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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 IC_{50} value of 5.62 µg/mL. Compounds 1 and 2 exhibited moderate anti-leishmanial activities against *Leishmania donovani* with IC_{50} 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 IC_{50} 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].

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).

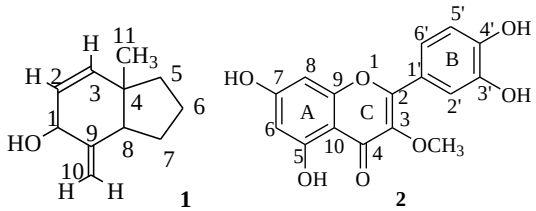
A glass column was packed with 1.5 kg of the silica gel under 20% CH_2Cl_2 in *n*-hexane. The adsorbed sample was carefully loaded onto the column. Elution was effected, first with the solvent system used for parking the column (20% CH_2Cl_2 in *n*-hexane) and then with solvent systems of increasing polarity upto 3% CH_3OH in CH_2Cl_2. 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-H_2SO_4 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 $CDCl_3$, acetone-d_6, MeOD and DMSO depending on solubility of the compound under analysis. Electronspray Ionization High-Resolution Mass Spectroscopy (EI-HRMS) spectra recorded on 70 ev, on SSQ 710 MAT mass spectrometer.

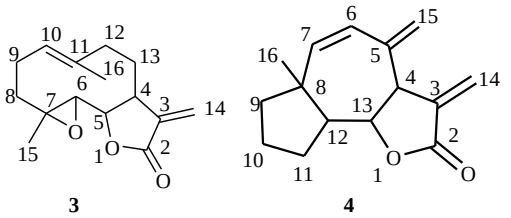
2.2 Plant material

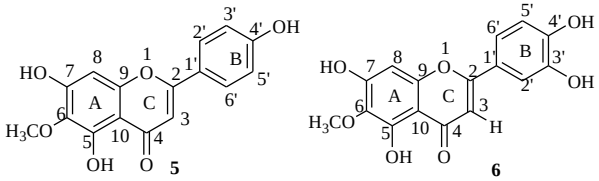
The fresh aerial parts of *T. camphoratus* were collected from Narok County, near Narok town (about 200 km from University of Nairobi on 27th January 2015 and identified by Mr. Patrick Mutiso, a Botanist of the University of Nairobi Herbarium, School of Biological Sciences (SBS), where a voucher specimen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

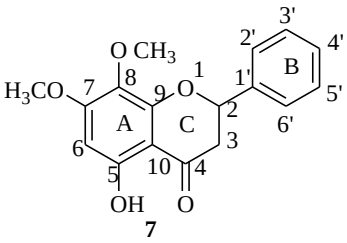
2.3 Extraction and isolation of compounds from the leaves of *T. camphoratus*

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 rotatory 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 (SiO_2, Merck grade 9385, pore size 60 Å, 230-400 mesh particle size) under 2% ethyl acetate (EtOAc) in *n*-hexane. Separation was effected using gravity column chromatography where the adsorbed extract was loaded onto a 1 kg SiO_2 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 severally using 2% methanol (CH_3OH) in CH_2Cl_2. The major band was carefully scratched from the plate, soaked in 4% MeOH in CH_2Cl_2 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 CH_2Cl_2) of the mother liquor of the fraction of the major column eluted with10% 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 upto 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-1*H*-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 CH_2Cl_2 in n-hexane. It has an R_f of 0.44 in 5% MeOH in CH_2Cl_2.

