Cytotoxic, Anti-inflammatory, and Leishmanicidal Activities of Diterpenes Isolated from the Roots of *Caesalpinia pulcherrima*

**Introduction**

*Caesalpinia pulcherrima* (L.) Sw. (*Caesalpiniaceae*), commonly called “Pride of Barbados” or “Paradise Flower”, is an evergreen shrub well distributed in tropical and subtropical regions of Africa, Asia, Australia, America, and the Caribbean. The plant is a common ornamental and has been used in traditional medicine as a stimulant, emenagogue, and arbotifacient [1, 2]. In addition, the plant has been found to be useful in the treatment of fever, malaria, diarrhea, asthma, and skin diseases [3]. A phytochemical investigation on the chloroform extract of *Caesalpinia pulcherrima* roots led to the isolation of ten known furanocassane diterpenoids, vouacapen-5α-ol (1), 8,9,11,14-didehydrovouacapen-5α-ol (2), 6β-cinnamoyl-7β-hydroxyvouacapen-5α-ol (3), pulcherrin A (4), pulcherrin B (5), pulcherrin J (6), pulcherrimin A (7), pulcherrimin B (8), pulcherrimin C (9), and pulcherrimin E (10). Chemical transformation of 3 and 7 gave compounds 6β-hydroxyisovouacapenol C (11), 6β-cinnamoyl-7β-acetoxyvouacapen-5α-ol (12), and pulcherrimin D (13). Cytotoxicity of compounds 1-13 was evaluated against three cancer cell lines (MCF-7, Hela, and PC-3). Anti-inflammatory potential of the compounds was evaluated via the oxidative burst assay using a luminol-amplified chemiluminescence technique. Leishmanicidal activity was tested against promastigotes of *Leishmania major* in vitro. Compounds 3, 4, 8, 9, and 10 were found active against all three cancer cell lines with IC_{50}s ranging from 7.02 ± 0.31 to 36.49 ± 1.39 µM. Compounds 8 and 13 exhibited a potent inhibitory effect on reactive oxygen species generated from human whole blood phagocytes (IC_{50} = 15.30 ± 1.10 µM and 8.00 ± 0.80 µM, respectively). Compounds 3, 9, and 13 showed significant activity against promastigotes of *L. major* (IC_{50} = 65.30 ± 3.20, 58.70 ± 2.80, and 55.90 ± 2.40 µM, respectively).
Chemotherapy, in addition to surgery, has proven to be useful in a number of different cancer types, but not without side effects. Some of these side effects compromise continued treatment, eventually leading to advanced stages of malignancy and mortality. As a result, there is a continued search for more effective and safer anticancer compounds, synthetic, and natural products have been promising in this regard.

The immune cells elicit their protective function against invading microbes and other foreign particles in a process called oxidative burst [14]. The term oxidative burst represents an early event during phagocytosis in which the immune cells generate reactive oxygen species (ROS) in response to soluble agonists [15, 16]. Oxidative burst therefore play a vital role in the host defense mechanism against intruders. However, in chronic inflammation, the overexpressed ROS causes damage to surrounding tissues and is associated with various inflammatory diseases [17]. Therefore, the inhibitors of phagocytes ROS generation can be potential anti-inflammatory agents [18]. Super oxide anion and other oxygen radicals such as hydrogen peroxide, hydroxyl radical, and hypochlorous acid are among the primary mediators of inflammation [17, 19, 20]. Their intracellular levels can be measured by a luminol-amplified chemiluminescence technique, which can be used to assess the immune function as well as to evaluate the anti-inflammatory potential and radical scavenging activity of various chemical and biological agents [21, 22].

