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# Isolation of Natural Product Hits from *Peperomia* species with Synergistic Activity against Resistant *Plasmodium falciparum* Strains

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***Authors' contributions***

*This work was done with the collaboration of all authors. Author MNN contributed to the study design, wrote the protocol, directed the bench work and drafted the manuscript. Author HMM performed the biological experiments. Author JAM designed and supervised the chemistry aspects of the work and contributed in writing the manuscript. Author DZ collaborated in the bench work, analyzed the data and also participated in drafting the manuscript. Author SBB conducted the bench chemistry work. Author VPKT conceived the study, designed and supervised the work and revised the manuscript. All authors read, corrected and approved the final manuscript.*

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## ABSTRACT

**Aims**: This study investigated the antiplasmodial activity of crude extracts, fractions and pure isolates of *P. vulcanica* and *P. fernandopoioana* (Piperaceae). Toxicity and interaction between the most active natural products were also assessed.
**Study Design:** Bioassay-guided approach was used to identify and further investigate the most active components against chloroquine-sensitive and resistant *P. falciparum* strains.
**Place and Duration of Study:** Departments of Biochemistry and Molecular Biology, Chemistry and Biotechnology Unit, Faculty of Science, University of Buea, Cameroon for one year.
**Methodology:** Test substances were prepared from the two plants and screened on four strains of *P. falciparum* (chloroquine-sensitive 3D7, multidrug resistant W2mef and Dd2, and a field isolate

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SHF4). Activity was determined by fluorescence microscopy and the parasite lactate dehydrogenase assay. The most active pure compounds were tested in combination and also tested in BALB/c mice for acute toxicity.
**Results:** The crude extracts showed moderate activity ($IC_{50}$ from 7.05 – 22.59 µg/mL). Eight of 16 compounds isolated from the hexane and methylene chloride extracts of *P. vulcanica* showed high activity ($IC_{50}$ from 0.89 - 3.23 µg/mL against W2mef). Four of the most active compounds tested in two different combinations showed synergism and two of them showed no signs of acute toxicity. Four fully characterized isolates**:** 5-Demethyltangeretin (**1**), Stigmasterol (**2**), Matairesinol dimethyl ether (**3**) and Peperovulcanone A (**4**) showed high to moderate activity ($IC_{50}$s ranging from 1.14 – 22.29 µg/mL).
**Conclusion:** These findings support the use of *P. vulcanica* in traditional medicine for the treatment of malaria and the plant material should be further evaluated towards development into a phytomedicine. Further exploration of the hits in combination with standard antimalarials may yield new efficacious antimalarial treatments.

*Keywords: Malaria; resistance; antiplasmodial; natural products; synergism; acute toxicity.*

## 1. INTRODUCTION

Chemotherapy remains the main tool against the heavy burden of clinical malaria in the absence of an efficacious vaccine [1]. However, virtually all drugs in current use are seriously threatened by increasing resistance with multidrug resistance in some cases [2]. Resistance has been significantly attenuated following the switch to combination therapy [3]. In spite of this approach there is evidence of emerging resistance to the World Health Organization (WHO)-recommended artemisinin-based combination therapies manifested as a decrease in clinical efficacy [4,5]. Hence there is an urgent need for new efficacious antimalarials. Presently, antimalarial discovery strategies include amongst others optimization of therapy with available drugs such as by combination therapy, chemical modification and development of analogs of existing antimalarials, screening of compounds used in treating other diseases, exploration of new chemotherapeutic targets, molecular modeling using virtual screening technology and docking and discovery of antiplasmodial natural products [6]. The study of medicinal plant preparations and their natural products has yielded important discoveries which could be exploited in addressing the need for new efficacious antimalarials. This is evidenced by the antiplasmodial activity recorded in several studies with isolation of natural product hits which may serve as templates for the derivation of suitable antimalarial leads [7]. In Cameroon, *Peperomia vulcanica* and *Peperomia fernandopoiana* of the Piperaceae family are used as medicinal plants by traditional medicine practitioners to treat febrile illnesses; particularly in the South West and North West regions where these plants grow at high altitudes. There are over one thousand species of *Peperomia* making it the largest genus of the Piperaceae; they are widely distributed across sub-tropical and tropical regions of the world but mostly in Central and Northern parts of South America [8]. They are herbs and most grow as epiphytes in rain forest habitats. Despite the existence of so many species, not many have been extensively studied. Several are used commonly as ornamental house plants. Very few such as *Peperomia pellucida* and *P. vulcanica* have been shown to have medicinal properties and also used as a spice [9,10]. *P. vulcanica* and *P. fernandopoiana* species are well known in Cameroon [11,12]. In a previous study of some plants used to treat fevers, considerable antiplasmodial activity was recorded against chloroquine-sensitive *Plasmodium falciparum* for *P. vulcanica* [12,13]. Antibacterial activity has also been reported for *P. vulcanica* and *P. fernandopoiana* in two studies [11,14]. These findings motivated this study which focused on investigating the antiplasmodial properties of the two *Peperomias* against sensitive, resistant and field parasite strains. We also attempted isolation and identification of the natural products which explain the antiplasmodial activity observed and their interaction. The acute toxicity of some of the most active isolates was also investigated.

