500 mg $kg^{-1}$, slightly toxic at 500-5000 mg $kg^{-1}$, practically non-toxic at 5000-15,000 mg $kg^{-1}$ and relatively harmless at $\geq$15,00 mg $kg^{-1}$.[35] However, it was reported[36] that any substance with $LD_{50} \geq 1000$ mg/kg is considered to be low toxicity or relatively safe.

According to previous findings by some authors, antiplasmodial activity was classified as highly active at $IC_{50} \leq 5$ µg/mL, promising activity at $\leq 15$ µg/mL, low activity at $\leq 50$ µg/mL and inactive at $> 50$ µg/mL.[37-39] The three fractions exhibited significant antiplasmodial activities however, the n-hexane:ethyl acetate fraction(1:1) (HEEA) exhibited the highest antiplasmodial activity against the chloroquine sensitive (D6) and chloroquine resistant (W2) *P. falciparum* clones at $IC_{50}$ 3.7 and 5.3µg/mL, respectively, while the methanol fraction (ME), exhibited the lowest antiplasmodial activities against the D6 and W2 *P. falciparum* clones at $IC_{50}$ 7.0 and 5.5 µg/mL, respectively. Due to the significant antiplasmodial activities exhibited by the three fractions, they were further assessed for their cytotoxicity activity in order to determine their selectivity index (see table 4).The significant antiplasmodial activity exhibited by these fractions against chloroquine sensitive and chloroquine resistant clones of *P. falciparum* could be due to the high presence of some active phytochemical components which may be acting in synergy with one another or singly to exert as implicated by Ogu *et al.*[31],in the *in vivo* antiplamodial activity of the aqueous extract of *C. pulcherrima* stem bark.

The HEEA exhibited the highest antiplasmodial activity against both clones of *P. falciparum* and as a result, was further subjected to column Chromatography to obtain compounds **1** and **2**.

Compound **1** (CP13) was isolated as white crystalline solid and displayed a molecular ion at 448.2617($M^+$) by HREIMS corresponding to a molecular formula $C_{29}H_{36}O_4$, signifying twelve (12) degrees of unsaturation. The MS, $^1$H-NMR and $^{13}$C-NMR also revealed the presence of trans cinnamoyl moiety. The $^1$H and $^{13}$C NMR spectroscopic data (see Table 6) were characteristics of the cassane-type diterpenoids. The spectroscopic data was compared with that of literature and was found to be Pulcherrin **J**.[40] The observed HMBC correlations of H-6 resonating at $\delta$ 5.43(t, J=3.0) to the carbonyl carbon of the cinnamyl side chain at $\delta$ 166.05 (C-1´) confirmed the location of the trans-cinnamoyloxy side chain at C-6. Thus compound **1** (CP 13) was assigned to be 6$\beta$-cinnamoyloxyvouacapen-5$\alpha$-ol and was named Pulcherrin **J**.

Compound **2** (CP93-123) was isolated as colourless prism and had a molecular formula of $C_{29}H_{36}O_5$ by HREIMS. The $^1$H and $^{13}$C are comparable to that of compound **1** except that the cinnamoyloxy group attached to C-6 in compound **1** has been interchanged and attached to C-7. In much the same way, the beta hydrogen attached to position 7 in compound **1** has been replaced by hydroxyl group. Compound **2** was successfully characterized through comparison of its spectra data with those reported in literature and was assigned as 6$\beta$-cinnamoyl-7$\beta$-hydroxy-vouacapen-5$\alpha$-ol.[41]

Compounds **1** and **2** were evaluated using the *in vitro* pLDH assay against the causative parasite of human malaria; *P. falciparum* [D6; chloroquine sensitive and W2; chloroquine-resistant strains]. The investigation of the isolated compounds revealed significant inhibition of parasites growth in D6 and W2 clones with $IC_{50}$ values of 10.25-10.62 µM and 10.25- 10.62 µM, for compounds **1** and **2**, respectively.

**Table 1:** Percentage yield of crude extract and fractions of *C. pulcherrima* stem bark.

| Crude extract and fractions | Percentage yield (%) (Stem bark) |
|---|---|
| Crude extract (CRU) | 8.80 |
| Hexane Fractions (HE) | 0.23 |
| Hexane/Ethyl acetate (1:1) (HEEA) | 19.41 |
| Ethyl acetate (EA) | 12.75 |
| Ethyl acetate/methanol (1:1) (EAME) | 63.87 |
| Methanol (ME) | 2.09 |

%yield of the crude was based on original weight of the powdered sample. While the %yield of fractions was based on 200g of crude extract.