Leishmaniasis is a parasitic protozoan infection caused by more than 20 species of the intracellular parasite *Leishmania*, with the most notable being *Leishmania major*, *Leishmania mexicana*, *Leishmania tropica*, *Leishmania braziliensis*, *Leishmania donovani*, and *Leishmania infantum* [23–25]. Though endemic in some tropical and subtropical countries, the disease currently affects more than 12 million people in more than 100 countries [25–27]. Toxicity and emergence of resistance to current antileishmanial agents is still a major challenge in the effective management of leishmaniasis. For this reason, there is an increasing search for less toxic and more effective alternatives for the management of leishmaniasis [23, 24, 28, 29]. A number of medicinal plants have been investigated and have shown some activity against various forms of the leishmania parasite [30–33].

In continuation of our study for the phytochemical and biological investigations of medicinal plants with potential anticancer properties, the methanol extract of *C. pulcherrima* roots was selected for investigation of cytotoxic activity against three cancer cell lines (MCF-7, HeLa, and PC-3). We report herein the isolation and structure elucidation of the cytotoxic compounds from the active fraction of *C. pulcherrima*. Cytotoxic activity represents a first step for screening potential bioactive substances. The isolated compounds were further evaluated for their ROS inhibitory as well as leishmanicidal activity.

### Results and Discussion

Column chromatographic fractionation of the cytotoxic chloroform extract of *C. pulcherrima* roots, followed by silica gel column chromatography, preparative TLC, and HPLC, led to the isolation of 10 furanocassane diterpenoids (1–10) (Fig. 1). Hydrolysis of 3 yielded 11, and acetylation of 3 and 7 yielded compounds 12 and 13, respectively.

The UV absorptions ($\lambda_{	ext{max}}$ at 230.0), IR absorption at 1452–1454 cm$^{-1}$, and $^1$H-NMR spectra (a pair of doublets at $\delta$ 6.12–6.20 and 7.19–7.22, $J$ = 1.5–2.0 Hz) of all of the compounds were characteristic of 2,3-disubstituted furanocassane-type diterpenoids [34–39]. On the basis of 1D and 2D NMR data, and comparison of $^1$H and $^{13}$C-NMR data with the literature, the structures of the compounds were identified as vouacapen-5α-ol (1),
8,9,11,14-didehydrovouacapen-5α-ol (2), 6β-cinnamoyl-7β-hydroxyvouacapen-5α-ol (3) [34], pulcherrin A (4), pulcherrin B (5) [40], pulcherrin J (6) [41], pulcherrimin A (7), pulcherrimin B (8), pulcherrimin C (9) [35], pulcherrimin E (10), 6β-hydroxyisovouacapenol C (11) [4], 6β-cinnamoyl-7β-acetoxyvouacapen-5α-ol (12) [42]. The 13C-NMR data of compound 3, 4, 8, 9, 10 significantly (p < 0.05) decreased MCF-7, HeLa, and PC-3 cell viability with IC50 ranging from 15.65 ± 0.21 to 36.49 ± 1.39 µM, 7.02 ± 0.31 to 28.35 ± 0.40 µM, and 15.64 ± 1.30 to 27.59 ± 0.26 µM for MCF-7, HeLa, and PC-3 cell lines, respectively. Compounds 7 and 13 showed activity against HeLa and PC-3 cell lines (IC50 = 14.50 ± 0.79 to 28.35 ± 0.40 µM), compound 12 showed activity against MCF-7 and HeLa cell lines (IC50 = 16.43 ± 0.69 and 20.41 ± 1.48 µM for MCF-7 and HeLa, respectively), while compound 1 showed mild activity against the HeLa cell line (IC50 = 35.45 ± 1.44 µM). Compound 3 was most the active against all three cell lines (IC50 = 15.65 ± 0.21 µM, 7.02 ± 0.31 µM, and 15.64 ± 1.30 µM for MCF-7, HeLa, and PC-3 cell lines, respectively) (Table 1).

Hydrolysis of compound 3 to 11 led to loss of activity against MCF-7 and PC-3 cell lines and a significant reduction in activity against the HeLa cell line. Similarly, compounds without substituents at C-6 and C-7 (compounds 1 and 2) or with only one substitution in either position (compounds 5 and 6) showed little or no cytotoxic activity.

