A novel monoterpenoid from leaf exudates of *Tarchonanthus camphoratus* with anti-leishmanial activities

Okemwa Evans Kenanda

Abstract

One new monoterpenoid; 2,3,3a,4,5,7a-hexahydro-7a-methyl-4-methylene-1H-inden-5-ol (1) along with five known compounds including; two sesquiterpenes, costic acid derivative (2), (-)-parthenolide (3), and four known methoxylated flavonoids; 5,7,3',4'-tetrahydroxy-3-methoxyflavone (4), 5,7,4'-trihydroxy-6-methoxyflavone (5), 5,7,3',4'-tetrahydroxy-6-methoxyflavone (6) and 5-hydroxy-7,8-dimethoxyflavanone (7) were characterized from the leaf exudates of *Tarchonanthus camphoratus*. The structures of these compounds were determined based on spectroscopic data analyses. The new compound exhibited good anti-fungal activity against *Cryptococcus neoformans* with an IC₅₀ value of 5.62 µg/mL. Compounds 1 and 2 exhibited moderate anti-leishmanial activities against *Leishmania donovani* with IC₅₀ values of 14.17 and 12.84 µg/mL, respectively, (vs 0.85 for pentamidine and 0.12 µg/mL for amphotericin B). Compound 6 and 7 also showed anti-leishmanial activities with IC₅₀ values of 26.24 and 23.15 µg/mL, respectively. All compounds tested were not cytotoxic at 5 µg/mL.

Keywords: Monoterpenoid, anti-fungal activity, anti-leishmanial activity, *Tarchonanthus camphoratus*

1. Introduction

*Tarchonanthus camphoratus* belongs to the family *Asteraceae* and grows to 2-9 m high. This plant has characteristic leaves that are grey green above and pale grey and felted underneath, with prominent venation on the underside [1]. It grows in semi-arid regions of Kenya and Ethiopia [1]. Studies have shown that plants growing in these xeric habitats exude relatively simple organic compounds onto the outer aerial surface to protect the internal tissues from the harsh environmental conditions [2]. These surface compounds have become the subject of study as promising plant and human disease-controlling agents [3].

![T. camphoratus](image)

Fig 1: *T. camphoratus*

1.2 Ethno-medical application of *T. camphoratus*

The leaves of this plant have a wide range of ethno-medical applications. When burnt and inhaled, the leaves cure blocked sinuses, asthma and headache (Pretorius, 2008). The boiled leave extract treats cough, toothache, abdominal pain, bronchitis. The highly scented leaves are also used for massaging the body as perfume [4]. The Maasai of Kenya and Tanzania, for example, use the leaves of this plant as a deodorant [5]. The plant also shows powerful insect repellent action [6].

2. Experimental

2.1 General experimental procedures

Column chromatography was done by adsorbing 140 g of surface exudate extract of aerial parts of *T. camphoratus* on 150 g of Merck silica gel (70-230 mesh).
A glass column was packed with 1.5 kg of the silica gel under 20% CH₂Cl₂ in n-hexane. The adsorbed sample was carefully loaded onto the column. Elution was effected, first with the solvent system used for parking the column (20% CH₂Cl₂ in n-hexane) and then with solvent systems of increasing polarity up to 3% CH₃OH in CH₂Cl₂. Purification of the collected fractions was done by further gravity column chromatography using both silica gel and Sephadex LH-20 matrix and then by re-crystallization and preparative TLC. Compounds were visualized by observing under UV light at 254 nm followed by spraying the plates with 1% vanillin-H₂SO₄ spray reagent and placing the plates in iodine tanks in order to view the compounds that were UV inactive. 1D and 2D NMR spectra were recorded in CDCl₃, acetone-d₆, MeOD and DMSO depending on solubility of the compound under analysis. Electrospray Ionization High-Resolution Mass Spectroscopy (EI-HRMS) spectra recorded on 70 ev, on SSQ 710 MAT mass spectrometer.

### 2.2 Plant material
The fresh aerial parts of T. camphoratus were collected from Narok County, near Narok town (about 200 km from University of Nairobi on 27th January 2015 and identified by Mr. Patrick Mutiso, a Botanist of the University of Nairobi Herbarium, School of Biological Sciences (SBS), where a voucher specimen (Okemwa-27/January, 2017) is preserved.

