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Sesquiterpenoids with antiplasmodial activities from *Warburgia stuhlmanii* Engl. root bark

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ABSTRACT

Phytochemical investigation of *Warburgia stuhlmannii* ethyl acetate root extract led to isolation of ten known compounds identified as polygodial (**1**), cinnamolide (**2**), warburganal (**3**), bemadienolide (**4**), ugandensidial (**5**), muzigadial (**6**), mukaadial (**7**), 6α-hydroxymuzigadial (**8**), ugandensolide (**9**) and deacetylugandensolide (**10**). Structures of the isolated compounds were determined by spectroscopic methods; NMR, IR, UV-Vis and spectrometric method, EI-MS, as well as comparison with literature data. In their antiplasmodial activities, the compounds had moderate activities against the chloroquin sensitive (**D10**) and chloroquin resistant (**W2**) strains of *Plasmodium falciparum*. Among the compounds, mukaadial (**7**) had the highest activities against both D10 and W2 strains of *P. falciparum*, with IC_{50} values of 5.2 µM and 5.8 µM, respectively, while muzigadial (**6**) though effective on D10 strain (IC_{50} = 5.6 µM) was less effective against W2 strain (IC_{50} = 16.4 µM).

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1. Introduction

Nowadays, human beings encounter a wide range of persistent and/or unknown diseases. Without doubt, one of the best remedies to overcome these diseases is to rely on medicinal and herbal plants with notable therapeutic potentialities and remarkably lower side effects (Bailly, C., 2021a; Bailly, C., 2021b). A large number of reports are found in the literature discussing this challenging case and representing the presence of many bioactive compounds possessing diverse pharmaceutical, pharmacological, medical as well as biological properties (Mohammadhosseini et al., 2019a; Mohammadhosseini et al., 2019b; Mohammadhosseini et al., 2021; Vignesh et al., 2021; Oloyede et al., 2021; Thagriki and Ray, 2022).

Warburgia is a genus of plants in the family Canellaceae. The species of this genus are highly valued within the traditional health systems, for managing stomachache, constipation, toothache, common cold, cough, fever, muscle pains, weak joints, measles and malaria (Kokwaro, 2009; Maroyi, 2014). *Warburgia stuhlmannii* Engl., one of the species of the genus *Warburgia* is widespread at East African coastline (Muchugi et al., 2009). The plant is a small ever green tree with leaves that are very glossy above, small fruits containing two or more seeds with an oily endosperm and a yellow to greyish black bark (Verdcourt, 1954). Traditionally, *W. stuhlmannii*, is used as a remedy for toothache, rheumatism, cough, management of constipation (Beentje, 1994) and malaria treatment (Muthaura et al., 2007).

Malaria is a disease caused by the protozoan parasites of the genus *Plasmodium* and is endemic in 91 countries, predominantly in Africa, Asia and Latin America with about 40% of the world's population at risk (WHO, 2001). About 241 million cases of malaria were reported in 2020 worldwide out of who 627,000 died with Africa contributing 95% of all the malaria cases and 96% of

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death arising from malaria (WHO, 2020).The disease is transmitted by female anopheles mosquitoes (Lindsay et al., 2000). Malaria parasites have however shown some level of resistance to most antimalarial drugs currently available (Anonymous, 2007) and most of the drugs in use have serious side effects. Development of resistance by *Plasmodium* strains against commonly used drugs such as chloroquine and the lack of affordable new drugs are some of the factors that limit the battle against malaria (Mehrotra et al., 2017). There is therefore a need for continuous research towards development of new classes of antimalarial drugs (Mehrotra et al., 2017) to which malaria parasites will not develop resistance and have none or minimal side effects.

