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Original Research

Reassessment of the polar fraction of *Stachys alopecuros* (L.) Benth. subsp. *divulsa* (Ten.) Grande (Lamiaceae) from the Monti Sibillini National Park: A potential source of bioactive compounds

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ABSTRACT

Background: The phytochemical analysis of *Stachys alopecuros* subsp. *divulsa*, an endemic Italian species, has been recently reported and has showed the presence of 8-O-acetylharpagide (2), harpagide (3), allobetonicoside (4), and 4′-O-galactopyranosyl-teuhircoside (5). In this paper, an in deep study of its glycosidic fraction with the aim to widen the knowledge on its secondary metabolites content is reported. **Materials and Methods:** Chromatographic techniques were used for the isolation of constituents while spectroscopic and spectrometric techniques were applied for the structures elucidation. **Results:** Besides the known constituents, all of them reconfirmed, ajugoside (1), reptoside (6) and 6-O-acetyl-ajugol (7) were also identified among the iridoids while the phenolic components resulted to be chlorogenic acid (8), β-arbutin (9), verbascoside (10), and stachysoside A (11), instead. **Conclusion:** The iridoid pattern of *S. alopecuros* subsp. *divulsa* has been expanded with the identification of not previously reported compounds as well as for the phenolic fraction. Except for the reconfirmed verbascoside (10), the other phenolic compounds were recognized for the first time in the studied species. The complete NMR assignment of compound (1) by means of bidimensional techniques is reported, and both the chemotaxonomic and pharmacological relevance of the isolated compounds is largely discussed.

KEY WORDS: Chemotaxonomy, chlorogenic acid, iridoid, NMR, phenylethanoid glycosides, β-arbutin

INTRODUCTION

The genus *Stachys* comprises more than 450 species mainly distributed in warm-temperate regions of the Mediterranean basin and South-Western Asia. It represents one of the largest genera of Lamiaceae family [1], and the majority of the species belonging to this genus have been largely used in the traditional medicine. *Stachys* spp. are currently indicated as "Mountain tea" in several regions reflecting their principal use in infusions and decoctions. The genus *Stachys* is represented by annual or perennial herbs known for their interesting biological activities, i.e., anti-inflammatory [2], antispasmodic, sedative, and diuretic [3-5]. Several phytochemical components, which may be responsible of the biological activities, have been isolated from *Stachys* species, i.e., iridoids [6,7], phenylethanoid glycosides, flavonoids [8,9], acidic metabolites, polysaccharides, and terpenoids [10].

In Italy, the genus *Stachys* encompasses about 30 species and subspecies among which five species are considered endemic. *Stachys alopecuros* subsp. *divulsa* is one of these. This plant is characteristic of the mountain habitat of central Italy, being distributed only in a few regions (Umbria, Marche, Abruzzo, Lazio, and Molise) [11], on stony mountain pastures, scrubs, and screes, and preferring a calcareous, and dry soil up to 2000 m a.s.l. *S. alopecuros* subsp. *divulsa* is a perennial plant with small, hirsute, and subcylindrical ascending stem. The opposite leaves are petiolate and densely hairy on the edge and along the veins. The flowers are gathered in small verticillasters held in a dense spike, yellow-white in color and blooming from June to August-September. The fruits, which are brown colored, are constituted by 4 ovate nuculae [12]. A previous study on this subspecies revealed the presence of phenypropanoid, saponin, and iridoid glycosides [6] which

are considered taxonomic markers for the genus and the family. In this paper, we reported a reassessment of the polar fraction constituents of this plant employing a patented method allowing the isolation of secondary metabolites of glycosidic nature.

MATERIALS AND METHODS

Plant Materials

The fresh plant materials (aerial parts) were collected at the flowering stage from a spontaneous population growing at the altitude of 1520 m a.s.l. in the territory of Pizzo Tre Vescovi, Marche, Italy (GPS coordinates: N 42°58′07″, and E 13°14′10″). The botanical recognition was performed by one of us (F.M.) using available literature [12], and a representative specimen has been deposited in the Herbarium Universitatis Camerinensis (CAME, included in the online edition of Index Herbariorum c/o School of Biosciences and Veterinary Medicine, University of Camerino, Italy) under the codex CAME 24825. A sample of the studied species is also stored in our laboratory (code number SAD-30062012) for any further reference.

Instruments

NMR spectra were recorded on a Varian (now Agilent Technologies) Mercury 300 MHz instrument and/or on a Bruker Avance III 400 MHz instrument using D_2O or CD_3OD as deuterated solvents: The chemical shifts were expressed in ppm using the HDO signal to set the ^{1}H-spectra acquired in D_2O, while the internal solvent signal (m5) at 3.31 ppm was set as reference for the spectra in CD_3OD.

MS spectra were performed on a Q-TOF MICRO spectrometer (Micromass, now Waters, Manchester, UK) equipped with an ESI source that operated in the negative and/or positive ion mode. The flow rate of sample infusion was 10 μL/min with 100 acquisitions per spectrum. Data were analyzed using the MassLynx software developed by Waters.

Solvents having RPE purity grade were all purchased from Sigma-Aldrich or Carlo Erba Reagenti, silica gel 60 (70-230 mesh ASTM) was purchased from Fluka.

Bidimensional NMR Experiments

Bidimensional spectra were performed on a Bruker Avance III 400 MHz instrument, operating at 9.4 T at 298 °K. Heteronuclear single quantum correlation (HSQC) experiments were acquired with a spectral width of 15 and 250 ppm for the proton and carbon, respectively, an average $^1J_{C\text{-}H}$ of 145 Hz, a recycle delay of 2 s and a data matrix of 4 K × 256 points. Heteronuclear multiple bond correlation (HMBC) experiments were acquired with a spectral width of 15 and 250 ppm for the proton and carbon, respectively, a long-range coupling constant of $^nJ_{C\text{-}H}$ of 8 Hz, a recycle delay of 2 s and a data matrix of 4 K × 256 points.

Extraction and Isolation of Polar Compounds

A portion of 390.0 g of hair-dried plant materials was extracted with a mixture of ethanol 96% and distilled water in ratio 8:2 v/v (1.4 L). The whole was left in maceration for 5 days so that the metabolites could come into solution. The procedure was repeated three times to achieve an exhaustive extraction. The solutions, having a dark green coloration, were gathered together in a same flask and then filtered. After filtration, the organic solvent was eliminated under reduced pressure at a temperature below 45°C until a water suspension was obtained. Throughout this last part, pH was checked on normal litmus paper, and this was about 8. This step is necessary to verify that pH is not too acid, meaning not under the value of 5.5, because an extreme acidity at this step may cause secondary reactions with the formation of several artifacts coming from the hydrolysis of glycosides and esters present in the extract. The water suspension was then frozen to −20°C and later lyophilized at the same temperature to preserve temperature-sensitive compounds possibly present. The final dried crude extract obtained from this methodology weighed 86.7 g and was dark green colored.

Adsorption Chromatography

The crude extract (86.7 g) was subjected to an active charcoal treatment. It was adsorbed on a stationary phase consisting in a mixture of charcoal/celite/polyamide (10:1:1) (60.0 g) until no reaction to vanillin/HCl spray reagent occured according to a method reported in the patent by Ballero *et al.* [13]. The resulting suspension was then stratified on a Gooch funnel and eluted first with H_2O to eliminate the mono- and disaccharides. These usually coelute with glycosidic constituents in silica gel chromatography and may interfere during the structure identification of the metabolites of interest. The desorption chromatography was conducted in steps using EtOH/H_2O mixtures gradually increasing in EtOH percentages (30, 60, and 95%). In this manner, three fractions at different polarity were collected, and after freeze drying 23.9, 4.70, and 0.82 g of solids were recovered, from the 30, 60 and 96% ethanolic elutions, respectively.

Silica Gel Column Chromatography

After a preliminary screening on thin layer chromatographys and paper chromatography, the separation step on silica gel column was first conducted on the 60% eluate because this showed the whole iridoid components and in comparable amount among them in respect to the other fractions. The 30% fraction, containing the more polar compounds, was partitioned in a second step. The 95% fraction did not contain iridoid glucosides and was not even considered.

The first chromatographic separation was performed on an aliquot of the 60% ethanolic elution for the weight of 4.0 g using 120.0 g of silica gel (ratio 1:30). The eluting system was a mixture of *n*-BuOH/H_2O (82:18 v/v). From this chromatographic separation (Scheme 1) 11 compounds were isolated and identified by comparison with data reported in literature and/or by comparison with pure compounds available in our laboratory:

Verbascoside (10, 12.4 mg) [14] from the assembly of fractions 8-15; stachysoside A (11, 11.2 mg) [8,15] in mixture with verbascoside (1:2) in the assembly of fractions 17-24; β-arbutin (9, 3.2 mg) [16] from the assembly of fractions 30-32; reptoside (6) [17] and 6-*O*-acetyl-ajugol (7) [18] in mixture in ratio 1:1.5 (25.0 mg) from the assembly of fractions 43-45; ajugoside (1, 113.7 mg) [17,19] as pure compound from the assembly of fractions 53-54; 8-*O*-acetyl-harpagide (2, 1.0 g) [20] from the assembly of fractions 70-88; chlorogenic acid (8) [21] in mixture with 8-*O*-acetyl-harpagide (2) in ratio 1:2 (109.4 mg) from the assembly of fractions 95-112; harpagide (3) [22] and chlorogenic acid (8) in mixture 3:1 (48.3 mg) from the assembly of fractions 118-122; allobetonicoside (4) and chlorogenic acid (8) in mixture 10:1 (29.1 mg) from the assembly of fractions 133-135; allobetonicoside (4, 100.7 mg) [23] as a pure compound from the assembly of fractions 144-163; 4′-*O*-galactopyranosyl-teuhircoside (5) [6] in mixture with allobetonicoside (4) in ratio 3:1 (24.7 mg) from the assembly of fractions 166-169.

A second chromatographic column was, instead, conducted on an aliquot of the 30% desorption solids (3.5 g). Similar compounds were isolated from this chromatographic step. In particular, harpagide (3), 8-*O*-acetylharpagide (2) as pure compounds, allobetonicoside (4) in mixture with 4′-*O*-galactopyranosyl-teuhircoside (5), and ajugoside (1) occurring in traces (relative quantities not estimated). On the other hand, phenolic compounds as well as reptoside (6) and 6-*O*-acetyl-ajugol (7), were not found.

Spectral Data of the Isolated Compounds

Ajugoside (1): NMR data see Table 1; ESI-MS: *m/z* 412.80 $[M+Na]^+$.