## 2. MATERIALS AND METHODS

### 2.1 Preparation of Test Substances

#### 2.1.1 Plant collection and preparation of crude extracts

The plants were collected whole in the month of April in 2011, from Mount Cameroon, South West region of Cameroon by Mr. Ndive Elias, a

botanist in the Limbe Biodiversity and Conservation Centre, Cameroon. The botanist identified the plants using voucher specimens in the herbarium of the Centre as *P. vulcanica* Baker and C.H. Wright, voucher NºS.C.A. 8829 and *P. fernandopoiana* C.D.C., voucher NºS.C.A. 8786. Crude extracts were prepared as described [11]. Briefly, the air dried plant materials were ground to fine powder and each plant powder (1.75 kg of *P. vulcanica* and 2 kg of *P. fernandopoiana*) was sequentially macerated at room temperature for 3 x 4 days each, in 6 L of hexane (Hex), 6 L of methylene chloride (MC) and 6 L methanol (MeOH) respectively. The mixture was filtered and the filtrate concentrated by rotary evaporation. The crude extracts were recovered, dried, weighed and stored at 4ºC until further use.

#### 2.1.2 Fractionation and isolation of pure compounds

Bioassay – guided fractionation was done only on active crude extracts to isolate the pure compounds for further antiplasmodial screening as earlier described [11]. The hexane crude extract was fixed on Celite and fractionated using Vacuum Liquid Chromatography (VLC) on silica gel and eluted with gradients of ethyl acetate (EtOAc [0 – 80%]) in hexane. Following thin layer chromatography (TLC), 8 main fractions were identified, three of which were active. Fraction 3 (obtained with 10% EtOAc - hexane) was further chromatographed on $SiO_2$ with a gradient of EtOAc in hexane to afford stigmasterol (130mg). Fraction 5 (obtained with 40% EtOAc - hexane) was chromatographed on $SiO_2$ and later passed through Sephadex LH20 to yield 5-demethyltangeretin (44mg) and white crystals of matairesinol dimethyl ether (500mg). Their structures were determined by spectroscopy (1D) and measurement of melting points and the data obtained compared with published data in the literature [11,15]. The spectroscopic data of three of four fully characterized isolates are as follows:

5-Demethyltangeretin (**1**): white crystals, mp 167-168ºC, $^{13}$C NMR (125 MHz, $CDCl_3$), δ(ppm): 55.4 (C4-OMe), 60.5 (C6-OMe), 61.4 (C8-OMe), 61.8 (C7-OMe), 103.2 (C-3), 106.1 (C-10), 114.6 (C-3', C-5'), 122.6 (C-1'), 128.2 (C-2',6'), 132.6 (C-8), 135.8 (C-6), 145.4 (C-9), 148.5 (C-5), 152.6 (C-7), 162.4 (C-4'), 164.0 (C-2), 183.0 (C-4).

Stigmasterol (**2**): white crystals, mp 165ºC, $^{13}$C NMR (125 MHz, $CDCl_3$), δ(ppm): 12.0 (C-29), 12.2 (C-18), 19.0 (C-26), 19.4 (C-19), 21.1 (C-11, C-21), 21.2 (C-27), 24.5 (C-15), 25.4 (C-28), 28.93 (C-16), 31.6 (C-2), 31.9 (C-7, C-8, C-25), 36.5 (C-10), 37.2 (C-1), 39.7 (C-12), 40.5 (C-20), 42.3 (C-4), 42.4 (C-13), 50.1 (C-9), 51.2 (C-24), 55.9 (C-17), 56.9 (C-14), 71.7 (C-3), 121.7(C-6), 129.3 (C-23), 138.3 (C-22), 140.7 (C-5).

Matairesinol dimethyl ether (**3**): white crystals, mp 127-128ºC, $^{13}$C NMR (125 MHz, $CDCl_3$), δ(ppm): 34.4 (C-5), 38.1 (C-6), 41.0 (C-3), 46.4 (C-2), 55.8 (2xOMe), 55.9 (2xOMe), 71.1 (C-4), 111.0 (C-5"), 111.3 (C-5'), 111.8 (C-2"), 112.3 (C-2'), 120.5 (C-6"), 121.3 (C-6'), 130.1 (C-1'), 130.4 (C-1"), 147.8 (C-4"), 147.9 (C-4'), 148.9 (C-3', C-3"), 178.6 (C-1).

The fourth compound, Peperovulcanone, was isolated as earlier reported [15].