### 2.3 Extraction and isolation of compounds from the leaves of T. camphoratus
The surface exudates of the fresh aerial parts (4 kg) of T. camphoratus were extracted by successively dipping into portions of ethyl acetate and acetone for short periods (~15s) to avoid extraction of internal tissue compounds. The extracts were filtered under pressure and solvent removed by rotary evaporator. This yielded 112 g of a black crude extract translating to 2.8% yield. An amount of 100 g of the extract was adsorbed onto 115 g of silica gel (SiO₂ Merck grade 9385, pore size 60 Å, 230-400 mesh particle size) under 2% ethyl acetate (EtOAc) in n-hexane. Separation was achieved using gravity column chromatography where the adsorbed extract was loaded onto a 1 kg SiO₂ column (15 cm x 10 cm). Stepwise gradient elution with mixtures of EtOAc in n-hexane starting with 2% EtOAc in n-hexane up to 18% in increasing order of polarities was carried out leading to 272 fractions of 300 ml each. The fractions were combined based on their thin layer chromatography (TLC) profiles into 28 fractions. The last fraction eluted with 18% EtOAc in n-hexane yielded a mixture of three compounds. The mixture was purified on preparative TLC by developing several times using 2% methanol (CH₃OH) in CH₂Cl₂. The major band was carefully scratched from the plate, soaked in 4% MeOH in CH₂Cl₂ and concentrated in vacuo using rotary evaporator, leading to 1 (184 mg). Compound 2 crystallized from the seventh fraction eluted with 10% EtOAc in n-hexane while 3 crystallized from the fifth fraction eluted with 8% EtOAc in n-hexane as white crystals. Compounds 4 and 6 were obtained by purification using PTLC (3% MeOH in CH₂Cl₂) of the mother liquor of the fraction of the major column eluted with 10% EtOAc in n-hexane. The fraction, eluted with 16% EtOAc in n-hexane was purified further using column chromatography eluting initially with 12% EtOAc in n-hexane up to 18% in increasing order of polarity. White crystals of 7 recrystallized from the first fraction and yellow ones of 5 from the third fraction of this minor column.

### 3. Results and Discussion

#### 3.1 Structure elucidation of new compounds
On extraction, the mass of the surface exudate extract was 9% yield /dry leaf weight from which the seven compounds. Structure elucidations of the compounds was accomplished through 1D and 2D NMR and mass spectrometric analyses, and also by comparison with published spectra of related compounds.

#### 3.1.1 2, 3, 3a, 4, 5, 7a-Hexahydro-7a-methyl-4-methylene-1H-inden-5-ol (1)
Compound 1 was isolated as a white compound from the surface exudates of aerial parts of T. camphoratus by column chromatography. It was crystallized from CH₂Cl₂ in n-hexane. It has an Rf of 0.44 in 5% MeOH in CH₂Cl₂. The structure of this natural compound was elucidated from analysis of its 1D and 2D-NMR spectral data. The 13C-NMR showed 11 carbon atoms suggesting a monoterpeneskeleton. Both 13C-NMR and DEPT analysis showed the presence of two quaternary and nine protonated carbons. Furthermore, the 13C-NMR, DEPT and HMBC indicated a methylene carbon at δc 103.5 suggesting a H₂C=CR₂ group. This also meant that the quaternary carbon at δc 150.4 was directly attached to the sp² methylene carbon which showed HMBC correlation with the protons at δH 4.67 and δH 5.18. This confirmed the diastereotopic nature of the protons. HMBC indicated their long range (J_HC) connectivity to sp³ carbons at δC 47.5 (C-8) and 70.0 (C-1). Because of its chemical shift, C-1 is oxygenated. 1H-NMR showed a broad singlet at δH 2.61 which was the proton of hydroxyl group attached to this carbon.
The $sp^3$ carbons appearing at $\delta_c$ 127.1 and 140.7 were found to be adjacent to each other and were assigned to C-2 and C-3 respectively in the monoterpenoid skeletal structure. From the HMOC spectrum, these carbons showed cross peaks with the protons appearing at $\delta_h$ 5.48 and 5.58 respectively. The protons attached to the carbons, showed COSY relationship. The proton at $\delta_h$ 5.58 corresponds to C-3. It appeared as a doublet of doublets ($J=6.4$ and $J=1.6$) resulting from magnetic interaction with protons of C-1 and C-2 protons. The coupling constant, $J=6.4$, proved that it is strongly coupled to C-2 proton and are cis to each other. Furthermore, their peaks exhibited a ‘roof’ effect. The proton on C-3 showed HMBC correlation with C-1 and C-8 confirming its placement at this position in the ring. This revealed the six-membered cyclic system in the structure.