Table 1: Complete NMR assignment of ajugoside (1) in D_2O

Ajugoside (1), (400 MHz, D_2O)				
Position	δ, ^1H	Multiplicity, J (Hz)	δ, ^{13}C (HSQC)	HMBC Correlations
1	5.94	d, *J*=1.1	93.73	$C_{5'}$, $C_{8'}$, $C_{1'}$
2	-	-	-	-
3	6.31	dd, *J*=6.4, 2.3	139.72	$C_{1'}$, $C_{4'}$, C_5
4	4.89	ddd, *J*=6.4, 2.1, 1.1	103.46	$C_{3'}$, $C_{5'}$, $C_{6'}$, C_9
5	2.85	br d, *J*=8.3	39.83	$C_{3'}$, C_8
6	4.18	d, *J*=4.0	75.55	$C_{7'}$, C_8
7a	2.25	d, *J*=15.4	46.74	$C_{5'}$, $C_{6'}$, $C_{8'}$, $C_{9'}$, C_{10}
7-b	2.18	dd, *J*=15.4, 4.3		
8	-	-	89.43	-
9	2.93	br d, *J*=8.5	47.48	$C_{1'}$, $C_{5'}$, C_8
10	1.56	s	21.49	$C_{7'}$, $C_{8'}$, C_9
Glucose				
1′	4.82	d, *J*=8.1	98.25	$C_{1'}$, $C_{2''}$, $C_{3'}$
2′	3.33	dd, *J*=9.3, 8.1	72.73	$C_{1''}$, $C_{3'}$
3′	3.56	m	75.65	$C_{1''}$, $C_{2''}$, $C_{4'}$, $C_{5'}$
4′	3.44	m	69.65	$C_{3''}$, $C_{6'}$
5′	3.52	m	76.26	$C_{4''}$, $C_{6'}$
6′a	3.98	dd, *J*=12.5, 2.2	60.77	$C_{4''}$, $C_{5'}$
6′b	3.78	dd, *J*=12.5, 6.0		
CH_3CO	2.08	s	174.14; 21.73	$C_{8'}$, C=O

HSQC: Heteronuclear single quantum correlation, HMBC: Heteronuclear multiple bond correlation

8-*O*-acetylharpagide (2): ^1H NMR (300 MHz, CD_3OD) δ: 6.40 (1H, d, *J* = 6.4 Hz, H-3), 6.08 (1H, br s, H-1), 4.92 (1H, d, J = 6.5 Hz, H-4), 4.60 (1H, d, *J* = 7.8 Hz, H-1′), 3.90 (1H, d, *J* = 11.8 Hz, H-6′a), 3.71 (2H, m, H-6′b; H-3′), 3.44-3.29 (2H, m, H-4′; H-5′), 3.22 (1H, t like, J = 8.4 Hz, H-2′), 2.86 (1H, br s, H-9), 2.18 (1H, d, *J* = 15.1 Hz, H-7a), 2.02 (3H, s, CH_3CO), 1.94 (1H, dd, *J* = 15.2, 4.4 Hz, H-7b), and 1.45 (3H, s, H-10).

^{13}C NMR (APT) (75 MHz, CD_3OD) δ: 173.3 (CH_3CO), 143.8 (C-3), 106.8 (C-4), 99.8 (C-1′), 94.5 (C-1), 88.6 (C-8), 78.0 (C-3′), 77.6 (C-5′), 77.4 (C-6), 74.4 (C-2′), 73.3 (C-5), 71.6 (C-4′), 62.8 (C-6′), 55.4 (C-9), 46.0 (C-7), 22.5 (CH_3CO), and 22.2 (C-10).

ESI-MS: *m/z* 429.04 $[M+Na]^+$; m/z 835.19 $[2M+Na]^+$.

Harpagide (3): ^1H NMR (300 MHz, CD_3OD) δ: 6.32 (1H, d, *J* = 6.3 Hz, H-3), 5.74 (1H, br s, H-1), 4.95 (1H, d, *J* = 6.3 Hz, H-4), 4.58 (1H, d, *J* = 7.9 Hz, H-1′), 3.90 (1H, d, *J* = 12.2 Hz, H-6′a), 3.75-3.60 (2H, m, H-6′b; H-3′), 3.44-3.33 (2H, m, H-4′; H-5′), 3.26-3.16 (1H, dd, *J* = 8.8, 8.2 Hz, H-2′), 2.56 (1H, br s, H-9), 1.90 (1H, dd, *J* = 13.8, 4.6 Hz, H-7a), 1.80 (1H, dd, *J* = 13.8, 3.6 Hz, H-7b), 1.25 (3H, s, H-10).

ESI-MS: *m/z* 387.14 $[M+Na]^+$.

Allobetonicoside (4): ^1H NMR (300 MHz, D_2O) δ: 6.43 (1H, d, *J* = 6.3 Hz, H-3), 6.11 (1H, br s, H-7), 5.94 (1H br s, H-1), 5.28 (1H, d, *J* = 8.3 Hz, H-1″), 5.01 (1H, d, *J* = 6.3 Hz, H-4), 4.75 (1H, d, *J* = 7.8 Hz, H-1′), 4.19 (1H, t, *J* = 2.6 Hz, H-9), 3.93 (2H, m, H-6′a; H-6″a), 3.83 (2H, m, H-6′b; H-6″b), 3.70-3.33 (8H, m, H-3′; H-3″; H-4′; H-4″; H5′; H-5″; H-2′; H-2″), and 2.28 (3H, s, H-10).

^{13}C NMR (75 MHz, D_2O) δ: 204.86 (C-6), 178.50 (C-8), 144.09 (C-3), 127.77 (C-7), 102.69 (C-4), 98.89 (C-1′), 96.58 (C-1″), 92.25 (C1), 77.45 (C-5), 76.18 (C-5′), 75.60 (C-3′), 73.57 (C-2′), 72.71 (C-4″), 71.20 (C-2″), 69.85 (C-3″), 69.42 (C-4′), 66.62 (C-4″), 61.03 (C-6″), 60.70 (C-6′), 54.85 (C-9), 17.62 (C-10).

ESI-MS: *m/z* 528.73 $[M+Na]^+$; m/z 544.69 $[M+K]^+$.

4′-*O*-galactopyranosyl-teuhircoside (5): ^1H NMR (300 MHz, D_2O) δ: 6.43 (1H, d, *J* = 6.4 Hz, H-3), 6.13 (1H, br s, H-7), 5.94 (1H, br s, H-1), 4.98 (1H, d, *J* = 6.4 Hz, H-4), 4.80 (1H, partially overlapped with solvent signal, H-1″), 4.73 (1H, d, *J* = 8.1 Hz, H-1′), 2.29 (3H, s, H-10).

ESI-MS: *m/z* 529.12 $[M+Na]^+$.

Reptoside (6): ^1H NMR (300 MHz, D_2O) δ: 6.40 (1H, d, *J* = 6.5 Hz, H-3), 5.90 (1H, br s, H-1), 5.02 (1H, dd, *J* = 6.4, 1.3 Hz, H-4), 4.75 (1H, d, partially obscured by the solvent signal, H-1′), 3.92 (1H, dd, *J* = 12.4, 1.9 Hz, H-6′a), 3.71 (1H, br d, *J* = 12.4 Hz, H-6′b), 3.26 (1H, dd, *J* = 8.3, 8.2 Hz, H-2′), 2.65 (1H, br s, H-9), 2.04 (3H, s, CH_3CO), 1.93 (2H, m, H-6a; H-7a), 1.71 (2H, m, H-6b; H-7b), 1.43 (3H, s, H-10).

ESI-MS: *m/z* 413.04 $[M+Na]^+$.

6-*O*-acetyl-ajugol (7): ^{1}H NMR (300 MHz, D_2O) δ: 6.34 (1H, dd, *J* = 6.4, 1.8 Hz, H-3), 5.50 (1H, d, *J* = 2.8 Hz, H-1), 4.89 (1H, dd, *J* = 6.4, 2.8 Hz, H-4), 4.81 (1H, obscured by the solvent signal, H-6), 4.70 (1H, d, *J* = 8.0 Hz, H-1′), 3.90 (1H, dd, *J* = 12.1, 1.6 Hz, H-6′), 3.68 (1H, br d, *J* = 12.2 Hz, H-6′b), 3.24 (1H, m, H-2′), 2.79 (1H, br t, *J* = 6.5 Hz, H-5), 2.34 (1H, dd, *J* = 7.9, 2.7 Hz, H-9), 2.12 (1H, br dd, *J* = 14.0, 6.5 Hz, H-7a), 2.08 (3H, s, CH_3CO), 1.93 (1H, dd, *J* = 13.8, 8.6 Hz, H-7b), 1.38 (3H, s, H-10).

ESI-MS: *m/z* 413.04 $[M+Na]^+$.

Chlorogenic acid (8): ^{1}H NMR (300 MHz, CD_3OD) δ: 7.56 (1H, d, *J* = 15.9 Hz, H-β′), 7.05 (1H, d, *J* = 1.9 Hz, H-2′), 6.96 (1H, dd, *J* = 8.2, 1.9 Hz, H-6′), 6.78 (1H, d, *J* = 8.2 Hz, H-5′), 6.26 (1H, d, *J* = 15.9 Hz, H-α′), 5.33 (1H, td, *J* = 9.1, 4.5 Hz, H-3), 4.17 (1H, dt, *J* = 6.2, 3.2 Hz, H-4), 3.73 (dd, *J* = 8.5, 3.1 Hz, H-5), 2.27-2.13 (2H, m, H-2a; H-6a), 2.13-1.98 (2H, m, H-2b;H-6b).

ESI-MS: *m/z* 377.30 $[M+Na]^+$.

β-arbutin (9): ^{1}H NMR (300 MHz, CD_3OD) δ: 7.07 (2H, d, *J* = 8.6 Hz, H-3, H-5), 6.69 (2H, d, *J* = 8.6 Hz, H-2, H-6), 4.95 (1H, partially obscured by HDO signal, H-1′), 3.96-3.79 (m, overlapped glucose protons), 3.77-3.54 (m, overlapped glucose protons), 3.18 (1H, br t, *J* = 7.9 Hz, H-2′).

ESI-MS: *m/z* 311.22 $[M+K]^+$.

Verbascoside (10): ^{1}H NMR (300 MHz, CD_3OD) δ: 7.59 (1H, d, *J* = 15.9 Hz, H-β″), 7.05 (1H, d, *J* = 1.4 Hz, H-2″), 6.96 (1H, dd, *J* = 8.2, 1.4 Hz, H-6″), 6.78 (1H, d, *J* = 8.2 Hz, H-5″), 6.69 (1H, br s, H-2′), 6.68 (1H, d, *J* = 8.0 Hz, H-5′), 6.56 (1H, dd, *J* = 8.0, 1.6 Hz, H-6′), 6.28 (1H, d, *J* = 15.9 Hz, H-α″), 5.19 (1H, d, *J* = 0.9 Hz, H-1‴), 4.38 (1H, d, *J* = 7.8 Hz, H-1), 2.79 (2H, br t, *J* = 7.3 Hz, H-β′), 1.09 (3H, d, *J* = 6.2 Hz, H-6‴).

ESI-MS: *m/z* 623.19 $[M-H]^-$; m/z 647.12 $[M+Na]^+$.