### 2.2 Biological Experiments

#### 2.2.1 Sources and culture of malaria parasites

Laboratory strains of *P. falciparium* 3D7 (chloroquine - sensitive) and W2mef were acquired from MR4 (Manassas, Virginia, USA). *P. falciparum* Dd2 was offered by Professor Alex Rowe of C.I.I.E University of Edinburgh, UK. W2mef and Dd2 are multidrug resistant clones of the W2 strain with resistance to chloroquine, quinine, mefloquine, sulfadoxine and pyrimethamine. A field isolate (SHF4) was obtained from a confirmed malaria patient prior to chemotherapy in a health centre in Buea, Cameroon.

The candle jar technique [16] was used to culture the parasites with modifications as described [17,18]. The parasites were grown using human red blood cells (O+) suspended in complete culture medium [containing 10.43 g sterile-filtered RPMI 1640 medium (Sigma Aldrich) supplemented with 25 mM HEPES, 5 g/L Albumax II (10 g/L for field strains), 2 mM L-glutamine, 15 µg/mL hypoxanthine, and 0.75 mL of 80 mg/mL gentamycin, incubated at 37ºC, in an atmosphere of 5% $CO_2$ in a HERA Cell 150 incubator (Thermo electron, Germany).

For field isolates, anticoagulated venous blood from a malaria patient after microscopic detection of the parasite was collected in heparin or citrate phosphate dextrose solution. The blood sample was washed in pre-warmed (37ºC) serum-free RPMI medium, centrifuged three times for 5 minutes at 1000 g and the buffy coat removed after each centrifugation. The washed blood was immediately transferred into a culture flask in

same medium and incubated as above. The patient blood was cryopreserved as described [17] when not immediately cultured.

**2.2.2 Bioassay of activity of test substances**

*2.2.2.1 Determination of antiplasmodial activity*

Stock solutions of all test substances were prepared by initially dissolving in dimethyl sulfoxide (DMSO, 100 - 200 µL), the volume made up with malaria culture medium giving a 0.2% final DMSO concentration. The solutions were sterile-filtered through 0.22 µm filter and stored at -20ºC until testing. Positive controls of 10 µg/mL chloroquine diphosphate (SIGMA) and 5 µg/mL artemether (SIGMA), were prepared following the same method. Antiplasmodial activity was assessed using the *in vitro* growth inhibition assay as described [17]. A primary screen was performed in triplicate wells at 100 µg/mL, to identify test substances with considerable activity (>50% inhibition of growth), which were re-tested in a secondary screen to determine their $IC_{50}$. Extracts which produced high inhibitions were fractionated, active fractions identified using the primary screen at 100 µg/mL and pure compounds isolated from them as described above. All assays were performed in 96-well microtitre plates with 100 µL of parasitized blood containing 1% parasitaemia added to the same volume of test substance in all required wells. Positive and negative (1% parasitized blood plus culture medium without test substance or drug) control wells were included and the plate was incubated under culture conditions as above. Inhibition in the primary screen was measured using fluorescence microscopy with acridine orange stain at x 400 magnification and the parasite lactate dehydrogenase (pLDH) assay in the secondary screen.

*2.2.2.2 Determination of parasitaemia*

Following the primary screen the number of infected erythrocytes (Ei) was counted using a counting grid in 20 microscopic fields per well with uniform spread of cells (with approximately 400 cells per grid area giving a total 24 000 cells per test concentration).

The percentage parasitaemia was obtained as follows:

$$[(Ei / 24\,000) \times 100] \quad (1)$$

and percentage inhibition per concentration was calculated using the formula below [17]:

$$[(\%\ \text{parasitaemia in control wells} - \%\ \text{parasitaemia of test wells}) / (\%\ \text{parasitaemia of the control wells})] \times 100. \quad (2)$$

In the secondary screen, extracts which fulfilled the criterion of >50% inhibition specified above were serially diluted and tested at final concentrations ranging from 3.125 -100 µg/mL. Growth inhibition was checked using fluorescence microscopy as described above and the plate was stored at -20ºC for the parasite lactate dehydrogenase assay. Sixteen pure compounds isolated from *P. vulcanica*, were tested in a primary screen at a single concentration of 40 µg/mL in duplicate wells, similarly as for the crude extracts. This was followed by a secondary screen at final concentrations of 0.625 – 40 µg/mL for the compounds with ≥50% inhibition of parasite growth in the primary screen and the pLDH assay was used to determine $IC_{50}$ values. The modified pLDH assay was used to determine the antiplasmodial activity as described [19], and optical densities were read using a plate reader (Emax-Molecular Devices Corporation, California, USA).

*2.2.2.3 Assessment of combinations of active compounds*

Pure compound interactions were investigated as previously described [20] on W2mef. Four of the most active compounds from *P. vulcanica* were tested in two pairs by combining compounds initially separated with solvents of different polarities (hexane and methylene chloride) during preparation of crude extracts. Solutions of the compounds were prepared same as above to give concentrations of 32 times the pre-determined $IC_{50}$s such that dilution would attain the $IC_{50}$ of the compound by the fourth two-fold serial dilution or approximately at the midpoint of the serial dilution of each compound. Solutions of each pair of compounds were combined in volume ratios per well as follows: 5:0 i.e. 5 volumes of one solution combined with zero volume of the other (100:0 µL), 4:1 (80:20 µL), 3:2 (60:40 µL), 2:3 (40:60 µL), 1:4 (20:80 µL) and 0:5 (0:100 µL) in separate duplicate wells and tested as above. Then the $IC_{50}$s of the two compounds alone and in combination were determined using the pLDH assay as above and isobolograms generated to determine the nature of their interaction [20].