The structure of this natural compound was elucidated from analysis of its 1D and 2D-NMR spectral data. The ^{13}C-NMR showed 11 carbon atoms suggesting a monoterpene skeleton. Both ^{13}C-NMR and DEPT analysis showed the presence of two quaternary and nine protonated carbons. Furthermore, the ^{13}C-NMR, DEPT and HMQC indicated a methylene carbon at δ_C 103.5 suggesting a $H_2C{=}R_2$ group. This also meant that the quaternary carbon at δ_C 150.4 was directly attached to the sp^2 methylene carbon which showed HMQC correlation with the protons at δ_H 4.67 and δ_H 5.18. This confirmed the diastereotopic nature of the protons. HMBC indicated their long range ($^3J_{HC}$) connectivities to sp^3 carbons at δ_C 47.5 (C-8) and 70.0 (C-1). Because of its chemical shift, C-1 is oxygenated. ^{1}H-NMR showed a broad singlet at δ_H 2.61 which was the proton of hydroxyl group attached to this carbon.

The *sp*^{2} carbons appearing at δ_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 monoterpene skeletal structure. From the HMQC spectrum, these carbons showed cross peaks with the protons appearing at δ_H 5.48 and 5.58 respectively. The protons attached to the carbons, showed COSY relationship. The proton at δ_H 5.58 corresponds to C-3. It appeared as a doublet of doublets (^3J=6.4 and ^4J=1.6) resulting from magnetic interaction with protons of C-1 and C-2 protons. The coupling constant, ^3J=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 ^3J HMBC connectivity to C-3, C-8 and C-5. The singlet at δ_H 0.83, integrated for three protons, was assuredly due to these protons.

From ^{13}-CNMR spectrum, the peak appearing at δ_C 37.6 was placed at C-5 following its $^3J_{CH}$ coupling with C-3 proton as indicated by HMBC spectrum. In HMQC, its protons appeared at different chemical shifts as multiplets in the range δ_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-1*H*-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_2Cl_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 δ_C 182.6. The peaks appearing δ_C 129.2 and 128.4 were assigned to C-2 and C-3 respectively. The methoxy carbon was downfield shifted typically appearing at δ_C 59.8 and the corresponding protons at δ_H 3.87(*s*).

Aromatic carbons of ring A, with oxygen substitution, appeared in their expected chemical shift ranges. C-5 was assigned to δ_C 156.7. The phenolic proton of hydroxy group bonded to this carbon was downfield shifted to appear at δ_H 13.23 in the lowfield region of ^{1}H-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 δ_C 164.4. As a consequence of electron withdrawing effect of heteroatomic oxygen C-9 was also observed at δ_C 153.1 ppm in the downfield region of ^{13}C-NMR spectrum. Non-substituted ArC, C-6 and C-8, appeared at δ_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 δ_C 104.8 of a quaternary aromatic carbon was certainly due to C-10.

Hydroxy substituted carbons of ring B gave rise to signals δ 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 δ_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 δ_C 7.47- 7.51. The chemicals shifts of this compound and their assignments are recorded in table 1.

3.1.3 (-)-Parthenolide (3)

The comopound had an R_f of 0.40 in 60 % CH_2Cl_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 showed the compound has four quaternary carbons and the rest protonated. One of the quaternary carbons is δ_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 δ_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*^{2} hybridized were far much downfield shifted due to deshielding by anisotropy found in unsaturated moieties. Protonated sp^{2}carbons, C−10 andC−14, were also observed at δ_C 125.2 and 121.2 respectively. Due to their diastereotopic nature, C-14 protons formed doublets at δ_H 6.31 (*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 δ_C 16.9 and 17.3 in ^{13}C-NMR. The corresponding protons caused singlets at δ_H 1.30 and 1.71 respectively, each having an integration of three protons. The ^{13}C-NMR and DEPT showed four methylene C−8, 9, 12 and 13 at δ_C 36.3, 24.1, 41.2 and 30.5respectively, within their chemical shift ranges. Protons of these carbons formed multiplets in the range δ_H 1.21- 2.43. Two methine carbons, C-4 and -5 were also observed at δ_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-dimethyleneazuleno[4,5-b]furan-2(3*H*,6a*H*,9b*H*)-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 reveeled that it is(*Z*)-3a,4,7,8,9,9a-Hexahydro-6a-methyl-3,4-dimethyleneazuleno[4,5-b]furan-2(3*H*,6a*H*,9b*H*)-one (4), a kown 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 δ_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 δ_C 167.7.