Stachysoside A (11): ^{1}H NMR (300 MHz, CD_3OD) δ: 7.60 (1H, d, *J* = 15.6 Hz, H-β″[caff.]), 7.06 (1H, br s, H-2″), 6.98 (1H, br d, *J* = 8.5 Hz, H-6″), 6.78 (1H, d, *J* = 8.5 Hz, H-5″), 6.70 (1H, br s, H-2′), 6.68 (1H, d, *J* = 8.0 Hz, H-5′), 6.57 (1H, br d, *J* = 8.1 Hz, H-6′), 6.28 (1H, d, *J* = 15.6 Hz, H-α″), 5.42 (1H, br s, H-1‴), 4.43 (1H, d, *J* = 7.9 Hz, H-1), 4.31 (1H, d, *J* = 7.2 Hz, H-1⁗), 2.81 (2H, d, *J* = 7.1 Hz, H-β′), 1.16 (3H, d, *J* = 6.0 Hz, H-6‴).

ESI-MS: *m/z* 755.20 $[M-H]^-$; m/z 779.16 $[M+Na]^+$.

RESULTS

The reassessment of the glycosidic fraction of *S. alopecuros* subsp. *divulsa* allowed the isolation of several compounds. The majority of them were iridoids, followed by a phenolic glucoside, two phenylethanoid glycosides, and a caffeoylquinic acid. They were identified as ajugoside (1), 8-*O*-acetylharpagide (2), harpagide (3), allobetonicoside (4), 4′-*O*-galactopyranosyl-teuhircoside (5), reptoside (6), 6-*O*-acetyl-ajugol (7) [Figure 1] for the iridoid glycosides constituents, and as chlorogenic

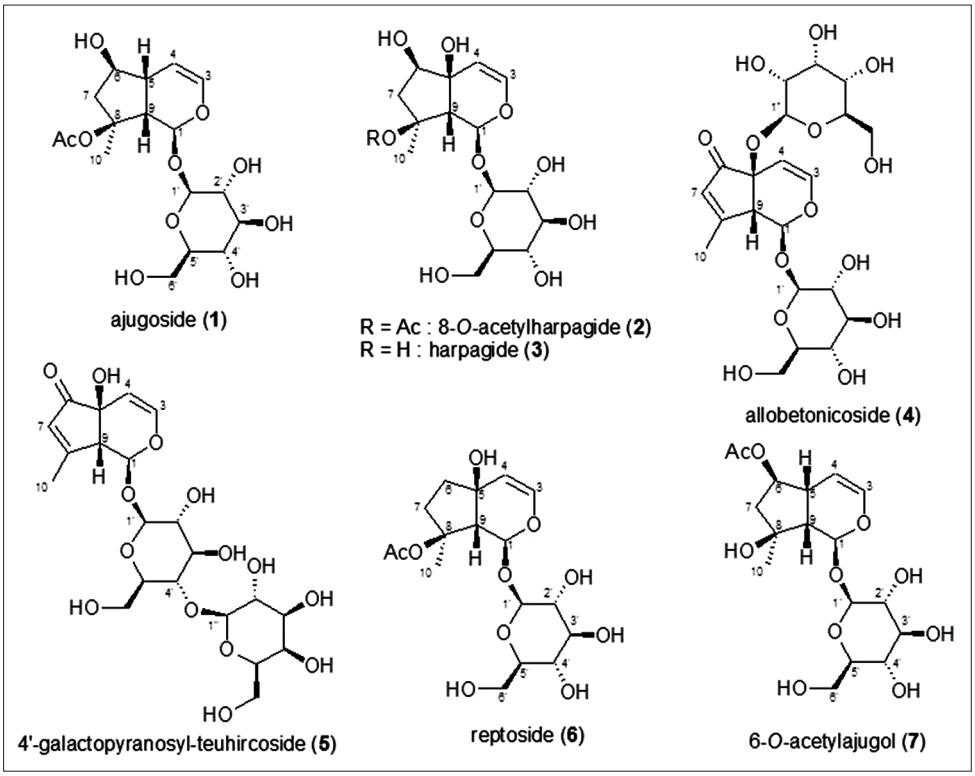


Figure 1: Iridoids from *Alopecurus alopecuros* subsp. *divulsa*

acid (8), β-arbutin (9), verbascoside (10), and stachysoside A (11) [(2-(3,4-dihydroxyphenyl)ethyl-*O*-α-L-arabinopyranosyl-(1→2)-*O*-6-deoxy-β-L-mannopyranosyl-(1→3)-4-*O*-[(2*E*)-3-(3,4-dihydroxyphenyl)-1-oxo-2-propen-1-yl]-β-D-glucopyranoside] [Figure 2] for the phenolic compounds. The presence of previously identified iridoid glycosides (2-5) has been confirmed, together with ajugoside (1), reptoside (6), and 6-*O*-acetyl-ajugol (7) which resulted to be new iridoid components for this species. Among the phenolic constituents, chlorogenic acid (8), β-arbutin (9), and stachysoside A (11) were isolated for the first time from the studied species [Figure 1].

DISCUSSION

Chemosystematic Implications of Isolated Compounds

The identified iridoids have all chemosystematic relevance. In particular, 2 and 3 are considered family markers, 4 is characteristic of the section Betonica, and 5 has never been reported in other

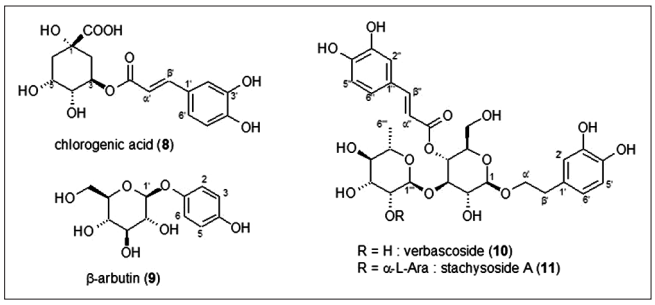


Figure 2: Phenolics from *Alopecurus alopecuros* subsp. *divulsa*

Stachys species so that it may be regarded as a chemotaxonomic marker at a subspecific level. Reptoside (6) resulted to be a rare metabolite in the *Stachys* genus because its occurrence has been reported only in a few species, namely *Betonica officinalis* (syn. of *Stachys officinalis*) [23] and *S. macrantha* [24]. 6-*O*-acetyl-ajugol (7), is also a rare metabolite in the Lamiaceae family since it has been found only in *Leonurus persicus* so far [18]. It is also worth of mention the presence of ajugoside (1) and reptoside (6) which are iridoids widely distributed in the species of the Ajugoideae subfamily. In fact, 1 was mainly reported in the *Ajuga* genus (e.g. *Ajuga tenorei* and *Ajuga chamaepitys*) [25,26], although its presence was also signaled in a few Lamioideae species as *Melittis melissophyllum* subsp. *melissophyllum* [19] and species of the genera *Stachys* and *Sideritis*. The occurrence of 1 in the *Stachys* genus is limited to a few species such as *B. officinalis* (syn. of *S. officinalis* (L.) Trevis.) [2], *S. macrantha* [24], *S. alpina* [2], *Stachys germanica* [2,27] and *Stachys recta* [2], and it has been identified also in the taxonomically related *Sideritis* genus like for instance in *Sideritis romana* [28], and *Sideritis perfoliata* subsp. *perfoliata* [29].

Considering their occurrence, compounds (1) and (6) may represent chemosystematic traits of proximity between Lamioideae and Ajugoideae subfamilies. The identification of 1 has been carried out by extensive NMR analysis on both mono - [Figures 3 and 4] and bidimensional [Figures 5 and 6] experiments since the assignment of resonances was not immediate for several signals, in particular for protons in 5, 6 and 9 positions on the iridoid skeleton as well as for protons in 3′ and 5′ positions of the glucose moiety [Figures 3 and 4].

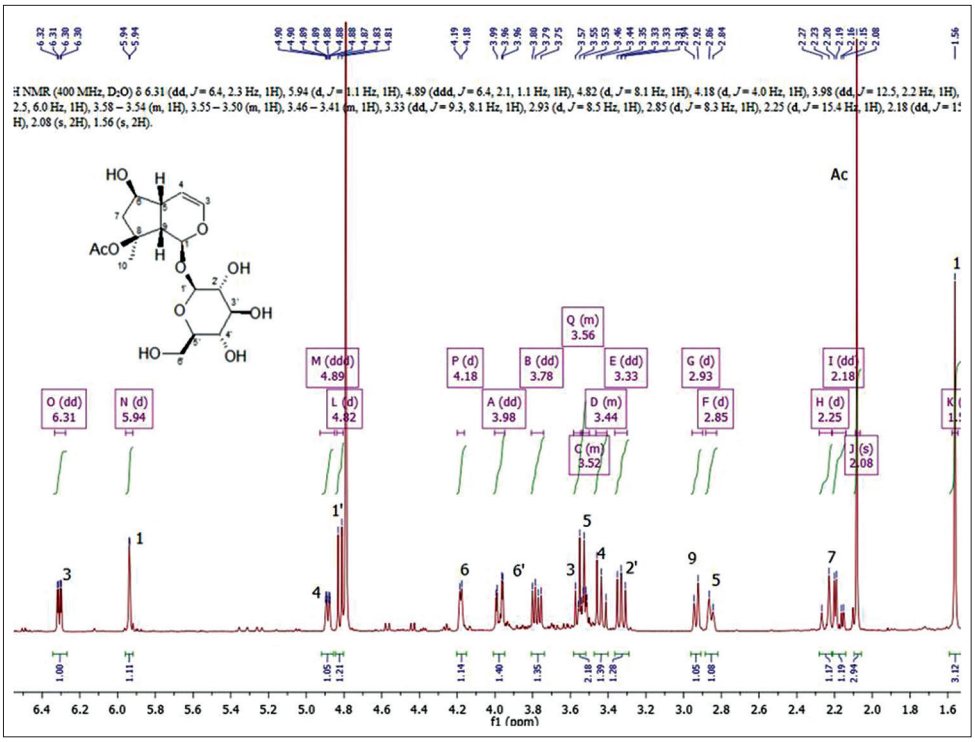


Figure 3: [1]H-NMR spectrum of ajugoside (1)

In particular, the resonance of H-6 was readily visible in the proton spectrum at 4.18 ppm, but the corresponding carbon signal was very near to a glucose carbon resonance and has been assigned by the direct HSQC correlation [Figure 5] with the signal resonating at 75.55 ppm. On the contrary, the assignment of protons and carbons in the position 5 and 9 was not immediate, and several long range correlations resulted to be crucial for discriminating the resonances of these nuclei. The signals relative to the positions 5 and 9 present resonances in the proton spectrum at 2.93 and 2.85 ppm, both as broad signals, and the study of the coupling constants gave no help for the structure elucidation. In the HSQC spectrum, a direct