The placement of methyl carbon at C-11 was made possible on the grounds that its protons had $J$ HMBC connectivity to C-3, C-8 and C-5. The singlet at $\delta_h$ 0.83, integrated for three protons, was assuredly due to these protons. From $^{13}$C-NMR spectrum, the peak appearing at $\delta_c$ 37.6 was placed at C-5 following its $J$CH coupling with C-3 proton as indicated by HMBC spectrum. In HMQC, its protons appeared at different chemical shifts as multiplets in the range $\delta_h$ 1.40-1.62. Signals due to protons at C-6 and C-7 formed multiplets that overlapped in the same region. The protons showed HMBC correlation to C-8 revealing the existence of a five membered cyclic ring. In view of the above spectral evidence and literature search, this compounds was determined as novel and named as 2, 3, 3a, 4, 5, 7a-Hexahydro-7a-methyl-4-methylene-1H-inden-5-ol. Table 1 below gives a summary of spectral data for compound 1.

### 3.1.2.5, 7, 3’, 4’-Tetrahydroxy-3-methoxychalcone (2)

This flavone was isolated from the surface exudates of the aerial parts of *Tarchonanthus camphoratus* amorphous white solid with an R$_f$ value of 0.41 in CH$_2$Cl$_2$ in- hexane. It was identified as 5,7,3’,4’-Tetrahydroxy-3-methoxychalcone, a known chalcone ([Hegaz et al., 2015]). Its $^{13}$C-NMR spectrum revealed the presence of sixteen carbons atoms with the carbonyl carbon of the ketone group appearing at $\delta_c$ 182.6. The peaks appearing $\delta_c$ 129.2 and 128.4 were assigned to C-2 and C-3 respectively. The methoxy carbon was downfield shifted typically appearing at $\delta_c$ 59.8 and the corresponding protons at $\delta_h$ 3.87(s).

Aromatic carbons of ring A, with oxygen substitution, appeared in their expected chemical shift ranges. C-5 was assigned to $\delta_c$ 156.7. The phenolic proton of hydroxy group bonded to this carbon was downfield shifted to appear at $\delta_h$ 13.23 in the lowfield region of 1H-NMR spectrum due to hydrogen bonding with carbonyl carbon that lengthens the O- H bond and deshields the proton. With the exception of carbonyl carbon, C-7 is most deshielded as a result appeared at $\delta_c$ 164.4. As a consequence of electron withdrawing effect of heteroatomic oxygen C-9 was also observed at $\delta_c$ 153.1 ppm in the downfield region of 13C-NMR spectrum. Non-substituted ArC, C-6 and C-8, appeared at $\delta_c$ 93.8 and 102.7. These are ArCs between oxygenated ArCs and experience strong shielding impacted by OH groups on the contiguous carbon atoms. The signal at $\delta_c$ 104.8 of a quaternary aromatic carbon was certainly due to C-10.

Hydroxy substituted carbons of ring B gave rise to signals $\delta$ 142.4 and 145.6 in ortho orientation with respect to each other and the chemical shifts are typical to this type of carbons. The protonated carbons of the aromatic ring were assigned to $\delta_c$ 113.2 (C-2), 115.7(C-5) and 119.2 (C-6) in the upfield end of the aromatic region. The corresponding protons were observed in the range of $\delta_c$ 7.47- 7.51. The chemicals shifts of this compound and their assignments are recorded in table 1.

### 3.1.3 (−)-Parthenolide (3)

This compound had an R$_f$ of 0.40 in 60% CH$_2$Cl$_2$ in n-hexane. Analyzing the spectral data showed it to be (−)- Parthenolide, a sesquiterpene that was earlier isolated from the same plant [Van Wyk et al., 1997]. The $^{13}$C-NMR revealed the presence of thirteen carbon atoms in the structure. Both $^{13}$C-NMR and DEPT$^1$ showed the compound has four quaternary carbons and the rest protonated. One of the quaternary carbons is $\delta_c$ 169.3. This chemical shift is typical for ketone group and was thus assigned to the carbonyl carbon in the skeletal structure. The remaining three quaternary carbons appearing at $\delta_c$ 134.6, 61.4 and 139.3 were caused by C–3, 7 and 11 respectively. C-7 is $sp^3$ hybridized but appeared lowfield because of being bonded to oxygen in the epoxide ring system. The C–3 and –11, which were $sp^3$ hybridized were far much downfield shifted due to deshielding by anisotropy found in unsaturated moieties. Protonated $sp^2$ carbons, C-10 and C-14, were also observed at $\delta_c$ 125.2 and 121.2 respectively. Due to their diastereotropic nature, C-14 protons formed doublets at $\delta_h$ 6.3 (J=2.8) and 5.62 (J=2.8). The proton bonded to C-10 was a doublet at 5.21 ppm (J=9.6). The coupling constant indicated strong magnetic interaction with the axial proton on C–9. Methyl C–15 and 16 distinctively emerged at $\delta_c$ 16.9 and 17.3 in $^{13}$C-NMR. The corresponding protons caused singlets at $\delta_h$ 1.30 and 1.71 respectively, each having an integration of three protons. The $^{13}$C-NMR and DEPT$^1$ showed four methylene C–8, 9, 12 and 13 at $\delta_c$ 36.3, 24.1, 41.2 and 30.5 respectively, within their chemical shift ranges. Protons of these carbons formed multiplets in the range $\delta_h$ 1.21- 2.43. Two methine carbons, C-4 and -5 were also observed at $\delta_c$ 47.6 and 82.5 respectively. The low chemical shift for the latter is due to its direct attachment to heteroatomic and electronegative oxygen. A summary of $^{1}$H- and $^{13}$C-NMR chemical shift assignments is given in Table 1.