### 2.2.3 Acute toxicity test

The test was performed in Balb/c laboratory mice (21.34±1.47g) of about 3 months old, divided in 3 groups of 6, equal in males and females. They all had access to food and water. The two most active pure compounds (MC3 and Hex 14 with $IC_{50}$s of 0.89 and 4.04 µg/mL respectively, the latter being the average value for Dd2 and W2mef) were tested at 10 × $IC_{50}$ per kg body weight of mouse [i.e. 8.9 mg/Kg body weight and 40.4 mg/Kg body weight respectively]. The required amount was initially dissolved in DMSO and made up to final volume with distilled water giving 0.25% DMSO in the solution. Each compound was administered to a separate group in a single dose i.e. 189.9 µg of MC3 or 862.1 µg of Hex 14 per mouse in a volume of 1mL on day zero by slow intraperitoneal injection using a 1mL syringe [21]. DMSO (0.25% per gram body weight) was also tested similarly in the control group. The physical activity, feeding and mortality were observed daily for 7 days [22].

## 2.3 Statistical Analysis

The $IC_{50}$s of crude extracts and pure compounds were determined from optical densities from the pLDH assay using the HN-NonLin V1.1 software (www.malaria.farch.net) and IC Estimator Run Regression version 1.1, an online calculator of $IC_{50}$ values. The antimalarial activity of test compounds were expressed as mean $IC_{50}$±S.D of the separate experiments performed in triplicate for crude extracts and in duplicate for pure compounds. The control wells optical densities were taken as 100% pLDH activity. A test was considered as having failed if the ratio of the optical density (OD) for the 100% well to the OD for the 0% well was less than 1.7. For the combination studies, the $IC_{50}$s obtained were used to calculate the fractional inhibitory concentration (FIC) values. The FICs of each compound for each combination ratio were calculated and isobolograms plotted. The isobologram which shows a concave curve indicates synergy while a convex curve indicates antagonism [20]. Combination indices of <1 and >1 confirm synergism and antagonism respectively. Student's t-test was used to compare the experimental and control groups of the acute toxicity before and after treatment, $P \leq 0.05$ was considered as statistically significant.

# 3. RESULTS

## 3.1 Identification of Isolated Compounds

Compound 1 was obtained as white crystals in hexane, mp 167-168ºC. Its $^1$H NMR spectrum showed four methoxy resonances at δ 3.82- 4.03 (12H, s, 4 × OMe). Other protons resonated at δ 12.75 (1H, s, 5-OH), 8.05 (2H, d, J = 9Hz, H – 2', 6'), 7.16(2H, s, 9Hz, H – 3', 5'), 6.90 (1H, s, H-3). Its $^{13}$C NMR signals above were in close agreement with literature values [23,24], thus enabling the assignment of the structure of 5-demethyltangeretin to compound 1. Compound 2 was obtained as white crystals in hexane, mp 165ºC. Its $^1$H NMR spectrum showed signals at δ(ppm): 0.67 (3H, s, $CH_3$-13), 0.81 (3H, d, J = 7.4 Hz, $CH_3$-26), 0.85 (3H, d, J = 7.4 Hz, $CH_3$-27), 0.91 (3H, d, J = 6.4 Hz, $CH_3$-20), 1.00 (3H, s, $CH_3$-10), 3.51 (1H, m, H-3), 5.03 (1H, dd, J = 14.4, 8.4 Hz, H-23), 5,13 (1H, dd, J = 14.4, 8.4 Hz, H-22), 5.35 (1H, m, H-6). By comparison of its 13C NMR data above with literature values [25], the structure of β-stigmasterol was assigned to compound 2. Compound 3 was obtained as white crystals in hexane, mp 127-128ºC. Its $^1$H NMR spectrum portrayed six proton signals in the region δ 6.46-6.75 which were attributed to two aromatic rings. Four methoxy groups resonated at δ 3.84 while other signals were observed at δ 4.10 (1H, dd, J= 9.0, 8.0 Hz, H-4α); 3.93 (1H, dd, J=10.5, 9.0, H-4β); 2.50 (1H, m, H-3) and 2.56 (1H, m, H-2). Its $^{13}$C NMR spectrum showed signals corresponding to twelve aromatic carbon atoms in addition to six other carbons at δ 178.5 9 (C-1), 46.4 (C-2), 41.0 (C-3), 71.1 (C-4), 38.1 (C-5) and 34.4 ( C-6 ). All these data enabled the structure of matairesinol dimethyl ether [26] to be assigned to Compound 3. Compound 4, Peperovulcanone A, was isolated in a previous study of the hexane extract of the same species [15]. The structures of these four compounds are shown in Fig. 1. Structure determination for the other compounds is being pursued.