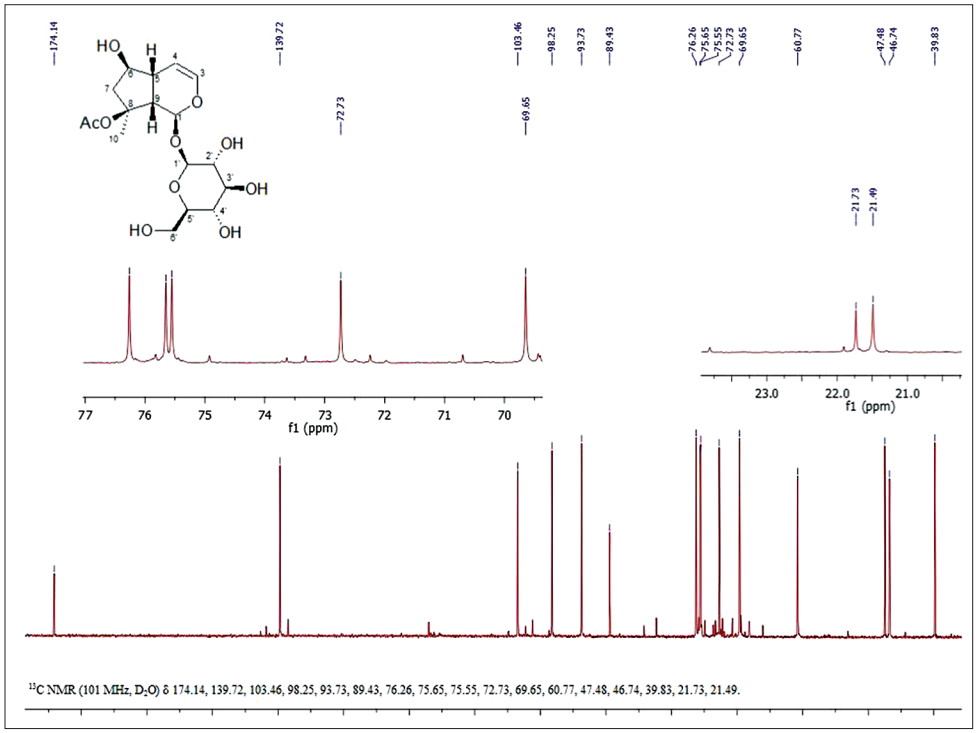


Figure 4: ^{13}C-NMR spectrum of ajugoside (1)

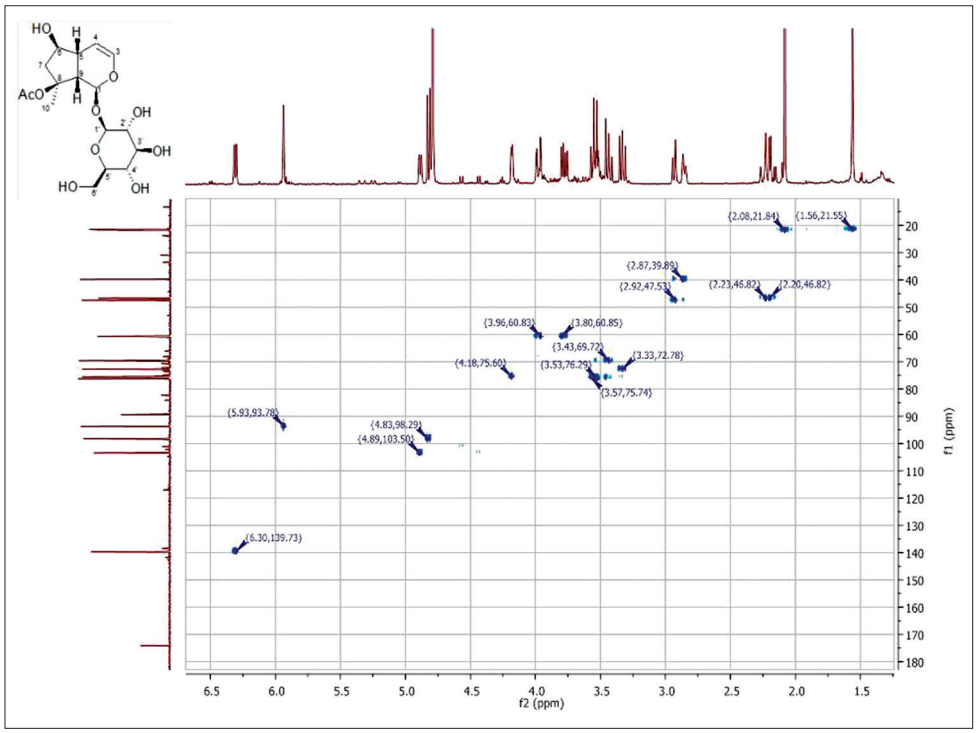
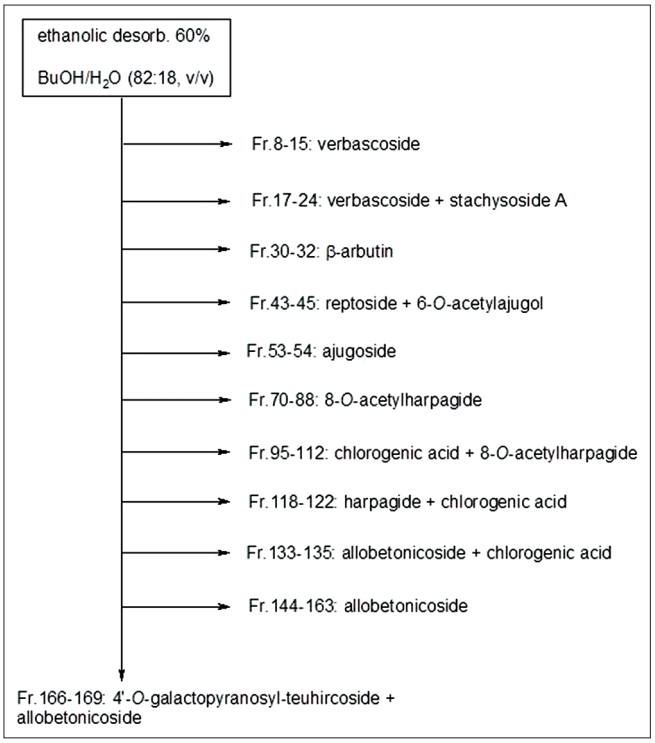


Figure 5: Heteronuclear single quantum correlation spectrum of ajugoside (1)

correlation between the proton signal at 2.93 ppm and the carbon resonating at 47.48 ppm was recorded as well as the correlation between the proton at 2.85 and the carbon at 39.83 ppm. This permitted to assign the respective carbons signals. In the HMBC experiments, [Figure 6] the only diagnostic correlation which permitted to differentiate the positions 5 and 9 was a long range correlation between H-3 (6.31 ppm) and the carbon resonating at 39.83 ppm, which could be assigned to C-5 of the iridoid skeleton, since almost all the proton signals correlated with both the carbons at 39.83 and 47.48 ppm. In fact, H-3 is relatively far from C-9, and this kind of signal could not be visualized with the coupling constant of 8 Hz set in the experiment. Using the HMBC correlations, it was also possible to differentiate the positions 3′ and 5′ on the glucopyranose unit. In particular, the resonances of the positions 1′, 2′, 4′, and 6′ (98.25, 72.73, 69.65, and 60.77, respectively) were readily assignable to the saccharidic moiety, while the signals relative to 3′ and 5′ might be differentiated on the basis of their correlation with near nuclei [Figures 5 and 6].

More specifically, from HMBC experiments the following long-range correlations were recognized: Between the proton signal at 3.33 ppm (H-2′) and the carbon at 75.65 (C-3′); the proton at 3.34 ppm (H-4′) with carbons at 76.26 (C-5′) and 75.65 (C-3′); the protons at 3.98-3.78 (H-6′) with carbon resonating at 76.26 (C-5′). The latter correlation is particularly crucial for the assignation of the C-5′ position because H-6′ cannot correlate with C-3′. The complete assignments of resonances are reported in Table 1.

Chlorogenic acid (8) has been isolated for the first time from *S. alopecuros* subsp *divulsa*, although it has been already found in several *Stachys* species as *S. tymphaea* [8], *S. lanata* [30], *S. officinalis* [31], *S. recta* [32], *S. byzantina*, *S. iberica* subsp. *iberica* var. *densipilosa* [33], and *S. glutinosa* [34]. In most of these species, the chlorogenic acid (8) resulted to be the main polyphenolic component occurring in the polar fraction just like in this case [Figure 2].



Scheme 1: Chromatographic steps on 60% ethonolic fraction and elution order of components

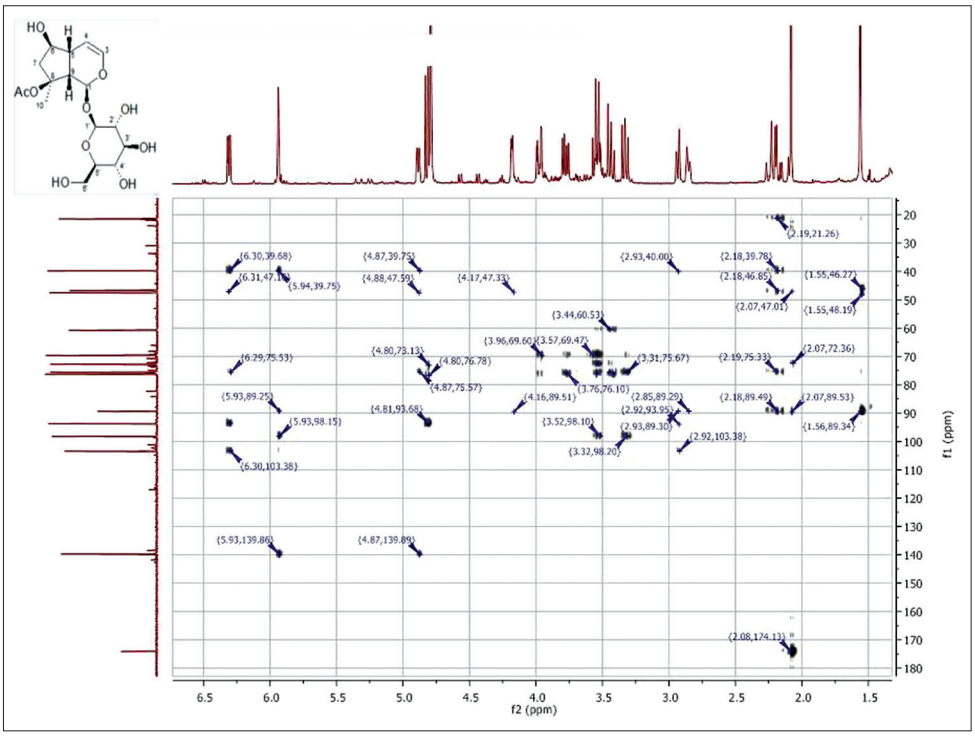


Figure 6: Heteronuclear multiple bond correlation spectrum of ajugoside (1)

Furthermore, β-arbutin (9) was a metabolite identified for the first time in this species. It proved to be a quite rare compound in the *Stachys* genus since its presence has been signaled only in *S. germanica* subsp. *salviifolia* [7]. The presence of verbascoside (10), a quite common phenyletanoid glycoside (PhGs) in Lamiaceae, has been confirmed, while stachysoside A (11), also known with the name lavandulifolioside, resulted to be as a new constituent of *S. alopecuros* subsp. *divulsa*. PhGs are compounds with a chemosystematic relevance in Asterids since their cooccurrence with iridoid glycosides, as in the studied case, has been largely confirmed [35]. The presence of 11 was noteworthy since it is not an ubiquitous compound, especially in the *Stachys* genus where it has been recognized only in a few species such as *S. lavandulifolia* [36], *S. sieboldii* [15], and *S. tymphaea* [8]. This compound resulted to be a constituent of other systematically related genera such as *Leonurus* [37,38], *Lagochilus* [39], and *Sideritis* [40].