### 3.1.4 (Z)-3a,4,7,8,9,9a-Hexahydro-6a-methyl-3,4-dimethyleazulenol[4,5-b]furan-2(3H,6aH,9bH)-one (4)

This is a novel compound that was isolated from surface exudates of aerial parts of *T. camphoratus*. It crystallized from DCM/hexane as a fine white powder with an R$_f$ of 0.58 in 30% ethyl hexane. It is highly soluble in methanol. Spectral analyses revealed that it is(Z)-3a,4,7,8,9,9a-Hexahydro-6a-methyl-3,4-dimethyleazulenol[4,5-b]furan-2(3H,6aH,9bH)-one (4), a known flavonoid ([^7]). Its structure was determined by 1D and 2D NMR spectroscopy. The total number of carbon atoms is in agreement with those exhibited by the 13C-NMR spectrum. In the $^{13}$C-NMR, the peak at $\delta_c$ 167.7 is characteristic of carbonyl carbon of an ester or carboxylic acid group. Since there was no hydroxyl proton corresponding to this carbonyl group in $^1$H-NMR, it was confirmed to be an ester group. Hence, the C-2 was assigned to the chemical shift value of $\delta_c$ 167.7.

The carbon at $\delta_c$ 69.6 was downfield shifted as compared to similar $sp^3$ hybridized methine carbons. This carbon was predicted to be attached to a heteroatom which is part of an ester group and was, therefore, assigned to C-13. From HMOC spectrum its corresponding proton was observed $\delta_h$
4.65. This proton showed long range $3J_{CH}$ connectivities with C-3, -5, and -8 and $3J_{CH}$ coupling to C-12. The $^1$H-NMR spectrum showed the methyl protons at $\delta_H 0.76$ ppm (s) and bonded to the carbon appearing at $\delta_C 19.3$ from HMBC cross peaks. The protons had long range HMBC ($^{1H}$H) coupling to C-9 ($\delta_C 37.7$), C-12 ($\delta_C 47.4$) and C-7 ($\delta_C 141.2$). From the proton integral ratio, position of chemical shift and DEPT, C-7 is a sp$^2$ CH carbon. The proton at $\delta_C 5.57$, from HMQC correlation, is directly attached to this carbon. It (C-7) appears as a doublet (J=12.0) meaning that C-6 ($\delta_C 128.8$) is also a sp$^2$ CH. The proton attached to this carbon appeared as a doublet at $\delta_H 5.48$ ppm. C-6 and C-6 proton signals exhibited ‘leaning effect; which indicated that they are vicinal sp$^2$ carbons and cis to each other. Furthermore, coupling constant suggested strong $3J_{HH}$ coupling with each other.

In $^{13}$C-NMR, the signals $\delta_C 104.2$ and 123.0 were due to C-15 and 14 respectively. The chemical shifts are typical for sp$^2$ CH$_2$ (also confirmed by DEPT) bonded to quaternary sp$^3$ carbon atoms. The quaternary carbons were observed at $\delta_C 146.1$ and 150.9 within their chemical shift range. HMOC showed the protons on C-14 caused a signal at $\delta_C 6.22$ in $^1$H-NMR. They also had $J_{CH}$ coupling to carbonyl C-2 and C-4 ($\delta_C 39.4$ ppm). The proton also had $J_{CH}$ connectivity to the carbon at 146.1 ppm. This justified the placement of this quaternary carbon at C-3 and the signal for the quaternary carbon at $\delta_C 150.9$ to C-5. From COSY spectrum, the CH$_2$ protons ($\delta_C 5.16$) on C-15 ($\delta_C 104.2$) coupled with C-4. The COSY spectrum also indicated that the three sp$^3$ methylene carbons, C-9, -10 and 11 were adjacent to each other. Protons on C-9, due to their diastereotopic nature, appeared as multiplets in the ranges $\delta_H 1.37$-1.45 and 1.59-1.62. The HMBC spectrum showed they had connectivities to C-7, 11, 12, and 16. Due to this relationship C-9 and C-11 were assigned to chemical shift values of $\delta_C 37.7$ and 29.4 respectively. Based on the above spectroscopic information and search from literature, this compound is new and is being reported for the first time. A summary of $^1$H- and $^{13}$C-NMR chemical shift assignments were recorded in Table 1.