## 3.2 Test Substances from *P. vulcanica* and *P. fernandopoiana*

The substances prepared and tested in this study are shown on Table 1.

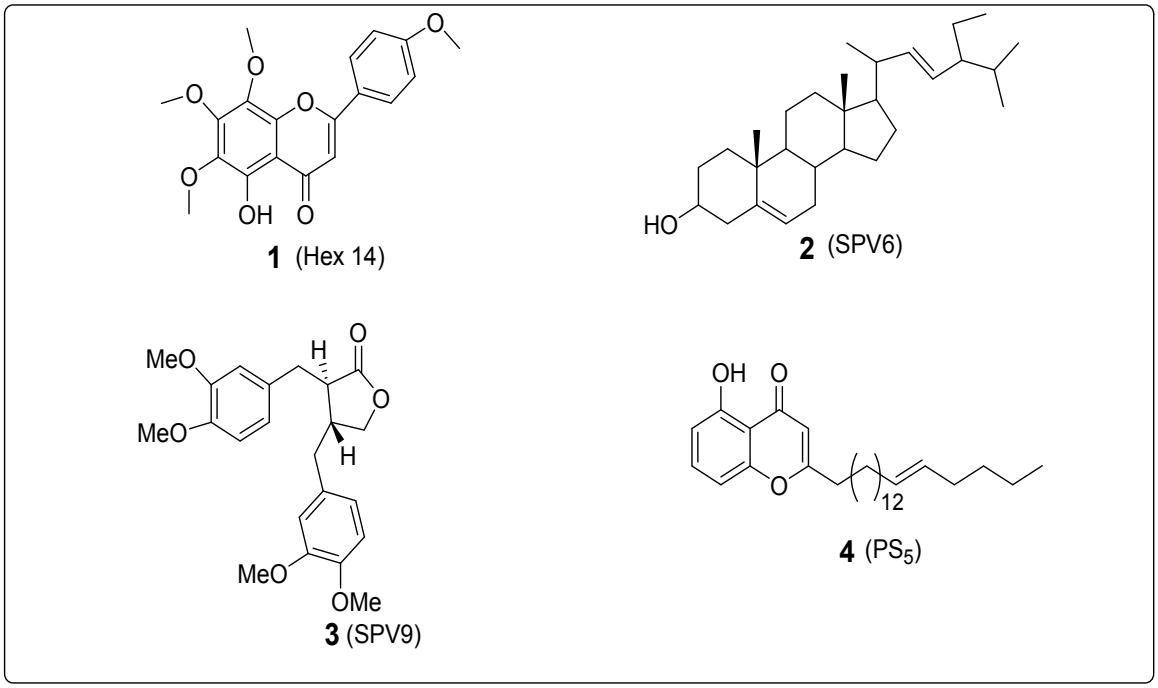


**Fig. 1. Chemical structures of four pure compounds isolated from the hexane crude extract of *Peperomia vulcanica***

*Compound codes and names: 1(Hex 14) = 5-Demethyltangeretin, 2 (SPV 6) = Stigmasterol, 3 (SPV 9) = Matairesinol dimethyl ether and 4 (PS5) = Peperovulcanone A. The hexane crude extract showed higher antiplasmodial activity than the methylene chloride and methanol extracts. The compounds demonstrated high to moderate antiplasmodial activity ($IC_{50}$s ranging from 1.14 to 22.29 µg/mL) against resistant Dd2 and W2mef strains of Plasmodium falciparum*

**Table 1. Yields of crude extracts and pure compounds isolated from *P. vulcanica* and *P. fernandopoiana* whole plants**

| Plant material (weight in Kg) | Extraction solvent | Crude extract | | |
|---|---|---|---|---|
| | | Weight (grams) | Yield (%w/w) | No. of compounds isolated |
| *Peperomia vulcanica* (1.75) | Hexane | 62.62 | 3.58 | 10 |
| | Methylene chloride | 66.21 | 3.78 | 6 |
| | Methanol | ND[a] | ND | ND |
| *Peperomia fernandopoioana* (2.0) | Hexane | 66 | 3.3 | ND |
| | Methylene chloride | 59.4 | 2.97 | ND |
| | Methanol | ND | ND | ND |

[a]*ND: Not done*

The yield of *P. vulcanica* crude extracts was higher than *P. fernandopoiana* for all solvents used (Table 1). On the basis of the considerable high inhibition observed in the primary screen, the hexane and methylene chloride extracts of *P. vulcanica* were fractionated and purified affording a total of 15 pure compounds, 9 from the hexane fractions (Hex 3, Hex 8, Hex 10, Hex 11, Hex 14, SPV 1, SPV 5, SPV 6 and SPV 9), and 6 from the methylene chloride fractions (MC 3, MC 16, MC 24, MC 26, MC 27 and MC 29).