Sesquiterpene lactones have previously been shown to possess activities against various *plasmodium* strains. In the study by Mehrotra et al., (2017), the sesquiterpene lactones; perthenolide and costunolide diepoxide showed the highest parasitaemia suppression (PI%) of 85.18% and 83.65%, respectively, after four days of treatment. This compared well with the activities of chloroquine and artesunate which suppressed the parasitaemia by 98.64% and 97.61%, respectively. On the other hand, Bordignon et al., (2017) established that the sesquiterpene lactones, *e.g.*, 8-(4′-hydroxy methacrylate)-dehydromelitensin, onopordopicrin and 8α-[4′-hydroxymethacryloyloxy]-4-*epi*-sonchucarplide isolated from *V. fimbrillifera* had high antiplasmodial activities with IC_{50} values of 2.96, 3.37 and 3.27 µg/mL, respectively, against the 3D7 strain of *P. falciparum*. From these results it is apparent that sesquiterpene lactones are active against the various strains of *Plasmodium*, hence the need for further study on antiplasmodial activities of sesquiterpene lactones is unavoidable.

2. Experimental

2.1. Instrumentation, fine chemicals and solvents

Melting points were determined on Gallenkhamp melting point apparatus. IR data were obtained on Bruker Vector 22 spectrophotometer (KBr pellet). Electron ionization (70 eV) mass spectral data were obtained on a MAT 8200 311 A Varian Bremen instrument. NMR data were measured on a JEOL NMR instrument operating at 360 and 90 MHz for ^{1}H NMR and ^{13}C NMR, respectively. Silica gel 60 (63-200 µm, Merck, Darmstadt, Germany) was used for gravity column chromatography (C.C.). Analytical thin layer chromatography was performed using aluminium precoated silica gel 60 F_{254} plates and chromatograms visualised by spraying with *p*-anisaldehyde-sulphuric acid mixture followed by heating at 100 °C. Solvents used were of analytical grade.

2.2. Plant material collection and identification

The root barks of *W. stuhlmannii* were collected from Kaembeni-Dida, Kilifi County, Kenya. Identification and authentification were done at the Kenya Forestry Research Institute (KEFRI), Gede regional centre Malindi, Kenya and voucher specimens (leaves, twigs and fruits) (MKA-WS-2010-02) were identified after comparison with authentic samples at the Kenya Forestry Research Institute headquarter, Muguga, Kenya.

2.3. Plant material preparation and solvent extraction

The root bark was air dried under a shade then ground into fine powder using a laboratory mill. One kilogram of the powdered plant material was successively extracted by cold percolation with ethyl acetate (3 x 3L). The solvent was added to the material then vigorously shaken on an orbital shaker, then set aside for 24 h. It was then filtered using Whatman No. 1 filter paper, and concentrated under reduced pressure to give brownish-green extracts in the yield of 80 g. The extract was divided into two portions; for bioassay tests (approximately 10 g) and the rest for isolation and characterization. Before isolation of the compounds, analytical thin layer chromatography (TLC) analysis was carried out for the determination of chemical profiles. The extracts were spotted on silica gel pre-coated TLC plates and sprayed with *p*-anisaldehyde-sulphuric acid mixture in methanol followed by heating at 100 °C for 2 to 3 minutes to detect the spots.

2.4. Isolation of phytoconstituents of the ethyl acetate extract of *W. stuhlmannii* root bark

65 g of the extract was subjected to column chromatography on silica gel (column size 5.0 x 60 cm, SiO_2 230-400 mesh ASTM, 500 g, pressure ≈ 1.0 bar) eluting with *n*-hexane containing increasing percentage of ethyl acetate (from 5% to 50%) and finally concluded with dichloromethane-methanol mixture (97:3; 95:5 and 9:1). A total of 85 fractions each 100 mL were obtained and their homogeneity monitored by the use of TLC with solvent systems *n*-hexane-ethyl acetate (95:5, 9:1, 4:1, 2:1 and 1:1) and dichloromethane-methanol (99:1 and 93:7). The fractions were combined depending on TLC profiles and those showing similar TLC spots were combined into five pools (1-V). Pool I (Fractions 2-7), moved with the solvent front (eluent; *n*-hexane-EtOAc, 95:5) and were combined. Removal of solvent under reduced pressure gave sweet smelling yellow oil (10 g) that was not used for further analysis. Pool II (Fractions 9-25) having been eluted with *n*-hexane-ethyl acetate (95:5 and 9:1) afforded three spots on TLC of R_f values 0.18, 0.40, and 0.56, respectively. From the combined fractions, needle shaped white crystals formed, which on re-crystallization (*n*-hexane-ethyl acetate, 9:1) afforded polygodial (**1**, R_f = 0.56, 100 mg). Evaporation of the mother liquor under reduced pressure afforded yellow brown gummy material (7 g) which on chromatographic purification (SiO_2: 200 g, column size: 3.0 x 50 cm, pressure ≈ 1.0 bar), solvent systems; *n*-hexane-ethyl acetate (97:3, 95:5, 9:1 and 4:1), collecting 50 mL each afforded 30 fractions. The fractions were pooled together depending on TLC profiles into three pools (pools A-C). Pool A (fractions 3-12) which gave single spot on TLC (R_f = 0.56) afforded further polygodial (**1**, 75 mg). Pool B (Fractions 14-18)