**Table 2:** Phytochemical analysis of the crude extract of *C. pulcherrima* stem bark.

| Phytochemicals | Inference | |
|---|---|---|
| | Aqueous Extract | Methanol Extract |
| Alkaloids | – | + |
| Saponins | + | + |
| Tannins | + | + |
| Phenols | + | + |
| Flavonoids | + | + |
| Terpenoids | + | + |

+ indicates presence of compound(s), - indicates absence of compound(s).

**Table 3:** Oral acute toxicity results of the crude extract of *C. pulcherrima* stem bark in mice.

| Group | Number of mice | Dosage (mg/kg) | Clinical signs | Mortality |
|---|---|---|---|---|
| 1 | 5 | 0(Control) | None | 0/5 |
| 2 | 5 | 1000 | None | 0/5 |
| 3 | 5 | 2000 | None | 0/5 |
| 4 | 5 | 4000 | Lethargic | 0/5 |
| 5 | 5 | 8000 | Lethargic | 2/5 |

The median lethal dose as calculated from equation 1 above is $LD_{50}$ = 5656.9 mg/kg.

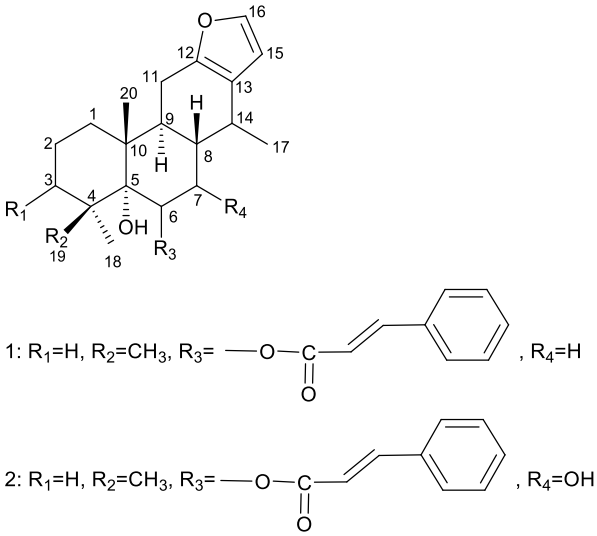


**Figure 1**: Molecular structure of Compounds **1** and **2.**

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Compound **2** ($IC_{50}$ value; 10.25 µM) showed a relatively higher antiplasmodial activity than compound **1** for both clones. The $IC_{50}$ values of the standard test drugs (artemisinin and chloroquine) (table 5) were significantly lower than those of the isolated compounds.

The Selectivity Indexes (SI) of the isolated compounds were observed to be relatively lower than those of the standard drugs, revealing that the standard drugs are relatively less toxic than compounds **1** and **2**.

**Table 4:** Activity of *C. pulcherrima* fractions against *P. falciparum*.

| | *P. falciparun* D6 | | *P. falciparun* W2 | | |
|---|---|---|---|---|---|
| | **$IC_{50}$ (µg/mL)** | ***SI*** | **$IC_{50}$ (µg/mL)** | ***SI*** | **VERO $IC_{50}$** |
| HEEA | 3.7 | 4.5 | 5.3 | 3.1 | 16.4 |
| EA | 8.2 | 2.7 | 7.1 | 3.2 | 22.4 |
| ME | 7.0 | >6.8 | 5.5 | >8.6 | >47.6 |

**Table 5:** Antimalarial screening of compounds **1** and **2** against *P. falciparum* D6 and W2 Clones and their selectivity indexes.

| | ***P. falciparun D6*** | | ***P. falciparum W2*** | | |
|---|---|---|---|---|---|
| **Metabolites** | **$IC_{50}$** (µM) | ***SI*** | **$IC_{50}$** (µM) | ***SI*** | **VERO $IC_{50}$** |
| **1** | >10.62 | 1.00 | >10.62 | 1.00 | >10.62 |
| **2** | >10.25 | 1.00 | >10.25 | 1.00 | >10.25 |
| **Artemisinin** | <0.0937 | >9.0 | <0.1062 | >9.0 | >16.86 |
| **Chloroquine** | <0.0937 | >9.0 | 0.4698 | >1.4 | >14.88 |