Bioactivities of Isolated Compounds

The isolated compounds are responsible for several interesting activities. In particular, iridoids are known to possess anti-inflammatory properties. Harpagide (3) and its derivatives are constituents of *Harpagophytum procumbens* D.C. (Pedaliaceae), a medicinal plant native to Namibia and commonly known as Devil's Claw. This plant has been widely studied for its anti-inflammatory and analgesic effects which were demonstrated also in *in vivo* models, principally in rats [41-43]. The target of harpagide and its derivatives is represented by the inhibition of COX-2, an enzyme deputed to the production of the proinflammatory mediators prostaglandins and leukotrienes. Recent docking studies have demonstrated that harpagide is a selective inhibitor of COX-2, binding its active site with a strong interaction and being stabilized by 10 hydrogen bonds [44]. This confirms, on a molecular base, the observed anti-inflammatory and analgesic actions exerted by plants containing harpagide derivatives. Beside the anti-inflammatory action, harpagide (3) results a modulator of Tumor necrosis factor α (TNF-α) secretion and induces the expression of several proteins involved in cell migrations. It shows an immunomodulatory effect facilitating the leukocyte migration in the inflamed tissues [45]. The latter is a very important role because the inflammatory process is not only responsible of pain and loss of function but it is also involved in the onset and progression of degenerative diseases, including cardiovascular disorders and cancer. In a similar way, 8-*O*-acetyl harpagide (3) also showed a dose-related inhibition of leukocyte adhesion and transmigration in a system based on human umbilical vein endothelial cells, and human monocytic leukemia cell line THP-1 previously activated with TNF-α [46].

It was also studied, the protective effect of harpagide (3) against bone loss in ovariectomized mouse model. It resulted that this iridoid stimulates the differentiation and maturation processes of osteoblasts and this conducted to an overall improved bone properties with the recovery of mineral density. The reduced levels of biochemical markers of bone loss in the serum of treated mice (alkaline phosphatase, osteocalcin, and C-terminal telopeptide) seems to be the major mode of action of harpagide (3) [47], thus demonstrating the potential role of this iridoid also in the prevention and treatment of age-dependent bone osteoporosis diseases.

From recent studies on iridoid activities, 8-*O*-acetylharpagide (2) and ajugoside (1) resulted to be useful compounds in the treatment of diabetes mellitus since they were able to stimulate the cellular glucose intake and lower the glucose blood level [38]. Another target of iridoids for the antidiabetic activity is represented by the inhibitory action, verified by docking studies, on glycogen phosphorylase-a [48]. In fact, several plant species rich in iridoids have been largely used in traditional medicine for the treatment of diabetes, e.g., *Scrophularia deserti* Del. [49] and *Leonurus sibiricus* L. [38].

The phenolic fraction of *S. alopecuros* subsp. *divulsa* was mainly composed by chlorogenic acid (8), which is also endowed with interesting activities [34]. This caffeoylquinic acid is a strong antioxidant [50], a good wound healer if topically applied on excisions [51] and has also antiviral action toward hepatitis B virus [52]. Furthermore, it shows antiproliferative action [34], inhibits carcinogenesis, strongly reduces the risk of cardiovascular diseases and significantly improves the metabolism of glucose. The latter is the main reason why it has recently drawn the attention from nutritionists for its high antidiabetic and anti-obesity properties [53]. The antioxidant and enzyme inhibitory activities in respect to acetylcholineserase, butrylcholinesterase, and α-amylase [33] may have a protective role in neurologic diseases such as Alzheimer's disease, and metabolic disorders as diabetes mellitus.

The other phenolic constituents also have interesting biological activities. In fact, β-arbutin (9) possesses a strong antibacterial activity and is traditionally used for urinary tract infections [54]. It is also a tyrosinase inhibitor and is employed as a skin whitener because it inhibits the melanin synthesis [55].

The phenylethanoid glycoside verbascoside (10) shows an antibacterial action especially toward *S. aureus* [56] and anti-inflammatory activity [57]. More recently, in an *in vivo* study, it resulted to be an inhibitor of xanthine oxidase which reduced uric acid concentration in rat serum. These results may suggest its potential use to treat hyperuricemy [58].

For what concerns the activity of stachysoside A (11), in literature there are mainly studies conducted on extracts obtained from species containing this compound. In this context, it is noteworthy the neuroprotective effects observed in *S. sieboldii* tuber extract which resulted to improve the memory dysfunction associated with vascular dementia or Alzheimer's disease in induced-ischaemia in mice [59]. The presence of antioxidant components resulted to have a primary role in neuroprotection and PhGs are well-known antioxidants. Species belonging to *Leonurus* genus, all containing (11) together with iridoids, have been largely used in the traditional Mongolian medicine for the treatment of diabetes mellitus [38] and in the treatment of neurological disorders such as anxiety, depression, and nervousness [37].

CONCLUSIONS

This phytochemical study on the endemic *S. alopecuros* subsp. *divulsa* shed light on three additional iridoid glucosides together with chlorogenic acid (8), β-arbutin (9), verbascoside (10), and stachysoside A (11) in the phenolic fraction. The presence of verbascoside has been already reported, while the other phenolics were identified for the first time in the studied species. The monoterpene glycoside pattern owned by *S. alopecuros* subsp. *divulsa* has been disclosed with respect to literature data, showing the presence of ajugoside (1), reptoside (6), and 6-*O*-acetyl-ajugol (7). All these compounds have chemosystematic relevance. In particular, 1 and 6 may represent a metabolic connection between Lamioideae and Ajugoideae subfamilies while 7 is a quite uncommon compound in the Lamiaceae family since it has been found only in *L. persicus* so far. We also took advantage of this occasion to report a complete NMR assignment for compound 1. From a therapeutic perspective, the isolated compounds are known for their antioxidant, antiproliferative, anti-inflammatory, analgesic, immunomodulatory, neuroprotective, and antidiabetic actions. Their occurrence in the studied species provides evidence of a possible natural source of bioactive compounds for extractive purposes but also of its potential as a natural remedy of medical importance.

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Original Research

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In vitro screening for protein tyrosine phosphatase 1B and dipeptidyl peptidase IV inhibitors from selected Nigerian medicinal plants

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ABSTRACT

Background/Aim: Protein tyrosine phosphatase 1B (PTP 1B) and dipeptidyl peptidase IV (DPP IV) have been identified as one of the drug targets for the treatment of Type-2 diabetes. This study was designed to screen for PTP 1B and DPP-IV inhibitors from some Nigerian medicinal plants. **Materials and Methods:** PTP 1B and DPP-IV drug discovery kits from Enzo Life Sciences were used to investigate *in vitro* inhibitory effect of crude methanolic extract of 10 plants; *Mangifera indica, Moringa oleifera, Acacia nilotica, Arachis hypogaea, Senna nigricans*, *Azadirachta indica, Calotropis procera, Leptadenia hastata, Ziziphus mauritiana,* and *Solanum incanum.* **Results:** The results indicated PTP IB inhibition by *S. nigricans* (68.2 ± 2.29%), *A. indica* (67.4 ± 2.80%), *A. hypogaea* (57.2 ± 2.50%), *A. nilotica* (55.1 ± 2.19%), and *M. oleifera* (41.2 ± 1.87%) were significantly ($P < 0.05$) higher as compared with standard inhibitor, sumarin while that of *L. hastata* (18.1 ± 2.00%) was significantly lower as compared with sumarin. The PTB 1B inhibition by *M. indica* (31.5 ± 1.90%) was not significantly ($P > 0.05$) different from that of sumarin. The DPP-IV inhibition by *S. incanum* (68.1 ± 2.71%) was significantly higher as compared with a known inhibitor, P32/98. *S. nigrican* (57.0±1.91%), *Z. mauritiana* (56.6±2.01%), *A. hypogaea* (51.0±1.30%), *M. indica* (44.6 ± 2.40%), *C. procera* (36.2 ± 2.00%), *A. nilotica* (35.4 ± 2.10%), and *A. indica* (33.6 ± 1.50%) show significantly ($P < 0.05$) lower inhibitions toward DPP-IV. **Conclusion:** The work demonstrated that these plant materials could serve as sources of lead compounds in the development of anti-diabetic agent(s) targeting PTP 1B and/or DPP-IV.

KEY WORDS: Dipeptidyl peptidase IV, inhibition, medicinal plants, protein tyrosine phosphatase IB, Type-2 diabetes mellitus

INTRODUCTION

Herbal medicines have gained popularity worldwide due to their natural sources, low-cost, and less toxicity. There are several plant species that have been reported to have antidiabetic effect in diabetic animal models [1-5] and humans [6]. In Nigeria, *Mangifera indica, Moringa oleifera, Acacia nilotica, Arachis hypogaea, Senna nigricans*, *Azadirachta indica, Calotropis procera, Leptadenia hastata, Ziziphus mauritiana,* and *Solanum incanum* are being used traditionally for the treatment of diabetes mellitus, but very little is known about the mechanism of actions of antidiabetic activity of these medicinal plants. However, reports show that a number of bioactive ingredients such as flavonoids [7], alkaloids [8], and saponin [9] have been reported to exert antidiabetic activity. Ojiako *et al.* [10] stated that interplay of these bioactive constituents in medicinal plants could be responsible for the hypoglycemic effect. Extracts of *M. indica* [11], *A. hypogaea* [12,13], *A. nilotica* [14], *A. indica* [15], *L. hastata* [16], *Z. mauritiana* [17], *C. procera* [18], and *M. oleifera* [19] have been reported to possess antidiabetic effects. Recently, efforts are being made toward elucidating the mechanism of actions of some of these medicinal plants and their active constituents.