showed a single spot on TLC and upon purification by crystallization in *n*-hexane-ethyl acetate (9:1) gave cinnamolide (**2**, R_f = 0.40, 40 mg). Fractions 21-30 constituted pool D, which afforded a single major spot on TLC and was further purified by crystallization in *n*-hexane-ethyl acetate (4:1) to give warburganal (**3**, R_f = 0.18, 60 mg). Pool III (fractions 27-50, 15 g) eluted mainly with *n*-hexane-ethyl acetate (7:3 and 2:1, 2.5 L) gave four major spots (R_f values 0.20, 0.18, 0.26 and 0.40) on TLC (solvent system *n*-hexane-EtOAc, 4:1, 2:1, 1:1). The fractions were combined then concentrated under reduced pressure and white needle like crystals crystallized out which on filtration and further purification by re-crystallization (*n*-hexane-ethyl-acetate; 4:1) afforded more of warburganal (**3**, 160 mg). The mother liquor was evaporated under reduced pressure to dryness, dissolved in *n*-hexane-EtOAc (4:1, 10 mL) and loaded onto silica gel packed column (SiO_2, 160 g). It was then eluted with *n*-hexane-ethyl acetate (9:1) followed by the same solvent systems in the ratios 17:3 and 4:1 to give bemadienolide (**4**, R_f = 0.26, 55 mg), ugandensidial (**5**, R_f = 0.20, 24 mg) and further cinnamolide (**2,** 60 mg). Pool 1V (fractions 53-73, 13 g) showed three spots of R_f values 0.20, 0.35 and 0.26 on TLC (solvent system *n*-hexane-EtOAc 3:1, 2:1, 1:1). Upon concentration under reduced pressure, was chromatographed over an open silica gel column (column size 3.5 × 60 cm, SiO_2 200 g, pressure ≈1.5 bar, solvent system *n*-hexane-EtOAc 17:3) followed by the same solvent system in the ratios 4:1, 5:1, 7:3 and 2:1, collecting 20 mL of each. A total of 150 fractions were sampled and their compositions determined by TLC (solvent system *n*-hexane-EtOAc, 17:3, 4:1, 5:1, 7:3 and 2:1). The fractions were combined into three minor pools (E-F). Pool E (fractions 15-30), eluted with *n*-hexane-EtOAc (4:1), gave a single spot of R_f value 0.20 on TLC and upon solvent evaporation under reduced pressure and crystallization (*n*-hexane-EtOAc 4:1) gave further ugandensidial (**5**, 85 mg). Pool F (fractions 32-78) also gave one spot on TLC and upon evaporation of the solvent under reduced pressure followed by crystallization (*n*-hexane-EtOAc, 7:3) afforded further bemadienolide (**4**, 25 mg). Pool G (fractions 80-125) gave one major spot on TLC, contaminated with other two minor ones and on concentration under reduced pressure, the major compound crystallized out as white amorphous powder which was further purified by recrystallization (*n*-hexane-EtOAc, 4:1) to give muzigadial (**6**, R_f = 0.35, 120 mg). Pool V (fractions 74-85, 7.5 g) gave four spots of R_f values 0.33, 0.29, 0.23 and 0.14 (solvent systems: *n*-hexane-EtOAc, 2:1 and CH_2Cl_2-MeOH, 97:3), was applied to silica gel column and eluted with *n*-hexane-ethyl acetate mixture (3:2 and 1:1) followed by CH_2Cl_2-MeOH (97:3). This led to isolation of mukaadial (**7**, R_f = 0.33, 150 mg), 6α-hydroxymuzigadial (**8**, R_f = 0.29, 65 mg), ugandensolide (**9**, R_f = 0.23, 34 mg) and deacetylugandensolide (**14**, R_f = 0.14, 250 mg).