These observations show that substitution at C-6 and C-7 in ring B of the vouacapene nucleus with a benzoyl or cinnamoyl group could be responsible for the observed cytotoxic activity. Compounds with the cinnamoyl substituents (compounds 3, 4, and 12) were more active compared to the benzoyl substituents (compounds 7, 9, and 10). The hydroxyl substituent at C-3 of ring A (compound 7) was found to be more active than the unsubstituted or acetylated counterpart (compounds 9 and 13) against HeLa and PC-3 cell lines, while unsaturation in ring A caused a slight increase in activity (compound 8, IC50 = 19.13 ± 0.88 µM), and acetylation led to a loss of activity against the MCF-7 cell line (compound 13, IC50 > 50 µM).

The anti-inflammatory activity of the compounds was evaluated by the oxidative burst assay using a luminol-amplified chemiluminescence technique. Zymosan-stimulated phagocytes elicit a chemiluminescence response in the presence of luminol [43, 44]. Luminol (5-amino-2,3-dihydro-1,4-phthalazindione), being of low molecular weight, can cross the cell membrane and reacts with myeloperoxidase-dependent intracellular oxygen metabolites released during the oxidative burst to form an excited state intermediate, which on relaxation emits light [22]. Presently, the ability of the compounds to suppress the release of ROS was used to assess their anti-inflammatory potential. The results showed that compounds 8 and 13 showed a potent inhibitory effect on oxidative burst with IC50 values of 15.3 ± 1.1 µM and 8.0 ±
Materials and Methods

General experimental procedures

Melting points were determined on a capillary melting point apparatus (Buchi). Optical rotations were measured on a Perkin-Elmer P-2000 polarimeter. IR spectra were recorded on KBr discs on an FTIR-8900 spectrophotometer (Shimadzu). UV spectra were recorded in MeOH on an Evolution 300B spectrophotometer (Thermo-Scientific). NMR spectra were determined on Bruker Avance AV-300, AV-500, and AV-600 spectrometers in CDCl₃. Chemical shifts are expressed in parts per million (ppm) using TMS as the internal standard. Optical rotations were measured on a Perkin-Elmer 241 spectropolarimeter. Mass spectra were recorded at an ionizing voltage of 70 eV (direct probe) on a double focusing magnetic sector mass analyzer (JEOL JMS-600H). HPLC was performed 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). HPLC was performed on a silica gel column (250 × 20 mm) and a UV detector (Japan Analytical Industry Co. Ltd.). Column chromatography was performed using Merck preparative silica gel (70–230 mesh). TLC analyses were performed on precoated silica gel 60 F₂₅₄ plates. The plates were visualized under UV (254 and 366 nm), and by spraying with ceric sulphate reagent and iodine vapor.

Plant collection

Fresh C. pulcherrima roots were collected within the campus of the University of Benin, Nigeria, in June 2014. The plant material was identified by Mr. Ugboogu O. A. and Mr. Shasanya O. S. of the Forestry Research Institute of Nigeria (FRIN) Ibadan where a specimen was submitted (voucher number FHI 109 969).

Extraction and isolation

Air-dried and powdered roots (2.9 kg) of C. pulcherrima Linn. were extracted with methanol (7.5 L) at room temperature for 7 days. The vacuum-dried extract (240 g) was suspended in MeOH:H₂O (4:1), and partitioned into petroleum ether, chloroform, and ethyl acetate soluble fractions by solvent-solvent extraction. The dried chloroform fraction (87.56 g) was fractionated by column chromatography (SiO₂, 8 cm×75 cm) eluting with hexane:CH₂Cl₂ (0:100 to 10:100) followed by HPLC [CH₂Cl₂:MeOH (99.5:0.5) 20 mL eluate] followed by recrystallization in MeOH to obtain compound 10 (30.4 mg, t₁₈ = 18 min).