Diabetes mellitus and its associated complications are the major cause of morbidity and mortality worldwide [20]. In particular, Type-2 diabetes mellitus is the most prevalent form of diabetes accounting for more than 80-90% of the total cases of diabetes [21,22]. Type-2 diabetes mellitus is associated with both macrovascular and microvascular complications that may result in tissue or organ damage. It is estimated that about 415 million people have diabetes in the world and more than 14 million cases in sub-Saharan African [23]. It is expected that this figure would be double in 2040. In Nigeria, there were more than 1.56 million cases of diabetes in 2015 and number of deaths related to adult diabetes (20-79 years) were estimated to be 40,815 [23].

Protein tyrosine phosphatases (PTB) are large family of surface proteins that are central modulators of tyrosine phosphorylation-dependent cellular activities [24,25]. Dipeptidyl peptidase IV (DPP IV) is a proteolytic enzyme that specifically deactivates glucagon-like peptide -1 (GLP-1), an incretin hormone which plays a significant role in the regulation of blood glucose level by stimulating the secretion of insulin, increasing β-cell mass and inhibit the secretion of glucagon [26]. PTP 1B and DPP-IV have been recognized as the best drug target for treatment of Type-2 diabetes mellitus [27,28]. Inhibition of PTP 1B increased the rate of phosphorylation of the insulin receptor and its substrate thereby promoting glucose transporters for the uptake of glucose by insulin sensitive cells while DPP-IV inhibitors maintain the level of active GLP-1 [26,28]. Therefore, medicinal products which contain essentially vast bioactive diversity may serve as potential sources of novel inhibitor(s) of PTP 1B and DPP-IV for the treatment of Type-2 diabetes mellitus. The study was designed to screen medicinal plants with PTP IB and DPP-IV inhibitory activities.

MATERIALS AND METHODS

Chemicals and Reagents

PTP IB and DPP-IV drug discovery assay kits used were products of Enzo® Life Sciences and other chemicals and reagent used were of analytical grade.

Plant Materials

A total of 10 plant materials were screened. These included plant extracts studied in our laboratory with *in vivo* hypoglycemic activities and other plant materials that are used by traditional medical practitioners in Northwest Nigeria for the management of diabetes mellitus. Information about antidiabetic plants was sourced by oral interview of traditional medical practitioners, diabetic patients using some of these plants and the general public. In this respect, the following plants were used (Table 1).

The plant materials were collected from farms around Sokoto, Katsina and Kwara States of Nigeria. The plant materials were identified and authenticated by a Taxonomist, Dr. Umar Abdullahi, from Botany Unit, Department of Biological Sciences, Usmanu Danfodiyo University, Sokoto Nigeria. Voucher specimens were deposited at the herbarium of the same institution.

Sample Preparation

The samples were shade dried and ground to powder using laboratory pestle and mortar, except groundnut seeds. The dried, ground powdered materials were stored in paper bags in desiccators until required. Ten g of the powdered samples were extracted in 100 mL of methanol for 72 h at room temperature, in brown cleaned reagent bottles, with intermittent mixing. At the end of the 72 h, the extracts were filtered using Whatman No. 1 filter paper. The filtrates were concentrated using rotary evaporator, and the concentrated filtrates were left to dryness in a drying cabinet and the materials obtained were stored in air tight labeled container at 4°C for further analysis. The groundnut seeds were soaked in distilled water for about 3 h and the extract filtered and the dissolved solutes (% w/v) determined. The extracted materials were reconstituted in DMSO at 10 mg/ml and used for the preliminary screening for PTP 1B and DPP-IV inhibitory activities.

PTP 1B Inhibition Assay

The kit components were thawed on an ice bath with the exception of BIOMOL RED™ that was stored at room temperature. The substrate ('IR5' Insulin receptor β, residues 1142-1153, Py1146) was reconstituted to a concentration of 1.5 mM by assay buffer and distilled H_2O. The assay buffer, 100 mM MES, pH 6.0 containing 300 mM NaCl, 2mM EDTA, 2 mM DTT and 0.1% NP-40 was diluted with equal volume of distilled H_2O and maintained on ice. The PTP 1B (human recombinant) was prepared in ×1 cold assay buffer. Stock of 10 mM of suramin (a known inhibitor) was prepared in assay buffer. The assay mixture was prepared in 96 well plate which contains 10 μg per 100 μL assay mixture of the sample. The plate reader was read at 620 nm, and all the assay protocol was done in accordance with manufacturer's instructions.

DPP IV Inhibition Assay

The crude extracts were screened for DPP-IV inhibition at 100 μg/mL in a total volume of 100 μL using DPP-IV drug discovery assay kits. The inhibitor (P32/98) was diluted in the assay 1 in 10 buffer (50 mM Tris, pH 7.5). The substrate (H-Gly-Pro-pNA) and DPP IV (BML-SE434-9090) were diluted in 1 in 50 μL of the assay buffer. The plant samples were reconstituted in 50 mM Tris buffer, pH 7.5, to give 1 μg/μL. The assay mixture was prepared in 96 well plates which contains 10 μg per 100 μL assay mixture of the sample. The assay mixture consists of 15 μL of DPP IV (17.3 μU/μl) and 50 μL of the substrate and was made up to 100 μL with the assay buffer while P32/98 was used in the place of extracts as a control. The blank was prepared using the substrate and the buffer only. The plate was read continuously at 405 nm, in a microplate reader at 1 min interval for 10 min. The percentage inhibition of the two enzymes by test extracts was calculated based on the activity in control well as 100% from three independent replicates.

Table 1: Medicinal plants screened

Botanical Name	Part used	Voucher number
Mangifera indica	Leaf	UDUS/VS/2011/30
Azadirachta indica	Leaf	UDUS/VS/2011/34
Moringa oleifera	Leaf	UDUS/VS/2011/31
Acacia nilotica	Seed	UDUS/VS/2011/32
Calotropis procera	Leaf	UDUS/VS/2011/28
Leptadenia hastata	Leaf	UDUS/VS/2011/35
Ziziphus mauritiana	Leaf	UDUS/VS/2011/36
Solanum incanum	Fruit	UDUS/VS/2011/22
Senna nigricans	Leaf	UDUS/VS/2011/33
Arachis hypogaea	Whole seed	UDUS/VS/2011/24

Data Analysis

The values are expressed as mean percentage inhibition ± standard deviation. The mean percentage inhibition was analyzed using one-way ANOVA with SPSS (Version 17.0), and $P < 0.05$ was considered statistically significant.

RESULTS

The results of the percentage inhibition of PTP 1B and DPP-IV of the crude methanol extract of medicinal plants used in the Northwest Nigeria are presented in Tables 2 and 3, respectively. The result shows that *S. nigricans* and *A. indica* show the highest PTP 1B inhibition of 68.2 ± 2.29% and 67.4 ± 2.80%, respectively, followed by *A. hypogaea* (57.2 ± 2.58%), *A. nilotica* (55.1 ± 2.19%), *M. oleifera* (41.2 ± 1.87%) which were significantly ($P < 0.05$) higher as compared with sumarin (30.1 2.00%). The PTP IB inhibition by *M. indica* (31.5 ± 1.90%) was not significantly ($P > 0.05$) different as compared with the standard inhibitor, sumarin while *L. hastata* with the least inhibition of 18.1 ± 2.00%. *C. procera, S. incanum,* and *Z. mauritiana* show no inhibition against PTP 1B activity which could serve as activators.

The results for DPP-IV inhibition indicated that *S. incanum* (68.1 ± 2.71%) was significantly ($P < 0.05$) higher as compared with a known inhibitor, P32/98 (63.1 ± 2.70%) while inhibition activity by *S. nigricans* (57.0 ± 1.91 %), *Z. mauritiana* (56.6 ± 2.01%) *Arachis hypogaea* (51.0 ± 1.30), *M. indica* (44.6 ± 2.40), *C. procera* (36.2 ± 2.00), *A. nilotica* (35.4 ± 2.10), and *A. indica* (33.6 ± 1.50%) were significantly ($P < 0.05$) lower as compared with P32/98. There was no inhibition of DPP-IV activity by *L. hastata* and *M. oleifera* which suggest the plants could act as activators of the enzyme.

Table 2: Percentage inhibition of crude methanol extract of different medicinal plants against PTP 1B

Plant materials	% inhibition
Mangifera indica	31.5±1.90
Azadirachta indica	67.4±2.80*
Calotropis procera	NI
Acacia nilotica	55.1±2.19*
Leptadenia hastate	18.1±2.00*
Solanum incanum	NI
Ziziphus mauritiana	NI
Senna nigricans	68.2±2.29*
Moringa oleifera	41.2±1.87*
Arachis hypogaea	57.2±2.58*
Sumarin	30.12±2.00

Data are expressed as Mean±SD, n=3 replicate, NI- no inhibition,*$p<0.05$ when compared with sumarin, standard inhibitor PTP 1B: Protein tyrosine phosphatase 1B

Table 3: Percentage inhibition of crude methanol extract of different medicinal plants against DPP IV

Plant materials	% inhibition
Mangifera indica	44.6±2.40*
Azadirachta indica	33.6±1.50*
Calotropis procera	36.2±2.00*
Acacia nilotica	35.4±2.10*
Leptadenia hastate	NI
Solanum incanum	68.1±2.71*
Ziziphus mauritiana	56.6±2.01*
Senna nigricans	57.0±1.91*
Moringa oleifera	NI
Arachis hypogaea	51.0±1.30*
P32/98	63.1±2.70

Data are expressed as Mean±SD, n=3 replicate, NI- no inhibition, *$p<0.05$ when compared with P32/98, standard inhibitor DPP IV: Dipeptidyl peptidase IV

DISCUSSION

The treatment of diabetes mellitus is considered a global challenge and evaluation of plant products with the aim of isolating antidiabetic agents is gaining popularity worldwide due to the presence of several bioactive constituents with minimal side effect. Selective inhibition of PTB 1B and DPP-IV has been suggested as novel therapeutic target for the treatment of Type-2 diabetes mellitus. In this study, inhibitory activities of ten medicinal plants on PTP 1B and DPP-IV were investigated. The result indicated that *S. nigricans*, *A. indica, A. hypogaea*, *A. nilotica*, *M. oleifera, M. indica,* and *L. hastata* possess significant potentials as sources of lead compounds for the development of PTP 1B inhibitors for the management of Type-2 diabetes mellitus. Similarly, *S. incanum, S. nigricans, Z. mauritiana, A. hypogaea, M. indica*, *C. procera, A. nilotica* and *A. indica* were active against DPP IV, which may serve as sources of inhibitors of the enzyme in the treatment of Type-2 diabetes mellitus. Natural inhibitors like berberine, an isoquinoline alkaloid has been reported to possess potent antidiabetic properties via inhibition of PTP 1B [29-31] and DPP-IV [32]. Papaverine, a structural analog of berberine which belongs to member of isoquinoline alkaloids have also been reported to exhibit potent PTP 1B inhibitory activity thereby lowering fasting blood glucose level *in vivo* [33]. Hydroalcoholic extracts of *Terminalia arjuna* and *Commiphora mukul* have been shown to possess significant DPP-IV inhibitory activity [34]. Although hypoglycemic effects of some of these plants screened have been reported, the mechanism of action has not been fully elucidated. It may be interesting to study whether the antidiabetic effect of these plants extracts acts via inhibition of PTP IB and/or DPP IV activities. Therefore, PTP 1B and DPP-IV inhibitory activities of some of these plants observed in this study indicate that they may serve as potent sources of hypoglycemic agent(s) for the treatment of Type-2 diabetes mellitus. Overexpression of PTP 1B is associated with the development of insulin resistance which could lead to Type-2 diabetes mellitus and obesity [35]. Of all the plants studied, *S. nigricans* had shown to be a better inhibitor of PTP 1B while *S. incanum* had a better effect against DPP-IV. The data reported in this study have shown that *S. nigricans, A. nilotica, A. hypogaea, A indica,* and *M. oleifera* are better inhibitors of PTB 1B than a known inhibitor, sumarin.