2.5. *In vitro* anti-plasmodium assay

Samples of pure compounds were assayed using an automated micro-dilution technique to determine the relevant IC_{50}. The chloroquine resistant *Indochina* (W2) and chloroquine sensitive *Sierra Leone* (D10) strains of *P. falciparum* were used in this assay with artesunate as the reference drug. Five milligrams of the test compounds were dissolved in five milliliters of dimethyl sulphoxide (DMSO) to give stock solutions of 1 mg/mL. In order to obtain the desired starting concentration, the stock solutions were diluted in the media RPMI 1640 and then 350 µL of the test compounds or reference drug solutions were loaded onto the wells of the first column of sterilized 96 well flat bottom culture plates. Two-fold serial dilutions were perfumed using a Biomek Automated Laboratory work station. The last column of the plate was used for the positive control which consisted of three complete medium with serum (CMS) containing parasites (PRB) and negative control which consisted of CMS and erythrocytes with no parasites (UNPRB). To each of the wells, 200 µL of the parasitized blood (1% parasitaemia) and 6% haemacrit were added then incubated for 24 h at 37 ºC in 6% CO_2. This resulted in the first wells of each compound to be tested at a concentration of 50 µg/mL. Exactly, 25 µL of [3H] hypoxanthine was added per well then incubated for further 18 h at 37 ºC and then frozen at -20 ºC for maximum lyses of erythrocytes. The contents of the plates were harvested using an automated cell harvester from Puckered Bioscience and then left to dry. Scintillation fluid was added to the plates, sealed and then counted on a liquid scintillation counter to give raw data representing the parasite counts (PRBC and UNPRBC).The midpoint (Y_{50}) was initially determined by Eqn. 1. Concentration of drugs causing 50% inhibition of (G-3H) hypoxanthine uptake (IC_{50}) was then carried out by interpolation after logarithmic transformation uptake of both concentration and radioactivity in counts per minute (CPM) using Eqn. 2 (Sixmith et al., 1984).

Y_{50} = [(PRBC − CPM value) − (UNPRBC − CPM value)]/2 (Eqn. 1)

IC_{50} = Antilog [logX_1 + (log Y_{50} − logX_1)(logX_2 − logX_1)]/2 (Eqn. 2)

Where IC_{50} = 50% growth inhibition of cultured parasites. Moreover, the terms X_1 and X_2 respectively account for the lower and higher concentration of the samples. Y_1 denotes the CPM value which corresponds with X_1, while Y_2 represents the CPM value corresponding with X_2.

3. Results and Discussion

3.1. Sesquiterpenoids isolated from *W. stuhlmannii* Engl. ethyl acetate root bark extract

In this study, phytochemical evaluation of *W. stuhlmannii* ethyl acetate root bark extract led to isolation of ten known compounds identified as polygodial (**1**) (Kubo et al., 1983), cinnamolide (**2**) (Kioy et al., 1990), warburganal (**3**) (Kioy et al., 1989), bemadienolide (**4**) (Canonica et al., 1969), ugandensidial (**5**) (Kioy et al., 1990), muzigadial (**6**) (Kioy et al., 1989), mukaadial (**7**) (Kioy et al., 1989), 6α-hydroxymuzigadial (**8**) (Wube et al., 2005), ugandensolide (**9**) (Kioy et al., 1989) and deacetylugandensolide (**10**) (Kioy et al., 1990) (see Fig. 1). Compound **8** is reported in this plant for the first time.