Column chromatography of fraction V (1.75 g) on SiO₂ (3 cm×60 cm, hexane:Me₂CO (98:2–80:20), 50 mL eluate) yielded three subfractions (VA–VC). Subfraction VA (136.1 mg) was subjected to HPLC [CH₂Cl₂:MeOH (98:2) at 4 mL/min] to obtain compound 10 (30.4 mg, t₁₈ = 18 min). Fraction VI (1.33 g) was recrchromatographed on a silica gel column (3 × 50 cm; CH₂Cl₂:MeOH (99.5:0.5–95:5) 20 mL eluate) followed by recrystallization in MeOH to obtain compound 7 (177.6 mg).

Hydrolysis of compound 3: 50 mg of 3 was refluxed with 5% KOH in MeOH for 3 h. After cooling, the mixture was extracted with CH₂Cl₂. The CH₂Cl₂ extract, on drying, yielded compound 11 (15 mg).

Acetylation of compounds 3 and 7: 50 mg each of compounds 3 and 7 were dissolved separately in a mixture of pyridine and acetic anhydride (2 mL each), and left overnight at room temperature. The resulting products after workup were separated by PTLC, which yielded compounds 12 (32 mg) and 13 (36 mg) from 3 and 7, respectively.
Cytotoxicity (MTT Assay)

Cell culture: Human breast cancer cell line (MCF-7) (ATCC: HTB-22), human cervical cancer cell line (HeLa) (ATCC: CCL-2), and human prostate cancer cell line (ATCC: CRL-1435) were cultured in Dulbecco’s modified Eagle’s medium (MCF-7 and PC-3) and Eagle’s minimum essential medium (HeLa) at 37 °C in a 5% CO₂ atmosphere. Confluent cells were trypsinized and plated in 96-well tissue culture plates (seeding density 8000 cells/well for MCF-7, 5000 cells/well for HeLa, and 10,000 cells/ well for PC-3) in 100 µL of medium.

Treatment with compounds: After 24 h, compounds were added in triplicate at 50 µM concentrations, and incubated for 48 h. The media was removed and 200 µL of MTT solution (0.5 mg/mL) were added to each well, and incubated at 37 °C for 3 h. The formazan crystals formed by the reduction of MTT were dissolved in 100 µL media served as the blank [45]. Doxorubicin (046 901, Sigma-Aldrich, purity ≥ 98% by HPLC) and pentamidine (ICN Biomedical Inc.; purity ≥ 98% by HPLC) were used as standards. The plates were incubated at 25°C for 72 h. The culture was examined microscopically on a Neubauer counting chamber [47].

The mortality rate was calculated by the following formula:

\[
\text{Mortality Rate(%) = } \frac{\text{Number of live cells in test}}{\text{Number of live cells in control}} \times 100
\]

IC₅₀ values were calculated using Ezfit 5.03 software (Perella Scientific). All assays were performed in triplicate.

Statistical analysis

Data are expressed as the mean ± S.E. M. of triplicate determination. Statistical significance was calculated by one-way analysis of variance, and, where applicable, differences between means were estimated by Duncan’s multiple range test.

\[ \frac{\text{mean O}_{\text{D}: \text{test compound}}}{\text{mean O}_{\text{D}: \text{control}}} = \frac{\text{mean O}_{\text{D}: \text{blank}}}{\text{mean O}_{\text{D}: \text{blank}}} \]

Inhibitory activity (%) = \[
\frac{\text{Control group RLU} - \text{Test Group RLU}}{\text{Control group RLU}} \times 100
\]

Leishmaniacal activity

Leishmania major was obtained from DESTO, and maintained in modified NNN biphasic medium using normal physiological saline. Leishmania parasite promastigotes were cultured in RPMI 1640 medium (Sigma), supplemented with 10% heat inactivated fetal calf serum (FCS) (PAA Laboratories GmbH). Parasites at the log phase were centrifuged at 2000 rpm for 10 min, and washed three times with saline. Parasites were diluted with fresh culture medium to a final density of 1 × 10⁶ cells/mL.