Furthermore, going by the result obtained it may be interesting to further exploit these natural products to investigate *in vivo* hypoglycemic effect, study the kinetics of the two enzymes and possibly isolate and characterize the bioactive component(s)

responsible for the inhibition. *S. nigricans* which have shown to be a better source of inhibitor of PTP 1B and to some extent DPP-IV inhibitors could be very promising sources of lead compound(s) for the treatment of Type-2 diabetes mellitus.

CONCLUSION

The results of this work indicated that these plants possess either inhibitory activity against PTP 1B and/or DPP-IV. *S. nigricans* possess significant potentials as sources of lead compound(s) in the development of inhibitors for PTP 1B and to some extent DPP-IV than others. It can then be concluded that these plant materials could serve as sources of lead compounds in the development of antidiabetic agent(s) targeting PTP 1B and/or DPP-IV.

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Original Research

Documentation of ethnomedicinal information and antimicrobial validation of *Thespesia populnea* used by Yanadi tribe of Ganugapenta village, Chittoor district, Andhra Pradesh, India

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ABSTRACT

Aim: This study aimed to document the traditional knowledge of medicinal plants and antimicrobial validation of *Thespesia populnea* used by Yanadi tribe of Chittoor district, Andhra Pradesh, India. **Materials and Methods:** The study was mainly focused on documentation of medicinal plants used by Yanadi tribe to treat different diseases with a standard questionnaire. These plants were cross-checked in Dr. Dukes Database and available literature to know the significance of this tribe on medicinal knowledge. Among the documented plants, *T. populnea* was selected for antimicrobial activity with disc diffusion assay. **Results:** Among the documented medicinal plants, herbs were the most utilized plants, followed by leaf part of the plants; paste form of medicinal preparation was the dominant one among the mode of preparations and oral administration was generally followed by this tribe. When checked these plants in Dr. Duke's Phytochemical and Ethnobotanical Database most of the medicinal plants were matched at least one medicinal use and most of them were correlated with existing literature. In antimicrobial activity, the microbial pathogens *Klebsiella pneumonia* among bacteria and *Rhizopus arrhizus* among fungi were most susceptible to methanol extract of *T. populnea*. **Conclusion:** From this study, we conclude that the preparation and dosage of the medicines by Yanadi tribe of this area is unique and the correlation of medicinal data with Duke's Database and existing literature reveals high medicinal significance of claimed data of this tribe and potential inhibitory activity of *T. populnea* could be studied further to isolate effective antimicrobial agents.

KEY WORDS: Antimicrobial activity, ethnomedicinal studies, Ganugapenta, medicinal plants, Yanadi tribe

INTRODUCTION

Since antiquated times people utilized plants for their daily needs, they practice the plant-based medicine to cure different ailments. These concerns are now asserted by modern civilization as "Ethnobotany" [1] defines the study of interaction between plants and people. The ancient people like tribes utilized plants for food, fodder, dyes, fibers and medicinal purposes collected

from forests without any destructive way, they gave up holy respect to the plants and conserved in the form sacred groves. The documentation of the above-mentioned actions except medicinal knowledge, practiced by various ethnic groups which apply the methods of ethnobotany and medical anthropology are known as ethnomedicine [2]. The earliest ethnomedicinal documentation was started in 1500's by knowing the medicinal value of Indian fever bark (*Cinchona officinalis*) by European people and in India this modern ethnomedicinal investigation was started during 1935 by Kirtikar and Basu [3]. This research was continued by different modern ethnologists in different places with different tribal communities of India still today [4-9]. The World Health Organization proclaims that 80% of the world population still relies on medicinal plants to cure different types of diseases either by the traditional way of treatment or isolation of novel active compounds from medicinal plants [10]. Among the world, India has rich and diverse cultural traditions associated with the use of medicinal plants in different traditional systems of medicine including Ayurveda, Homeopathy, Siddha, and Unani [11]. Therefore, India is a botanical garden of the world and a goldmine of well recorded and traditionally well-practiced knowledge of herbal medicine [12]. The ethnic people residing in different places of India still dependent on medicinal plants to treat various ailments. The people who have a treasure house of knowledge on medicinal plants keep subtly and passed that knowledge only for their generations. The ethnomedicinal investigation helps to ecologists and wildlife managers to ensure and establish the local knowledge, which ultimately helps to pharmacists and pharmacognosists to prepare novel pharmaceutics [13].

The ethnic group "Yanadi" is native to Chittoor district of Andhra Pradesh, India. Some of the researchers documented traditional knowledge of Yanadi tribe inhabited in surrounding villages of Chittoor district [14-20]. There is no proper report on medicinal knowledge of Ganugapenta village so far. Hence, the present investigation is mainly focused on medicinal knowledge of the Yanadi tribe of Ganugapenta village is situated in Chittoor district of Andhra Pradesh, India. This knowledge is transferred orally from generation to generation and dwindling rapidly due to the lack of interest among the younger generations. Therefore, this study is led to document the indigenous knowledge of this ethnic group. Based on the documented information the medicinal plant, *Thespesia populnea* is being used for many ailments by the Yanadi tribe of Ganugapenta village and was chosen to assess growth inhibitory efficacy against different bacterial and fungal pathogens.

MATERIALS AND METHODS

Study Area

The selected village Ganugapenta is situated in Chittoor district of Andhra Pradesh, India with the geographical coordinations such as 13°30′16″ N latitude and 79°8′49″ E longitudes with an elevation of 1425 feet above the mean sea level [Figure 1]. The village gets the highest precipitation from monsoon months with an annual average rainfall of 729 mm. The studied forest area comes under dry deciduous forests of Eastern Ghats covered with timber yielding trees, thorny shrubs and herbaceous flora

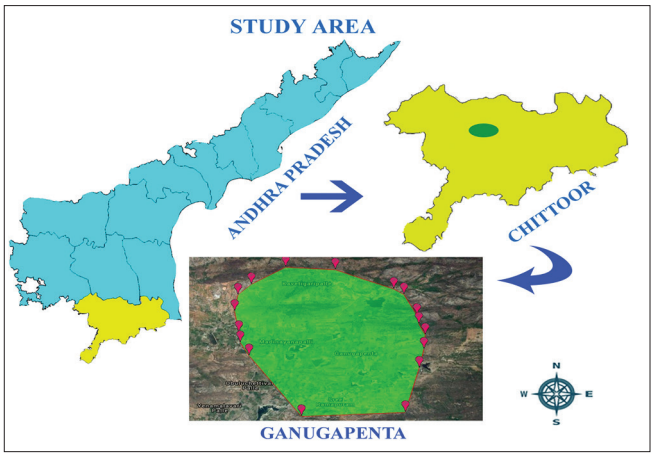


Figure 1: Mapping representation of study area

occupied with an area of 1125.38 hectares and possesses richest knowledge on usage of plants to treat various ailments by simple or in combination with other ingredients.

Data Collection

Frequent field visits were conducted during 2014-2016 for collection of ethnomedicinal information from Yanadi tribe inhabited in the forest areas of Ganugapenta and their surrounding villages. The 80% of data were collected from Ganugapenta village and remaining from surrounding villages like Kavetigaripalle and Yallankivaripalle situated on the north side, Madinayanapalli from west side, Sreeramapuram from the south side, finally east side of Ganugapenta having an enormous range of deciduous forest with high hill tops. Most of the medicinal plants are available on the east side of Ganugapenta which is the major source for collection of medicinal plants. The ethnic group cultivates and earns money from rice and black-eyed pea, cowpea, groundnut, black gram, green gram, red gram and also gets their wages from farm works in the surrounding villages. The tribal villages inhabited by 945 families with 4300 people; among them, 1912 of males, 1952 of females and 436 children are residing in thatched houses, thatched huts and in concrete roofed houses. Most of the ethnomedicinal data were collected from 22 traditional medicine practitioners such as Chengaiah (60Y), Chinnabba (50Y), Jayaramaiah (52Y), Nagaiah (70Y), Sankaraiah (61Y), Narasimhulu (55Y), Siddaiah (70Y), Ramaiah (70Y), Krishnaiah (56Y), Bosanna (52Y), Srinivasulu (58Y), Yerraiah (61Y), Ramakrishnaiah (52Y), Gopal (60Y), Veeraswami (60Y), Venkataswamy (54Y), Venkatesu (53Y), Muniah (64Y), Chinakka (60Y), Nagaratnamma (56Y), Kamalamma (65Y), and Parvatamma (54Y). Among the 22 practitioners 18 members are from men's, 04 members are from women's and their age range from 50 to 70 years. The younger generations are not interested to participate and if as they participate, we personally observed that they do not have sufficient knowledge about on medicinal plants. During the field visits, the plants were collected based on the information provided by the tribe in their own language with the help of standard questionnaire [Figure 2]. The collected medicinal plants were locally identified to know the vernacular name

PROFORMA FOR COLLECTING FIELD DATA ON MEDICINAL PLANTS

I. Tribe: Name of the TMP
Gender: Male/Female Age: below 15/15-40/ 40 above
Experience: below 5/15-10/10 above Locality: Altitude:
Knowledge gained from: Knowledge transferred to:
Occupation:
II. Name of the Diseases:
III. Number of diseases cured:
IV. Data on the plant:
a) Scientific Name: b) Vernacular Name (s) (Specify the dialect):
c) Family: d) Habitat: H/S/C/T
e) In case of Tree Species: Height and Girth and Bark nature f) flower color:
g) Fruit characteristics: h) Small: i) Latex Present: Yes/No
j) Collection and identified: k) Photograph: l) availability:
V. Description of the drug:
a) Time of the collection: Morning/Afternoon/Evening/Night
b) Method of preparation of the drug: (1) Natural form (2) Crushed (3) Juice
(4) Decoction (5) Poultice (6) Soft paste (7) Solid preparation (8) Powder
i) Internal application (Chewing, Ingestion, Inhalation, Ticking)
ii) External application (Lotion, Bath Ointment, Poultice)
c) Ingredients used: single/mixed
d) Mode of administration:
e) Preservation of the drug: Y/N (Duration----------)
f) Plant part used as Medicine: (i) Root (ii) Stem (iii) Leaf
(iv) Flower (v) Fruit (vi) Seed (vii) Root bark (viii) Stem bark
(ix) Latex (x) Gum.
Percentage of the plant parts used for the preparation of drugs for 100 gr
V. Therapeutic indications:
a) Dosage b) Person

Content	Duration			Child	Adult	Old
	day	Month	Year			

c) Diet restrictions: Y/N
d) Patient Treatment With in the Tribal /other than Tribal
e) Side effects: Y/N/Unknown
VI. Reason of the plant for considering as medicine
a). Magico – religious belief b) traditional c) personal experience of healers
d) Strong belief on herbal drug e) Tales f) proverbs
g) Satisfaction level on particular drug: satisfied/ partially satisfied/ not satisfied
h) No. of persons treated:
i) No. of persons cured:
j) Other information:

Figure 2: Questionnaire for collecting information on medicinal plants

from medicinal practitioner and bring back to the laboratory to prepare herbarium specimens. These herbariums were cross-checked with the help of herbaria already deposited in Department of Botany, Sri Venkateswara University, local flora of Chittoor district [21] and Gamble volumes [22]. The documented ethnomedicinal data were cross-checked with Dr. Dukes Phytochemical and Ethnobotanical Database [23] and with the existing literature. Voucher specimens were prepared for the collected medicinal plants and were deposited in the Herbarium wing, Department of Botany, Sri Venkateswara University, Tirupati.