**Table 6:** $^{13}C$ and $^{1}H$ NMR data of Compounds **1** and **2**.

| **Position** | **1**<br>**Pulcherrin J** | | **2**<br>**6*β*-cinnamoyl-7*β* hydroxy-vouacapen 5*α*-ol** | |
|---|---|---|---|---|
| | **$\delta_C$ (400 MHz) $(D_3CCOCD_3)$** | **$\delta_H$ (400 MHz)** | **$\delta_C$ (300 MHz) $(D_3CCOCD_3)$** | **$\delta_H$ (400 MHz)** |
| 1a | 35.11 | 1.40 (m) | 35.52 | 1.40 (m) |
| 1b | | 1.75 (m) | | 1.65 (m) |
| 2a | 18.77 | 1.45 (m) | 19.04 | 1.41 (m) |
| 2b | | 1.75 (m) | | 1.72 (m) |
| 3a | 38.51 | 1.04 (m) | 38.43 | 1.02 (m) |
| 3b | | 1.92 (m) | | 1.86 (t, *J* = 12.8) |
| 4 | 39.62 | - | 49.97 | - |
| 5 | 76.47 | - | 78.04 | - |
| 6 | 72.53 | 5.43 (bs) | 74.50 | 5.65 (d, *J* = 4.0) |
| 7a | 32.25 | 1.50 (m) | 68.93 | 4.28 (m) |
| 7b | | 2.31 (m) | - | |
| 8 | 31.41 | 2.07 (m) | 39.10 | 1.97 (m) |
| 9 | 38.46 | 2.54 (m) | 38.02 | 2.48 (m) |
| 10 | 41.99 | - | 41.00 | - |
| 11a | 22.27 | 2.43-2.44 (m) | 22.44 | 2.48 (m) |
| 11b | - | 2.44-2.46 (m) | - | - |
| 12 | 150.16 | - | 150.23 | - |
| 13 | 123.10 | - | 122.94 | - |
| 14 | 31.88 | 2.54 (m) | 28.23 | 3.04 (q, *J* = 12, 6.4) |
| 15 | 110.18 | 6.21 (d, *J* = 1.6) | 110.47 | 6.23 (d, *J* = 1.6) |
| 16 | 141.10 | 7.27 (bs) | 141.26 | 7.29 (d, *J* = 1.6) |
| 17 | 17.83 | 0.96 (d, 6.8) | 17.45 | 1.04 (d, *J* = 7.2) |
| 18 | 28.11 | 1.01 | 28.18 | 1.10 (s) |
| 19 | 26.20 | 1.26 | 25.81 | 1.19 (s) |
| 20 | 17.15 | 1.48 | 17.77 | 1.46 (s) |
| 1′ | 166.05 | - | 166.89 | - |
| 2′ | 119.62 | 6.00 (d, *J* = 16) | 120.06 | 6.58 (d, *J* = 16) |
| 3′ | 145.33 | 7.70 (d, *J* = 16) | 145.11 | 7.71 (m) |
| 4′ | 135.20 | - | 135.48 | - |
| 5′/9′ | 128.95 | 7.66 (m) | 129.77 | 7.41 (m) |
| 6′/8′ | 129.66 | 7.42 (m) | 129.00 | 7.62 (m) |
| 7′ | 131.09 | 7.42 (m) | 131.08 | 7.41 (m) |

## Conclusion

The study revealed that *C. pulcherrima* is rich in phytochemicals, all of which have been reported to exhibit different physiological activities. The fractions and the isolated compounds exhibited moderate-significant inhibition of *Plasmodium falcipurium*, thus validating the ethnomedicinal usage of different parts of the plants in the management of malaria and fever-related diseases. However, findings from our study indicate that the stem bark of *C. pulcherrima* may be toxic at doses > 5000 mg/kg b. wt, following a single oral administration and as revealed by the SI results. This study, to the best of our knowledge, is the first report of the antiplasmodial assessment of the fractions and the isolated compounds from *C. pulcherrima* stem bark on *P. falciparum*.

## Conflict of interest

The authors declare no conflict of interest.

## Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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