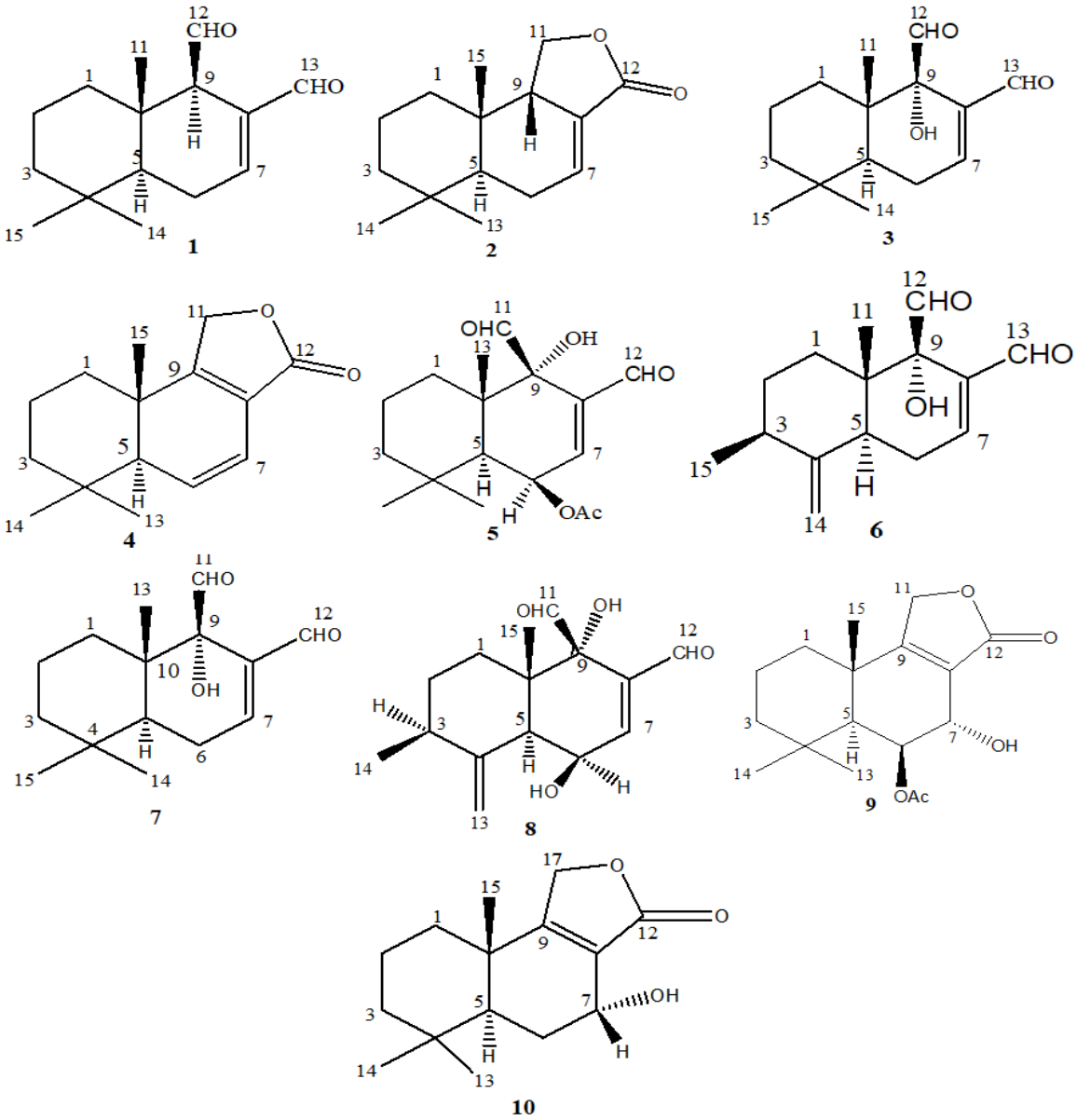

Fig. 1. Structures of compounds **1-10** isolated from *W. stuhlmannii* Engl. root bark.