The carbon at δ_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 HMQC spectrum its corresponding proton was observed δ_H

4.65. This proton showed long range $^{3}J_{CH}$ connectivities with C-3, -5, and -8 and $^{2}J_{CH}$ coupling to C-12.

The ^{1}H-NMR spectrum showed the methyl protons at δ_H 0.76 ppm (*s*) and bonded to the carbon appearing at δ_C 19.3 from HMQC cross peaks. The protons had long range HMBC ($^{3}J_{HC}$) coupling to C-9 (δ_C 37.7), C-12 (δ_C 47.4) and C-7 (δ_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 δ_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 (δ_C 128.8) is also a sp^2 CH. The proton attached to this carbon appeared as a doublet at δ_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 $^{3}J_{HH}$ coupling with each other.

In ^{13}C-NMR, the signals δ_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^2 carbon atoms. The quaternary carbons were observed at δ_C 146.1 and 150.9 within their chemical shift range. HMQC showed the protons on C-14 caused a signal at δ_C 6.22 in ^{1}H-NMR. They also had $^{3}J_{CH}$ coupling to carbonyl C-2 and C-4 (δ_C 39.4 ppm). The proton also had $^{2}J_{CH}$ 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 δ_C 150.9 to C-5. From COSY spectrum, the CH_2 protons (δ_C 5.16) on C-15 (δ_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 δ_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 δ_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 *Tarchonanthus camphoratus.* It was isolated as yellow crystals with R_f 0.46 in 2:5 EtOAc: 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*- disubstituted 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 δ_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 δ_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 δ_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 electronegative heteroatomic oxygen in a six-membered ring system. DEPT indicated that C-3 is protonated. The quaternary carbon appearing at δ_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 δ_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 δ_C 164.4 and δ_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 δ_H 6.55 suggesting a 1,2,3,4,5-pentasubstituted benzene skeleton. This helped formulate ring A. There was another singlet at δ_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 13^{C}-NMR spectrum, the signal at δ_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^2 quaternary carbon bonded to heteroatomic oxygen in a six-membered ring system was observed at δ_C 164.5. The signal at δ_C 102.8 of a protonated carbon was assigned to C-3. Its proton, as mentioned earlier, was observed at δ_H 6.61.

For ring A, three oxygenated carbons were observed within their expected chemical shift ranges. The signals δ_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 δ_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 δ_C 94.6 with its corresponding proton appearing as a singlet at δ_H 6.55. From DEPT spectrum, the signal at δ_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 δ_C 113.7 and 116.5 respectively with non-substituted C-6' in the *meta* position appearing slightly lowfield at δ_C 119.4. The quaternary C'-1 of the ring was assigned to chemical shift at δ_C 122.0. Aromatic protons in this ring system appeared between δ_H 6.88-7.38. It was found to ne nepetin 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 CH_2Cl_2 with an R_f of 0.34 in 30% EtOAC in n-hexane.

The structure of this compound was determined by 1D and 2D NMR spectroscopy. From ^{13}C-NMR revealed the presence of seventeen carbon atoms which was consistent with the proposed structure. In ^{1}H-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_H 2.71-2.76 (J_{vic}= 12.0, J_{gem}=4.0), and 2.95-3.03 (*dd*, 1H, CH_2 J_{vic}= 12.0, J_{gem}=4.0). The coupling constants indicated strong vicinal and geminal coupling. Furthermore, they had long range connectivities to carbonyl carbon (C=O) at δ_C 190.4 and the more shielded methine carbon at δ_C 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 δ_C 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_H 3.31-3.35. The proton appeared downfield of methylene protons due to its close proximity to a benzene ring and the heteroatom oxygen. Long range connectivities (^3J) were observed between the proton and carbonyl carbon and the non-substituted carbons, C-2'/6' (δ_C 125.7) of ring B. It also showed ^2J HMBC with methylene carbon, which resonated at δ_C 44.9 and the quaternary carbon (δ_C 139.5) of ring B. COSY spectrum, also indicated its correlation with the methylene protons.