3.1.5 5, 7, 4'-Trihydroxy-6-methoxyflavone (5)

This compound was successfully isolated from surface exudates of *Tarziconanthus camphoratus*. It was isolated as yellow crystals with $R_f$ of 0.43 in 1:1 EtOAc in n-hexane. Its structure was elucidated from NMR spectroscopy and comparison with spectral data of related compounds and was identified as hispidulin previously isolated from the same plant by Van Wyk et al. (1997).

The $^{13}$C-NMR spectrum revealed that it has sixteen carbon atoms. From DEPT spectral analysis, the compound has nine quaternary carbons and the rest being protonated. The $^1$H-NMR spectrum revealed two sets of protons exhibiting AABB spin system. This implicated a para- substituted benzene moiety. They were doublets at 6.90 (J=6.8) and 7.84 (J=6.4) ppm. The corresponding symmetric carbons of twice intensity were assigned to signals at $\delta_C 116.3$ and 128.8 with C-3/5'. They were upfield shifted due to the strong shielding effect from OH group on C-4'. This explains the existence of ring B with substitution at the para position.

For ring C, the chemical shift at $\delta_C 182.2$ was typical for carbonyl carbon of either ketone or aldehyde and was assigned to C-4. From $^{13}$C-NMR spectrum, the signals at $\delta_C 164.4$ and 102.7 were assigned to C-2 and C-3. C-2 was so downfield shifted because it is a sp$^2$ and bonded to an electronnegative heteroatomic oxygen in a six-membered ring system. DEPT indicated that C-3 is protonated. The quaternary carbon appearing at $\delta_C 104.5$ is undoubtedly assigned to C-10. It is usual for quaternary ArC between 1,3-diortho oxygen substituted ArC to resonate at approximately $\delta_C 100.0$.

In $^1$H-NMR, the presence of a singlet at 6.55 ppm, in the aromatic region, revealed the existence of a 1, 2, 3, 4, 5-pentasubstituted benzene ring. This proton was attached to C-8 of ring A. Another singlet appeared in this region (at 6.65 ppm) but this was due to the proton bonded to C-3. Furthermore, the $^{13}$C-NMR spectrum showed peaks at $\delta_C 164.4$ and $\delta_C 182.5$ assigned to C-2 and C-4 respectively. These peaks were downfield shifted due to oxygenation. Their exact chemical shifts are given in Table 2.

3.1.6 6,7,3'4'-Tetrahydroxy-6-methoxyflavone (6)

This a flavone that was isolated from the surface extract of *Tarchonanthus camphoratus* aerial parts. It is a yellow compound with $R_f$ of 0.43 in 1:1 EtOAc in n-hexane.

The $^{13}$C-NMR spectrum exhibited 16 signals which was consistent with the proposed structure (Appendix 6). The $^{13}$C NMR spectrum showed no overlapping of signals; all peaks were almost of equal intensity. The 1H-nmr spectrum showed a singlet at $\delta_H 6.55$ suggesting a 1,2,3,4,5-pentasubstituted benzene skeleton. This helped formulate ring A. There was another singlet at $\delta_H 6.61$ corresponding to C-3 of ring C. The DEPT spectrum indicated ten quaternary carbons with ring A and C accounting for seven of them. The remaining three carbons are C-1', -3' and -4'. Both $^1$H-NMR and $^{13}$C-NMR revealed no symmetric substitution in the structure (no overlapping of signals). Hence, to avoid symmetry, the OH groups were attached to C-3' and C-4'.

From 13C-NMR spectrum, the signal at $\delta_C 182.5$ was assigned to C=O moiety of a ketone which typically appears at this chemical shift value. Therefore, the chemical shift was undoubtedly due to C-4. C-2, a sp$^3$ quaternary carbon bonded to heteroatomic oxygen in a six-membered ring system was observed at $\delta_C 164.5$. The signal at $\delta_C 102.8$ of a protonated carbon was assigned to C-3. Its proton, as mentioned earlier, was observed at $\delta_H 6.61$.