3.2. Antiplasmodial tests

Compounds isolated from *W. stuhlmannii* Engl. (root bark) were tested for antiplasmodial activities against the chloroquine sensitive (D_{10}) and the chloroquine resistant (W2) strains of *Plasmodium falciparum* which ranged between low to moderate. Artesunate was used as positive control. Among the isolated compounds, mukaadial (**7**) was the most potent against both D10 and W2 strains of *P. falciparum* with IC_{50} values of 4.3 µM and 5.8 µM, respectively, but lower than those of the positive control (0.8 µM and 0.5 µM) when tested against the D10 and W2 strains, respectively. These results were in agreement with the results of Wube et al., (2010) given as 6.4 µM for D10 and 7.9 µM for the W2 strain. Muzigadial (**6**) was more active against the D10 strain IC_{50} = 5.6 µg but less active against W2 strain of *P. falciparum*, IC_{50} = 16.4 µM. These activities were however lower than those of the positive control (0.8 µM and 0.5 µM) against D10 and W2 strains, respectively, but were in agreement with results of Grace et al., (2010) in which numerical values of 0.31 µM and 1.18 µM were obtained for D10 strain and W2 strain of *P. falciparum*, respectively. Ugandensidial (**5**) was more active against the W2 strain than the D10 strain, with IC_{50} values of 8.2 µM and 30.2 µM, respectively but lower than those of the positive control. Similar results were obtained by Wube et al., (2010), given as IC_{50} = 7.6 µM for W2 and IC_{50} = 29.8 µM for D10 strain. The sesquiterpene lactone, 6α-hydroxymuzigadial (**8**) was more active (IC_{50} = 12.2 µM) against the W2 strain than the D10 strain (IC_{50} = 29.8 µM) though the activities were lower than those of the positive control. Ugandensolide (**9**) and bemadienolide (**4**) both showed low activities against the D10 and W2 strains of *P. falciparum* (Table 1).

Table 1
Antiplasmodial activities of compounds isolated from ethyl acetate root bark of *W. stuhlmannii* Engl..

Tested compound	IC_{50} values for isolated compounds (µM/mL)	
	Activity against D10 strain	Activity against W2 strain
Polygodial (**1**)	> 102.5	> 102.5
Cinnamolide (**2**)	42.5 ± 1.5	22.3 ± 1.2
Warburganal (**3**)	6.4 ± 1.3	18.6 ± 1.4
Bemadienolide (**4**)	> 92.3	> 92.3
Ugandensidial (**5**)	30.2 ± 2.2	8.2 ± 1.8
Muzigadial (**6**)	5.6 ± 1.6	16.4 ± 1.8
Mukaadial (**7**)	4.3 ± 1.1	5.8 ± 1.6
6α-Hydroxymuzigadial (**8**)	29.8 ± 1.2	12.2 ± 1.4
Ugandensolide (**9**)	> 98.4	> 98.4
Deacetylugandensolide (**10**)	63.5 ± 1.5	50.6 ± 1.3
Artesunate	0.8	0.5

4. Concluding remarks

The present study evaluated for the first time the antiplasmodial potential of compounds isolated from the EtOAc extract of *W. stuhlmannii* Engl. *R*oot bark. Ten compounds characterized as polygodial (**1**), cinnamolide (**2**), warburganal (**3**), bemadienolide (**4**), ugandensidial (**5**), muzigadial (**6**), mukaadial (**7**), 6α-hydroxymuzigadial (**8**), ugandensolide (**9**) and deacetylugandensolide (**10**) were isolated. Compound **8** is reported in this species for the first time. Compound **7** exhibited the highest antiplasmodial activities against the D10 and W2 strains of *P. falciparum*, while the remaining nine compounds showed varying antiplasmodial activities against the two strains. Results from this study have validated the traditional use of root bark of this plant in the management of malaria. Other strains of *P. falciparum* should be used to test the antiplasmodial activities of compounds isolated from the root bark of this plant.

Conflict of interest

The authors declare that there is no conflict of interest.

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