The ^{13}C NMR signal for the non-substituted aromatic carbon on ring A was typically observed at δ 92.8. From HMQC correlation, the corresponding proton was a singlet at δ_C 6.15 in the aromatic region of ^{1}H-NMR spectrum. Furthermore, HMBC experiment clearly indicated its $^3J_{HC}$ connectivity to the methoxy substituted carbon (C-8) and quaternary carbon (C-10) appearing at δ_C 129.3, and 104.2 respectively. There was also HMBC correlation of this proton with the 1,3-*diortho* oxygenated aromatic carbons, C−9 and −7 appearing at δ_C 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 δ_C 125.7 and δ 128.3. C'-4 of this ring was assigned the chemical shift at δ_C 128.2. From HMQC, The corresponding protons to these carbons appeared in the region of δ_H 7.34-7.53 as multiplets integrating for five protons. Table 10 shows the ^{1}H- and ^{13}C-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 IC_{50} 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 IC_{50} values of 14.17 ± 0.3 and 12.84 ± 0.3 µg/mL respectively. These activities were relatively lower than the standard drugs, pentamidine (IC_{50} = 0.85 µg/mL) and amphotericin B (IC_{50} = 0.12 µg/mL). Furthermore, 6 and 7 also showed anti-leishmanial activities with an IC_{50} value of 26.24 ± 0.4 and 23.15 ± 0.4 µg/mL respectively, therefore inactive. All compounds were not cytotoxic upto 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% N_2, 5% O_2, 5% CO_2), 10% human serum and 6% hematocrit of A+ red blood cells. Once cultures reached a parasitemia level of 3% with at least a 70% ring stage development, parasites were transferred to a 96 well micro-titer plate with wells pre-coated with sample. The samples were serially diluted across the plate to provide a range of concentrations used to accurately determine IC_{50} values. Plates were incubated in a mixed gas incubator for 24 hours. Following the specified incubation time, (^{3}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 (^{3}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 IC_{50} to D6 or W2 IC_{50}) 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 (ATTC, 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.

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Table 1: Natural compounds isolated from *Tarchonanthus camphoratus*