For ring A, three oxygenated carbons were observed within their expected chemical shift ranges. The signals $\delta_C 153.1$, 157.7 and 152.8 were assigned to C-5 C-7 and C-9 respectively. However, methoxylated C-6 was downfield shifted to appear at $\delta_C 131.8$ due to strong shielding from hydroxy groups in both ortho positions. The non-substituted ArC, C-8, was responsible for the peak at $\delta_C 94.6$ with its corresponding proton appearing as a singlet at $\delta_H 6.55$. From DEPT spectrum, the signal at $\delta_C 104.5$ was due to a quaternary carbon and is typical for a ArC between 1,3-diortho oxygen substituted ArCs. This was certainly due to C-10.

In ring C, due to asymmetric substitution, none of the six carbons overlapped. As result of strong shielding effect of hydroxyl group on ortho carbons, C-2' and C-5' were assigned to relatively upfield chemical shifts $\delta_C 113.7$ and 116.5 respectively with non-substituted C-6' in the meta position appearing slightly lowfield at $\delta_C 119.4$. The quaternary C-1 of the ring was assigned to chemical shift at $\delta_C 122.0$. Aromatic protons in this ring system appeared between $\delta_H 6.88$-7.38. It was found to ne neperitin which was isolated from this plant by Van Wyk et al. (1997). Its NMR chemical shift assignments are recorded in Table 2.
3.1.7 5-Hydroxy-7,8-dimethoxyflavone (7)

This compound was isolated from the internal tissue extract. It crystallized as a yellow compound that crystallized from MeOH in CH2Cl2 with an Rf of 0.34 in 30% EtOAC in n-hexane. The structure of this compound was determined by 1D and 2D NMR spectroscopy. From 13C-NMR revealed the presence of seventeen carbon atoms which was consistent with the proposed structure. In 1H-NMR spectrum, the methylene and methine protons of ring C exhibited a typical ABX spin system. As a consequence of diastereotopic nature of the methylene protons in the Azole ring, they were observed as doublet of doublets in the ranges of δ 2.71-2.76 (JH,JH= 12.0, Jgem=4.0), and 2.95-3.03 (dd, 1H, CH2; JH,JH=12.0, Jgem=4.0). The coupling constants indicated strong vicinal and geminal coupling. Furthermore, they had long range connectivities to carbonyl carbon (C =O) at δc 190.4 and the more shielded methine carbon at δc 79.1 which was downfield shifted due to its attachment to heteroatomic oxygen. This is expected for methine carbons of in a five-membered heterocyclic ring which resonate in the region of δc 77-110.

The methine proton, due to coupling with both axial (J=12.0) and equatorial (J=4.0) methylene protons, also appeared as a doublet of doublets in the region of δ = 3.31-3.35. The proton appeared downfield of the methylene protons due to its close proximity to a benzene ring and the heteroatomic oxygen. Long range connectivities (2J) were observed between the proton and carbonyl carbon and the non-substituted carbons, C-2'/6' (δc 125.7) of ring B. It also showed 2J HMBC with methylene carbon, which resonated at δc 44.9 and the quaternary carbon (δc 139.5) of ring B. COSY spectrum, also indicated its correlation with the methylene protons.

The 14N NMR signal for the non-substituted aromatic carbon on ring A was typically observed at δ 92.8. From HMOC correlation, the corresponding proton was a singlet at δc 6.15 in the aromatic region of 1H-NMR spectrum. Furthermore, HMBC experiment clearly indicated its 3HC connectivity to the methoxy substituted carbon (C-8) and quaternary carbon (C-10) appearing at δc 129.3, and 104.2 respectively. There was also HMBC correlation of this proton with the 1,3-dioortho oxygenated aromatic carbons, C-9 and C-7 appearing at δc 156.8 and 158.0 respectively.

The intense signals of the two pairs of equivalent carbons, C-2'/6' and 3'/5', on ring B appeared at δc 125.7 and δ 128.3. C-4 of this ring was assigned the chemical shift at δc 128.2. From HMQCC, the corresponding protons to these carbons appeared in the region of δH 7.34-7.53 as multiplets integrating for five protons. Table 10 shows the 1H- and 13C-NMR chemical shift assignments. Its was previously isolated from aerial parts of Tarchonanthus camphoratus [reference].