### 3.3 Antiplasmodial Activity of Test Substances and Combinations

The antiplasmodial activities of the crude extracts and pure isolates are shown on Tables 2 and 3.

**Table 2. Antiplasmodial activity of *Peperomia vulcanica* crude extracts**

| Test Substance[a] | 3D7[b] | | W2mef[b] | | Dd2[b] | | SHF4[b] | |
|---|---|---|---|---|---|---|---|---|
| | %[c] | $IC_{50}$[d] | % | $IC_{50}$ | % | $IC_{50}$ | % | $IC_{50}$ |
| **$PV_{Hex}$** | 99.6 | 11.56±0.9 | 83.6 | 9.3±3.9 | ND[e] | 7.1±0.0 | 78.7 | 19.6±2.5 |
| **$PV_{MC}$** | 99.2 | 19.3±4.1 | 56.3 | 24.4±4.8 | ND | 49.5±4.0 | 78.9 | 8.1±1.2 |
| **$PV_{MeOH}$** | 98.9 | 16.8±2.5 | 63.5 | 14.9±2.9 | ND | 15.5±0.0 | 89.2 | 14.8±7.1 |
| **ART** | 95.5 | ND | 99.12 | 0.05±0.0 | ND | 0.13±0 | 92.14 | ND |
| **CQ** | ND | 0.26±0.05 | ND | 0.16±0.0 | 68.8 | 0.16±0.0 | ND | 0.1±0.01 |

[a] *Test substance code: $PV_{Hex}$ = Hexane extract, $PV_{MC}$ = Methylene chloride extract, $PV_{MeOH}$ = Methanol extract, ART = Artemether, CQ = Chloroquine*
[b] *P. falciparum strains: 3D7 (chloroquine-sensitive), W2mef and Dd2 (multi-drug resistant), SHF4 is a field isolate.*
[c] *Percentages of inhibition of parasite growth at a single extract concentration of 100 µg/mL in the primary screen.*
[d] *$IC_{50}$ in µg/mL is the mean of three experiments. Parasite growth in the negative control ranged from 1.5 - 4.24% (except Dd2 with <1.2%) after 48 hours incubation of initial 1% parasitaemia.*
[e] *ND: Not done. $IC_{50}$s for the crude extracts of P. fernandopoioana were not determined due to the relatively low activity (48 to 59% inhibition of parasite growth at 100 µg/mL) in the primary screen.*

**Table 3. Antiplasmodial activity of pure compounds from hexane and methylene chloride extracts of *Peperomia vulcanica* on resistant strains**

| Compound code[a] | $IC_{50}$ (µg/mL)[c] | | Compound code[a] | $IC_{50}$ (µg/mL) |
|---|---|---|---|---|
| | W2mef | Dd2 | | W2mef |
| Hex 3 | 9.67±8.57 | 1.14±0.12 | SPV 9 | 12.1±6.36 |
| Hex 8 | 6.69±9.49 | 2.58±1.65 | PS5[b] | 22.29±4.09 |
| Hex 10 | 6.24±6.18 | 1.19±0.22 | MC 3 | 0.89±0.01 |
| Hex 11 | 11.19±9.42 | 1.49±0.41 | MC 16 | 14.13±10.68 |
| Hex 14 | 6.94±8.18 | 1.14±0.02 | MC 24 | 1.74±0.45 |
| SPV 1 | 30.06±0.41 | ND[d] | MC 26 | 16.70±136 |
| SPV 5 | 16.66 | ND | MC 27 | 5.31±2.12 |
| SPV 6 | 22.06 | ND | MC 29 | 3.23±2.85 |

[a] *Compound codes Hex, SPV and PS5: compounds isolated from fractions of the hexane crude extract, and MC: compounds isolated from the methylene chloride crude extract.*
[b] *PS5 is Peperovulcanone isolated in a previous extraction*
[c] *$IC_{50}$ is the mean of two experiments.*
[d] *ND: Not done. Positive control $IC_{50}$ (µg/mL): artemether = 0.05±0 against W2mef and 0.13±0 against Dd2; chloroquine = 0.13±0 for both parasites*

Activities were classified following a scale used in another study [27] with some modification based on our experience as follows: high ($IC_{50}$ < 5 µg/mL), moderately active (5 < $IC_{50}$ < 30 µg/mL), weak activity (30 < $IC_{50}$ < 50 µg/mL), and inactive (> 50 µg/mL). Five of the 10 pure compounds isolated from active fractions of the hexane extract showed high activity against Dd2 ($IC_{50}$ = 1.14 - 2.58 µg/mL) and moderate activity against W2mef (Table 3).

The other 5 demonstrated low activity. Hex 10 had the highest activity against W2mef ($IC_{50}$ = 6.24 µg/mL) while Hex 3 and 14 had the highest activities against Dd2 ($IC_{50}$ = 1.14 µg/mL). Of the six compounds isolated from the methylene chloride extract, MC 3 showed the highest activity ($IC_{50}$ = 0.89 µg/mL) against W2mef (Table 3).