1(MEOD)						2(Acetone-d_6)			3(Acetone-d_6)		
PS	**δ_C (Hz)**	**δ_H (Hz)**	**HMQC**	**HMBC**	**DQF-COSY**	**PS**	**δ_C (Hz)**	**δ_H (Hz)**	**PS**	**δ_C (Hz)**	**δ_H (Hz)**
1	70.0	4.63 (*t*, 1H, *J*=4.0)	4.63 (*t*, 1H, *J*=4.0)	C-3, -10	5.49, 5.61						
2	127.1	5.49 (*dd*, 1H, *J*=1.6; 6.4)	5.49 (*dd*, 1H, *J*=1.6; 6.4)	C-4, 9	4.63	2	149.2		2	169.3	
3	140.7	5.61 (*t*, 1H, *J*=6.4)	5.61 (*t*, 1H, *J*=6.4)	C-1,4,5,8	5.49	3	128.4		3	134.6	
4	37.8					4	182.6		4	47.6	2.35-2.43 (*m*)
5	37.6	1.40-150 (*m*, 1H, CH_2) 1.60-1.62 (*m*, CH_2)	1.40-150 (*m*, 1H, CH_2) 1.60-1.62 (*m*, CH_2)	C-3,	1.71, 1.63	5	156.7	13.24 (*s*, 1H, ArOH)	5	82.5	3.84 (*t*, 1H, CH, *J*=6.8)
6	26.7	1.71 (*m*, 1H) 1.63 (*m*, *J*=9.2)	1.71 (*m*, 1H) 1.63 (*m*, *J*=9.2)		1.4, 163	6	102. 7	6.59 (*s*, 1H, CH, ArH)	6	66.4	2.80 (*d*, 1H, CH, *J*=2.4)
7	29.2	1.63 (*m*, 1H)	1.63 (*m*, 1H)		1.71	7	164.4		7	61.4	
8	47.5	2.23 (*d*,1H, *J*=12.0)	2.23 (*d*,1H, *J*=12.0)		5.18, 1.48	8	93.8	6.60 (*s*, 1H, CH, ArH)	8	36.3	1.70-1.76 (*m*, 2H, CH_2)
9	150.4					9	153.1		9	24.1	2.13-2.00 (*m*, 2H, CH_2)
10	103.5	4.67 (*s*), 5.18(s)	4.67 (*s*), 5.18(s)	C-1, C-8	2.24, 5.18	10	104.8		10	125.2	5.21 (*d*, 1H, CH, *J*=9.6)
11	18.2	0.84 (*s*, CH_3)	0.84 (*s*, CH_3)	C-11, 4 or 5, 6		1'	122.8		11	139.3	
						2'	113.2	7.47 (*d*, CH, ArH, *J*=8.0)	12	41.2	2.13-2.00 (*m*, 2H, CH_2)
						3'	145.6	3.14, 9.50 (*s*, (*broad*), 1H, ArH)	13	30.5	1.21-1.27 (*m*, 2H, CH_2)
						4'	142.4		14	121.2	6.31 (*d*, 1H, CH_2, *J*=2.8) 5.62 (*d*, 1H, CH_2, *J*=2.8)
						5'	115.7	7.00 (*d*, CH, ArH, *J*=8.0)	15	17.3	1.30 (*s*, 3H, CH_3)
						6'	119.2	7.51 (*d*, 1H, ArH, *J*=4.0	16	16.9	1.71 (*s*, 3H, CH_3)
						3-OCH_3	59.8	3.87 (*s*, 3H, CH_3)			

KEY: PS-position,

Table 2: Natural compounds isolated from *Tarchonanthus camphoratus*

4(CDCl3)			5			6(DMSO)			7(CDCl3)		
PS	**δ_C (Hz)**	**δ_H (Hz)**	**PS**	**δ_C (Hz)**	**δ_H (Hz)**	**PS**	**δ_C (Hz)**	**δ_H (Hz)**	**PS**	**δ_C (Hz)**	**δ_H (Hz)**
2	168.7		2	164.4		2	164.5 (1C, *q*, C-O)		2	79.1 (1C, CH)	3.31-3.35 (1dd, CH_2 J_{ax}= 12.0, J_{eq}=4.0)
3	146.1		3	102.7 (1C, CH, sp^2 C)	6.70 □*s*, 1H, CH)	3	102.8 (1C, sp^2 CH)	6.61 (*s*, 1H, CH)	3	47.0 (1C, CH_2)	2.71-2.76 (*dd*, 1H, CH_2 J_{vic}= 12.0, J_{gem}=4.0) 2.95-3.03 (*dd*, 1H, CH_2 J_{vic}= 12.0, J_{gem}=4.0)
4	39.4	2.50-2.56 (*dd*, *J*=12.0, *J*=8)	4	182.5 (1C, *q*, C=O)		4	182.5 (1C, *q*, C=O)		4	190.4 (1C, *q*, C=O)	