3.2 Bioactivities

All the seven compounds were evaluated for their in vitro anti-plasmodial, anti-leishmanial, anti-fungal and anti-bacterial activities. For anti-plasmodial activity, the compounds were evaluated against the D6 (chloroquine sensitive) and W2 (chloroquine resistant) strains of P. falciparum. All tested compounds demonstrated no anti-plasmodial activity. The anti-bacterial and anti-fungal activities of the target compounds were also tested using the agar-diffusion method. The compounds were evaluated against standard strains of Escherichia coli, Pseudomonas aeruginos, M. intracellulare, C. albicans, C. glabrata, C. krusei, A. fumigates, C. neoformans, S. aureus. Staphylococcus aureus acted as an example of Gram positive bacteria and Escherichia coli Gram negative bacteria and C. albicans, C. glabrata, C. krusei, A. fumigates and C. neoformans as representatives of fungi. Most compounds showed no anti-microbial activities against the tested microbes. However, compound 1 showed interesting anti-fungal activity against Cryptococcus neoformans standard strain with an IC50 value of 5.62 ± 0.2 µg/mL. Compounds 1 and 2 exhibited moderate anti-leishmanial activities and cytotoxicity against Leishmania donovani and Vero cells with IC50 values of 14.17 ± 0.3 and 12.84 ± 0.3 µg/mL respectively. These activities were relatively lower than the standard drugs, pentamidine (IC50 = 0.85 µg/mL) and amphotericin B (IC50 = 0.12 µg/mL). Furthermore, 6 and 7 also showed anti-leishmanial activities with an IC50 value of 26.24 ± 0.4 and 23.15 ± 0.4 µg/mL respectively, therefore inactive. All compounds were not cytotoxic up to the maximum concentration tested (5 mg/mL).

3.2.1 In vitro anti-plasmodial activity assay

The in vitro activity against Plasmodium falciparum of extracts and pure compounds were evaluated for 50% growth inhibition of cultured parasites by automated micro-dilution [8]. Two commonly used P. falciparum strains for drug sensitivity assays, chloroquine sensitive sierra Leone I (D6) and chloroquine resistant Indo-China 1 (W2) were grown in continuous culture supplemented with mixed gas (90% N2, 5% O2, 5% CO2), 10% human serum and 6% hemocrit of A+ red blood cells. Once cultures reached a parasitaemia level of 3% with at least a 70% ring stage development, parasites were transferred to a 96 well micro-titer plate with wells precoated with sample. The samples were serially diluted across the plate to provide a range of concentrations used to accurately determine IC50 values. Plates were incubated in a mixed gas incubator for 24 hours. Following the specified incubation time, (H)-hypoxanthine was added and parasites allowed to grow for an additional 18 hours. Cells were processed with a plate harvester (Tom Tec) onto a filter paper and washed to eliminate unincorporated (H)-hypoxanthine. Filters were measured for activity in a microtiter plate scintillation counter (Wallac). In addition to the P. falciparum strains, samples were tested on the VERO mammalian cell line as an indicator of general cytotoxicity. The selectivity indices (SI) (ratio of VERO IC50 to D6 or W2 IC50) were calculated.

3.2.2 In vitro anti-leishmanial activity assay

The in vitro test was performed as described by Hoet et al. [9]. Amphotericin B (a commercial anti-leishmaniasis drug) and pentamidine were used as positive controls in all experiments with an initial concentration of 1.0 µg/mL. First stock solutions of crude extracts and compounds were prepared in DMSO or in ethanol/water (2:1) for water extracts at 20 mg/mL. The solutions were further diluted in the medium to give 0.2 mg/mL stock solutions. Extracts and compounds were tested against standard strain Leishmania donovani in eight serial three-fold dilutions (final concentration range: 100–0.05 µg/mL) in 96-well microtiter plates.

3.2.3 In vitro anti-microbial activity assay

The anti-microbial susceptibility assays were done using CLSI method [10]. The positive controls were Ciprofloxacin (≥98% purity assessed by HPLC, ICN Biomedicals, Ohio) for bacteria and amphotericin B (≥ 80% purity assessed by HPLC, ICN Biomedicals, Ohio). The test organisms, C. albicans (ATCC 90028), C. glabrata (ATCC 90030), C.
krusei (TCC 6258), A. fumigatus (ATCC 90906), C. neoformans (ATCC 9011), S. aureus (ATCC 29213), Methicillin-resistant S. aureus (ATCC 33591), E. coli (ATCC 35218), P. aeruginosa (ATCC 27853) and M. intracellulare (ATCC 23068) were obtained from the American Type Culture Collection, ATCC (Manassas, VA).