The two combinations of the four most active compounds were as follows: Hex 14 from hexane extract with $IC_{50}$ = 6.94 µg/mL combined with MC 3 from methylene chloride extract with $IC_{50}$ = 0.89 µg/mL, and Hex 3 from hexane extract with $IC_{50}$ = 1.14 µg/mL combined with MC 24 from methylene chloride extract with $IC_{50}$ = 1.74 µg/mL; all $IC_{50}$s were recorded against W2mef. These combinations demonstrated synergistic antiplasmodial effect against W2mef. The $IC_{50}$s of the individual compounds in the four combination wells were considerably lower than the $IC_{50}$s of the compounds alone; this resulted in concave curves in the isobolograms (Fig. 2). The combination indices of 0.69 and 0.75 for the two

combinations respectively further confirm the shapes of the isobolograms [20].

Meanwhile, the three crude extracts from *P. vulcanica*, produced greater than 50% inhibition of the four parasite strains, at 100 µg/mL in the primary screen (Table 2). Overall, the hexane crude extract ($PV_{Hex}$) produced the highest inhibition (78 - 99.6%) and the methylene chloride extract ($PV_{MC}$) showed the lowest inhibition (56.39 - 99.22%). The two crude extracts from *P. fernandopoiana* produced relatively low inhibition (48 to 59% at 100 µg/mL) hence were not further investigated.

The secondary screen of the three crude extracts from *P. vulcanica* which showed >50% inhibition in the primary screen revealed moderate antiplasmodial activity against the four parasite strains. Overall, the activity was highest for $PV_{Hex}$ ($IC_{50}$s ranging from 7.05 - 19.67 µg/mL), followed by $PV_{MeOH}$ ($IC_{50}$s range: 14.78 - 16.84 µg/mL) and lowest for $PV_{MC}$ ($IC_{50}$s range: 8.11- 49.56 µg/mL) (Table 2). The fractions from two of the active crude extracts of *P. vulcanica*, $PV_{Hex}$ and $PV_{MC}$, tested against 3D7 and SHF4 strains of *P. falciparum* showed very high activities with growth inhibition ranging from 85 to 100% confirming the high inhibition by the crude extracts in the primary screen mentioned above.

### 3.4 Acute Toxicity of Pure Compounds Hex 14 and MC3

At the end of seven days post administration of the test compounds, the average weight of all the groups of animals increased by 9.4%, 4.3% and 3.6% for the control, Hex 14 and MC 3 groups respectively, but this was not significant (*P*>0.05). No difference in physical activity and appetite between test and control groups was observed. Also no death was recorded in any of the groups during the 7 days study period.

## 4. DISCUSSION

This study was motivated by the considerable antiplasmodial activity previously reported in the crude extract of *P. vulcanica* [12,13]. Hence it was focused on isolating and identifying the antiplasmodial principles in *P. vulcanica* and *P. fernandopoiana*. Of the sixteen compounds from *P. vulcanica*, eight hits were identified with high antiplasmodial activity ($IC_{50}$s from 0.89 - 3.23 µg/mL); these compounds should be further investigated to fully establish their antimalarial potential. Furthermore, this study is the first to report antiplasmodial activity for two of the isolated compounds i.e. 5-Demethyltangeretin (**1**), ($IC_{50}$ = 6.94 µg/mL against W2mef and 1.14 µg/mL against Dd2) and Peperovulcanone A (**4**),