5	150.9		5	153.1 (1C, *q*, ArC-OH)	12.97 (*s*, 1H, ArOH)	5	153.1 (1C, *q*, ArC-OH)	12.98 (*s*, 1H, CH, ArOH)	5	157.9 (1C, *q*, ARC-OH)	5.47 (*d* (*seudo*), 1H, ArOH)
6	128.8	5.48 (*d*, CH, *J*=8.0)	6	131.8 (1C, *q*, ArC-OCH_3)		6	131. 8(1C, *q*, ArC-OCH_3)		6	92.6 (1C, CH, ArC)	6.15 (*s*,1H, CH, ArH)
7	141.2	5.57 (*d*, *J*=12.0)	7	157.6 (1C, *q*, ArC-OH)	5.51 (*s*, 1H, ArOH)	7	157.7 (1C, *q*, ArC-OH)		7	158.0 (1C, *q*, ArC−OCH_3)	
8	38.0		8	94.7 (1C, CH, ArC)	6.58 (*s*, 1H, CH, ArH)	8	94.6 (1C,CH, ArC)	6.55 (*s*, 1H, ArH)	8	129.3 (1C, *q*, ArC−OCH_3)	
9	37.7	1.37-1.45 (*m*, 1H, CH_2)	9	152.8 (1C, *q*, ArC-O)		9	152.8 (1C, *q*, ArC-O-)		9	156.8 (1C, *q*, ArC—O)	
10	26.8	1.37-1.45 (*m*, 1H, CH_2) 1.62-1.69(*m*, 1H, CH_2)	10	104.5 (1C, *q*, ArC)		10	104.5 (1C, *q*, ArC)		10	104.2 (1C, *q*, ArC)	
11	29.4	1.37-1.44 (*m*, 1H, CH_2) 1.62-1.69(*m*, 1H, CH_2)	1'	121.7 (1C, *q*, ArC)		1'	122.0 (1C, *q*, ArC)		1'	139.1 (1C, *q*, ArC)	
12	47.4		2'/6'	129.8 (2C, CH, ArC)	6.92 (*d*, 2H, ArH, *J*=6.8)	2'	113.7 (1C, CH, ArC)	6.88-7.38 (*m*, 3H, CH, ArH)	2'/6'	125.7 (2C, CH, ArC)	7.34-7.53 (*m*, 5H, CH, ArHs)
13	69.6	4.65 (1H, CH)	3'/5'	116.4 (2C, CH, ArC)	6.88 (*d*, 2H, ArH, *J*=6.8)	5'	116.5 (1C, CH, ArC)		3'/5'	128.3 (2C, CH, ArC)	
14	123.0	6.22 (*s*,2H, CH_2)	4'	161.5 (1C, q, ArC-OH	6.79 (*s*, 1H, ArOH)	3'	150.1 (1C, *q*, ArC-OH)	3.47 (*s*, 1H, CH, ArOH)	4'	128.2 (1C, CH, ArC)	
15	104.2	5.16(*s*, 2H, CH_2)	6-OCH_3	60.4 (1C, CH_3, OCH_3)	3.73 (*s*, 3H, CH_3)	4'	146.1 (1C, *q*, ArC-OH)	3.82 (*s*, 1H, CH, ArOH)	7-OCH_3	54.8 (1C, CH_3, OCH_3)	3.36, 3.79 (*s*, 6H, CH_3)
16	19.3	0.76(*s*, 3H, CH_3)				6'	119.4 (1C, CH, ArC)				
						6-OCH_3	60.4 (1C, CH_3, OCH_3)	3.73 (*s*, 3H, CH_3)			
									8-OCH3	60.1 (1C, CH_3, OCH_3	

Table 3: Anti-leishmanial activity assay data

Sample/compound	*L. donovani* IC_{50} µg/mL*	*L. donovani* IC_{90} µg/mL**	Sample /compound	*L. donovani* IC_{50} µg/mL*	*L. donovani* IC_{90} µg/mL**
Pentamidine	0.85	1.75	67	23.15	33.69
Amphotericin B	0.12	0.15	68	<1.6	NA
51	NA	NA	72	NA	NA
52	NA	NA	73	NA	NA
58	5.92	16.09	75	33.98	NA
59	5.24	7.39	EL	8.9	NA
61	14.17	<40	LA	11.75	24.91
60	6.29	10.66	DHA	14.37	28.59
64	12.84	NA			
66	26.24	39.25			

*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

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