In a 96-well microtiter plate, medium (180 µL) was added in different wells, and 20 µL (250 µg/mL) each of the test compounds were added to the medium in different wells, and serially diluted. Then, 100 µL of parasite culture were added in each well. To serve as a control, medium without a test compound was used. Amphotericin B (MP Biomedical Inc.; purity ≥ 98% by HPLC) and pentamidine (ICN Biomedical Inc.; purity ≥ 98% by HPLC) were used as standards. The plates were incubated at 25°C for 72 h. The culture was examined microscopically on a Neubauer counting chamber [47].

The percent of inhibition or decrease in cell viability was calculated using the following formula:

\[
\% \text{ Inhibition} = 100 - \frac{\text{mean O}_{\text{D}: \text{test compound}} - \text{mean O}_{\text{D}: \text{control}}}{\text{mean O}_{\text{D}: \text{blank}}} \times 100
\]
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\[ M^+ \] (16) for Ca_{3}H_{36}O_{8}. UV: (MeOH) \( \lambda_{\text{max}} \) 230.0. IR: (KBr) \( \nu_{\text{max}} \) 3496.7, 2937.4, 1724.2.

\textbf{Pulcherrimin E (10):} White solid, m. p. 217–220°C, \( [\alpha]_{D}^{23} + 67.7 \) (c = 0.16, CHCl_{3}). \( R_{f} \) 0.425 (Hexane:Me_{2}CO 7:3). HREI-MS: \( m/z \) (rel. int.) 630.2476 \([M^+] \) (16) for Ca_{3}H_{36}O_{10}. UV: (MeOH) \( \lambda_{\text{max}} \) 230.0. IR: (KBr) \( \nu_{\text{max}} \) 3539.0, 2958.6, 1724.2.

\textbf{6β-Hydroxyisovouvacapenol C (11):} White solid, m. p. 233–235°C, \([\alpha]_{D}^{23} + 130.3 \) (c = 0.10, CHCl_{3}). \( R_{f} \) 0.375 (hexane:Me_{2}CO 4:1). El-Ms: \( m/z \) (rel. int.) 334.3 \([M^+] \) (36) for Ca_{3}H_{36}O_{8}. UV: (MeOH) \( \lambda_{\text{max}} \) 229.0. IR: (KBr) \( \nu_{\text{max}} \) 3539.1, 2958.6, 1724.2.

\textbf{6β-Cinnamoyl-7β-acetoxyvouvacapen-5α-ol (12):} White solid, m. p. 122–124°C, \([\alpha]_{D}^{23} + 100.7 \) (c = 0.15, CHCl_{3}). \( R_{f} \) 0.50 (hexane:Me_{2}CO 8.5:1.5). El-Ms: \( m/z \) (rel. int.) 506.4 \([M^+] \) (20) for Ca_{31}H_{38}O_{6}. UV: (MeOH) \( \lambda_{\text{max}} \) 282.0. IR: (KBr) \( \nu_{\text{max}} \) 3520.0, 2923.9, 1722.3.

\textbf{Pulcherrimin D (13):} White solid, m. p. 204–206°C, \([\alpha]_{D}^{25} + 48.7 \) (c = 0.12, CHCl_{3}), \( R_{f} \) 0.625 (CH_{3}Cl:MeOH 9:8:0.2). El-Ms: \( m/z \) (rel. int.) 630.0 \([M^+] \) (10) for Ca_{3}H_{36}O_{10}. UV: (MeOH) \( \lambda_{\text{max}} \) 230.0 and (IR: (KBr) \( \nu_{\text{max}} \) 3496.0 (O-H), 2960.5, 1726.2, 1276.8.

**Supporting information**

Cytotoxic activity of the extract and fractions of \textit{C. pulcherrima} root bark, \( ^{1}H \)- and \( ^{13}C \)-NMR data of compounds 1–13, and \( ^{1}H \)-NMR spectra of compounds 1–13 are available as supporting information.

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**Conflict of Interest**

The authors declare no conflict of interest.