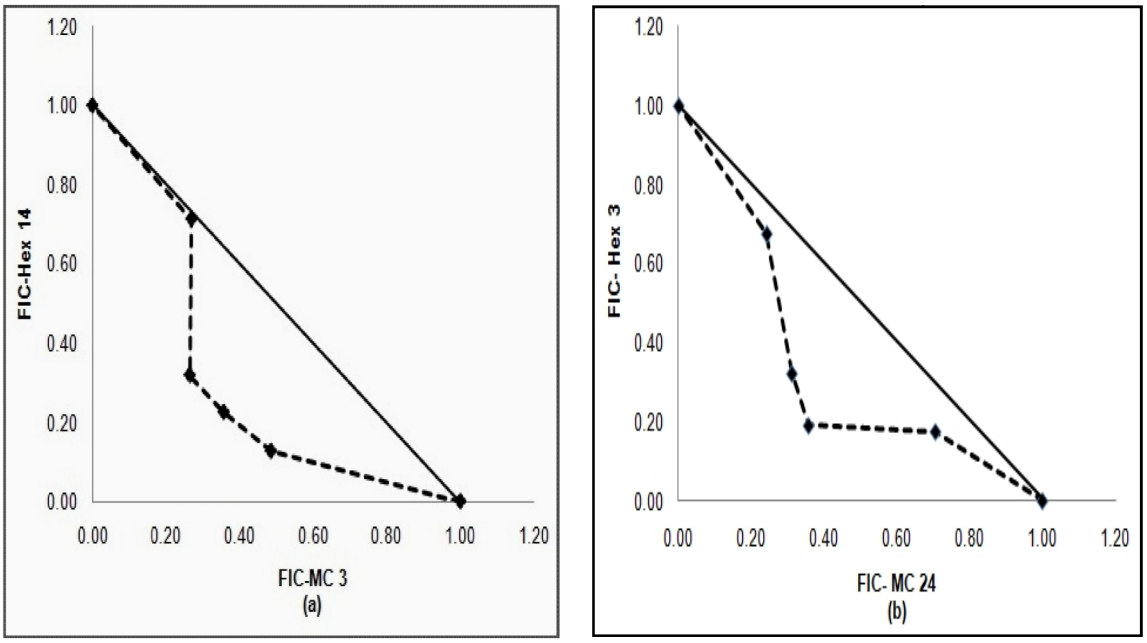


**Fig. 2. Isobolograms of synergistic antiplasmodial activity of compounds from *Peperomia vulcanica* against resistant *P. falciparum* W2mef**

*Effect of (a) Combination of Hex 14 (5-Demethyltangeretin) from hexane and MC 3 from methylene chloride extracts, (b) Combination of Hex 3 from hexane and MC 24 from methylene chloride extracts against W2mef*

($IC_{50}$ = 22.29 µg/mL), which had been isolated and described in previous studies [11,15], Fig. 1. Four compounds [Hex 3, Hex 10, Hex 11, Hex 14 (5-Demethyltangeretin)] had $IC_{50}$s of around 1 µg/mL against Dd2 while one compound (MC 3) also had an $IC_{50}$ of around 1 µg/mL against W2mef; this activity is fairly close to that of artemether (<1 µg/mL), the standard antimalarial used as positive control in this study (Table 3).

Four of the most active compounds in two separate combinations showed synergism against W2mef (Fig. 2). The combinations were made considering the polarities of the compounds i.e. a less polar compound from the hexane extract combined with a moderately polar one from the methylene chloride extract. The observed synergistic effect probably resulted from the drugs acting at different targets given their different polarities. This finding is significant because presently the rationale of malaria chemotherapy is combination therapy, which prevents or delays emergence of resistance to antimalarials and achieves greater therapeutic efficacy in contrast to monotherapy [3]. However, this should be demonstrated *in vivo* in an animal model which is more predictive of the outcome in humans. Other studies of combinations of natural products have reported various types of interaction including synergism. Synergistic and antagonistic interactions were reported between different pairs of products from the stem bark of *Kigelia africana* (*Bignoniaceae*), against the W2mef strain [20]. Synergism has also been demonstrated between the standard antimalarials, chloroquine and artemisinin, and the natural product harmine [28]. Hence investigation of the interaction between the highly active products from *P. vulcanica* and standard antimalarials may reveal new efficacious combinations.

Following the primary and secondary bioassay-guided screening, active crude extracts, fractions and compounds were identified. The strategy and experimental design used in this study distinguishes test substances with high and weak activity early in the discovery process hence focusing the search for suitable candidate antimalarial molecules on the most active substances. This approach has a high probability of identifying a hit for lead development. Secondly, it is rapid and ensures optimal use of resources in the search process. The crude extracts from *P. vulcanica* were the most active compared to those from *P. fernandopoiana* (Table 2) suggesting that the latter may be excluded from the concoctions used to treat malaria traditionally. Following the secondary screen extracts from *P. vulcanica* showed moderate activity ($IC_{50}$ values of 7.05 µg/mL against Dd2 and 9.29 µg/mL against W2mef, Table 2); thereby confirming the earlier report of considerable activity against the *P. falciparum* F32 (chloroquine-sensitive) strain with $IC_{50}$ of 37.5 µg/mL [13]. Overall, the secondary screen showed that all the *P. vulcanica* crude extracts were suitable for further analysis. Thus the hexane and methylene chloride extracts were fractionated and the methanol extract will be fractionated subsequently. Each extract showed high activity on at least one parasite strain. This suggests that the bioactive components are of different chemical classes and or are present in different quantities. The compounds which were highly active against resistant strains are suitable candidate molecules for further investigation whereas the moderately active compound could be subjected to medicinal chemistry exploration to discover more suitable antimalarial analogues.

Following the acute toxicity test the smaller increase ($P$>0.05) in body weight in the test groups compared to the control was probably due to compromised appetite and or inhibition of a growth process by the test compounds. The absence of acute toxicity observed in this study is consistent with a previous study on the hexane and methylene chloride crude extracts [11] and suggests the two most active compounds may have a low risk of toxicity.

## 5. CONCLUSION

The high antiplasmodial activity and the apparent lack of toxicity of *P. vulcanica* supports its use in traditional treatment of malaria and the plant material should be further studied towards development into an antimalarial phytomedicine. The highly active compounds are potential candidate molecules which should be further investigated in combination with standard antimalarials in view of developing new antimalarials for effective treatment of clinical malaria.

## CONSENT

Not applicable.

## ETHICAL APPROVAL

The authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as

specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee".

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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