3.2.4 In vitro cytotoxicity analysis

Monkey kidney fibroblasts (VERO) were obtained from the American type culture collection (ATCC, Rockville, MD). The cell viability studies were done against the fibroblasts. The cells were seeded at a density of 25,000 cells / well and incubated for 24 h in 96-well microplates. Samples at different concentrations were added and plates were further incubated for 48 h. The number of viable cells were determined using neutral red according to 11 DMSO and Doxorubicin (98-102% purity assessed by HPLC) were used as positive and negative controls, respectively.

Acknowledgement

The authors wish to acknowledge to Mr. Patrick C. Mutiso for identification and collection of plant materials. The authors also wish to thank the National Commission for Science and Technology and Innovation (NACOSTI), Kenya and International Science Programme (ISP), Uppsala University, through KEN-02 project for providing grants that supported this research. ID and 2D NMR, MS and antimicrobial assays were supported by the USDA ARS specific Cooperative Agreement No. 58-6408-1-603 and NIH, NIAID, Division of AIDS, Grant No. AI 27094, respectively.

Table 1: Natural compounds isolated from Tarchonanthus camphoratus

<table>
<thead>
<tr>
<th>1(MEOH)</th>
<th>2(Acetone-d$_6$)</th>
<th>3(Acetone-d$_6$)</th>
</tr>
</thead>
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<tr>
<td>PS</td>
<td>$\delta_c$ (Hz)</td>
<td>$\delta_t$ (Hz)</td>
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<tr>
<td>1</td>
<td>70.0</td>
<td>4.63 (t, 1H, J=4.0)</td>
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<tr>
<td>2</td>
<td>127.1</td>
<td>5.49 (dd, 1H, J=1.6; 6.4)</td>
</tr>
<tr>
<td>3</td>
<td>140.7</td>
<td>5.61 (t, 1H, J=6.4)</td>
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<tr>
<td>4</td>
<td>37.8</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>37.6</td>
<td>1.40-150 (m, 1H, CH$_2$)</td>
</tr>
<tr>
<td>6</td>
<td>26.7</td>
<td>1.71 (m, 1H)</td>
</tr>
<tr>
<td>7</td>
<td>29.2</td>
<td>1.63 (m, 1H)</td>
</tr>
<tr>
<td>8</td>
<td>47.5</td>
<td>2.23 (d,1H, J=12.0)</td>
</tr>
<tr>
<td>9</td>
<td>150.4</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>103.5</td>
<td>4.67 (s, 5.18(s)</td>
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<tr>
<td>11</td>
<td>18.2</td>
<td>0.84 (s, CH$_3$)</td>
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<tr>
<td>12</td>
<td>168.7</td>
<td></td>
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<tr>
<td>13</td>
<td>146.1</td>
<td>102.7 (1C, CH, sp$^2$ C)</td>
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<tr>
<td>14</td>
<td>39.4</td>
<td>2.50-2.56 (dd, J=12.0, J=8)</td>
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KEY: PS-position,

Table 2: Natural compounds isolated from Tarchonanthus camphoratus

<table>
<thead>
<tr>
<th>4(DC13)</th>
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<th>6(DMSO)</th>
<th>7(CDC13)</th>
<th>8(DMSO)</th>
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<td>2</td>
<td>164.5 (1C, q, C-O)</td>
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<td>3</td>
<td>146.1</td>
<td>102.7 (1C, CH, sp$^2$ C)</td>
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<td>102.8 (1C, sp$^2$ CH)</td>
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<td>4</td>
<td>39.4</td>
<td>2.50-2.56 (dd, J=12.0, J=8)</td>
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<td>Sample/compound</td>
<td>L. donovani IC₉₀ µg/mL*</td>
<td>L. donovani IC₉₀ µg/mL**</td>
<td>Sample /compound</td>
<td>L. donovani IC₉₀ µg/mL*</td>
</tr>
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<td>-----------------</td>
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<td>------------------------</td>
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<tr>
<td>Pentamidine</td>
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<td>Amphotericin B</td>
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<tr>
<td>51</td>
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<td>NA</td>
<td>72</td>
<td>NA</td>
</tr>
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<td>NA</td>
<td>NA</td>
<td>73</td>
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<td>33.98</td>
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<td>7.39</td>
<td>EL</td>
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<td>&lt;40</td>
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<td>26.24</td>
<td>39.25</td>
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</table>

*The concentration (µg/ml) that affords 50% inhibition of growth
**The concentration (µg/ml) that affords 90% inhibition of growth

NA = not active

EL = Ethyl linoleate
LA = Linoleic acid
DHA = cis-4,7,10,13,16,19-Docosahexaenoic acid ethyl ester
ND = no data or not determined

References