Antimicrobial Validation

Based on the ethnomedicinal information received from the Yanadi tribe, the medicinal plant *T. populnea* is enormously using in the preparations of herbal medicines to treat various diseases. Hence, the plant was selected for antimicrobial validation against different microorganisms. The leaves of *T. populnea* were collected from the Ganugapenta village forest area, cleaned with distilled water and shade dried and grounded with a kitchen blender. The dried 100 g of plant leaf powder was soaked in 500 ml of methanol and moderately shaken for 24 h on a shaker (Tanco HRD3 Shaker, India). The contents were filtered with Whatman No. 1 filter paper and again re-extracted with the same quantity of fresh solvent. The obtained filtrates were combined and dissipated with a rotary evaporator (Royal Scientific 137 B, India) at 38°C to separate the solvent from mixture. The remaining residue was dried to yield a granulate form of nearly 1.45% of powder (dry weight/material dry weight ×100) on lyophilization (Terroni lyophilizer, Brazil). The obtained powder was stored in airtight containers at 4°C for further studies. For antimicrobial activity, five bacterial species (*Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumonia*, *Proteus vulgaris*, and *Pseudomonas aeruginosa*) and five fungal pathogens (*Aspergillus flavus*, *Aspergillus niger*, *Curvularia lunata*, *Fusarium oxysporum*, and *Rhizopus arrhizus)* were procured from the Department of Microbiology, Sri Venkateswara University, Tirupati. The disc diffusion assay was followed for the checking antimicrobial activity [24]. The Whatman No. 1 filter paper (6 mm) was loaded with 20 μl of various concentrations like 25, 50, 100 μg/ml of leaf extract and kept for 10-15 min for drying under sterile conditions. Nutrient agar medium for bacteria and potato dextrose agar medium for fungi were prepared and solidified under sterile conditions.

Triplicates of treated discs were placed on solidified media and were incubated at 37 ± 2°C for 24 h and the results were tabulated.

RESULTS

The study revealed that the Yanadi tribe of Ganugapenta forest area using 54 medicinal plants belonging to 35 families to treat 40 ailments. The scientific name, vernacular name, voucher specimen number, family, part used, life form, mode of preparation, administration, and actual use of medicinal plants were listed in Table 1, and the important photographs of them were given in Figure 3. Among the documented medicinal plants, the Yanadi tribe used more plants from herbs, followed by shrubs, trees, and the stragglers for their preparation of medicine [Figure 4]. Majority of the medicines prepared from leaf part of the plant followed by root, stem bark, whole plant, stem, seed, flower, fruit, rhizome, and root tuber [Figure 5]. Among the different mode of medicinal preparations, paste form of medicine preparation is widely used method for the documented plants followed by juice, powder, decoction, crushed form, fresh form, capsule, fumes, and tonic form [Figure 6]. Oral administration of medicine is a predominant method used by this tribe followed by topical application and inhalation through nostrils [Figure 7]. In this study, we observed that the Yanadi tribe admixtures camphor for the preparation of medicines with *Glossocardia boswellia*, gingelly oil in the case of *Haldina cordifolia* and *Martynia annua*, jaggery in the case of *M. annua* and *Pterolobium hexapetalum*, lime in the case of *Cardiospermum halicacabum*, milk in the case of *Ocimum gratissimum* and *Sarcostemma acidum*, pepper and salt in the case of *Pavetta indica*, turmeric powder in the case of *Anisochilus carnosus*, *Capparis sepiaria*, *C. halicacabum*, *Cassia tora*, *Cleome viscosa*, *Dactyloctenium aegyptium*, *Jasminum angustifolium*, and *Sansevieria roxburghiana* and hot water in the case of *Curculigo orchioides*, *Maba neilgherrensis*, *Rhus mysorensis*, and *Sida spinosa*. Utilization of these admixtures may help to elevate the actual use of medicine. The camphor enhances the relief from rheumatic pains, the utilization of gingelly oil, milk, hot water may helpful to easy intake of medicines and may act as lubricants, utilization of turmeric powder and lime may enhance the actual life of medicine and their activity. Pepper and salt may give a taste to the medicine prepared from bitter parts of the plant.

The medicinal uses of the documented plants were cross-checked with Dr. Dukes Phytochemical and Ethnobotanical Database. Among the 54 medicinal plants documented from the Yanadi tribe, 20 medicinal plants were matched with this database for at least one use and the remaining most of the plants were correlated with recent scientific literature. Among the documented 54 medicinal plants, the plant *T. populnea* was utilized mostly by Yanadi tribe of Ganugapenta forest area to cure various ailments such as cuts, skin diseases, inflammations, stomachache, dysentery, lesions on the face and wounds. Notwithstanding these, the Yanadi tribe inhabited in different parts of Andhra Pradesh like, Tirumala hills of Chittoor district prepared paste from stem with the help of goat milk to treat dandruff [25], leaf paste to treat arthritis [26], inhabitants of Kailasagirikona prepared eye drops from flowers to soothe watering of eyes [27], the leaf paste was utilized as analgesic and antiseptic medicine by Sriharikota Island inhabitants [28]. Rather than this Yanadi tribe, the other tribes like Kanni tribes of Tamil Nadu state prepared juice from fruit to treat skin diseases, fistula, inflamed joints and insect bites [29], decoction from stem bark and leaves were used to treat leprosy [30]. Irula tribe of the same state to a prepare paste from leaves to treat skin diseases [31], juice from stem bark to treat snake bite [32], crushed form of fruit to treat insect bites [33]. The Hill

Table 1: Documentation of ethnomedicinal information from Yanadi tribe of Ganugapenta forest area

Scientific, vernacular name and voucher specimen No.	Family	Part used and life from	Mode of preparation, administration and medicinal use
Acacia chundra Rottler. (Sundra) NP 206	*Mimosaceae*	Stem bark (T)	Oral administration of stem bark decoction twice a day up to 3 days for the treatment of worm infection
Allmania nodiflora L. (Errabadhiaku) NP 234	*Amaranthaceae*	Leaf (H)	Paste form of plant leaves with a pinch of jaggery given orally for the treatment of worm infection
Andrographis serpyllifolia Rottl. (Pamu nelavemu) NP 207	*Acanthaceae*	Root (H)	A spoon of root decoction administered orally twice a day for the treatment of snakebite
Anisochilus carnosus (L.f.) (Sarugudu) NP 209	*Lamiaceae*	Leaf (H)	External application of leaf paste prepared with turmeric powder for the treatment of sores and ring worms
Aristolochia bracteata Retz. (Tella eswari) NP 230	*Aristolochiaceae*	Leaf (Cl)	External application of leaf paste for 3-4 weeks for the treatment of leprosy
Aristolochia indica L. (Nalla eswari) NP 235	*Aristolochiaceae*	Root (Cl)	A spoon of root decoction administered orally twice a day for 3 days for the treatment of snakebites
Canthium dicoccum (Gaertn). (Nalla balasa) NP 233	*Rubiaceae*	Stem bark (T)	Oral administration of stem bark decoction twice a day for the treatment of fever
Capparis sepiaria L. (Nelaupili) NP 236	*Capparidaceae*	Leaf (St)	External application of leaf paste with the admixture of turmeric powder for the treatment of skin diseases
Caralluma lasiantha (Wt). (Godugu jamudu) NP 239	*Asclepiadaceae*	Stem (H)	2-3 inches fresh form of stem pieces administered orally increases the desire of appetite in the case of lean children
Caralluma umbellata Haw. (Kundena kommulu) NP 257	*Asclepiadaceae*	Stem (H)	2-3 inches of fresh form of stem pieces administered orally once a day for 3 days for the treatment of stomachache and gastric ulcers
Cardiospermum halicacabum L. (Budda teega) NP 265	*Sapindaceae*	Leaf (H)	Crushed form of plant leaves along with lime and turmeric powder applied externally for 7-10 days for the treatment of rheumatic pains
Cassia montana Heyne ex. Roth. (Konda thangedu) NP 289	*Caesalpiniaceae*	Leaf (T)	Crushed form of leaf poultice externally to treat bone fracture
Cassia tora L. (Thatipumokka) NP 250	*Caesalpiniaceae*	Leaf (H)	External application of leaf paste prepared along with turmeric powder once a day for 3 days for the treatment of skin itches and inflammations
Cereus pterogonus Lam. (Bonthajemudu) NP 219	*Begoniaceae*	Stem (Sh)	Oral administration of tonic prepared by mixing the stem along with jaggery given once a day for 7 days for the treatment of cardiac diseases
Cleome felina L.f. (Erra vomintaku) NP 211	*Cleomaceae*	Whole plant (H)	Oral administration of 2-3 ml of whole plant decoction thrice a day for 3 days for the treatment of worm infection
Cleome viscosa L. (Kukka vaminta) NP 231	*Cleomaceae*	Seed (H)	External application of seed paste prepared with the admixture of turmeric powder was used for the treatment of rheumatic pains
Curculigo orchioides Gaertn. (Nelathati) NP 218	*Hypoxidaceae*	Root tuber (H)	Powder form of root tuber given orally with a glass of hot water for the treatment of diabetes and piles
Dactyloctenium aegyptium (L.) Willd. (Nela ragi) NP 222	*Poaceae*	Whole plant (H)	Crushed form of whole plant along with a pinch of turmeric powder is applied externally for the treatment of wounds
Diospyros melanoxylon Roxb. (Beediaku) NP 269	*Sapotaceae*	Stem bark (T)	Poultice of crushed form of stem bark applied externally for the treatment of wounds
Ehretia laevis Roxb. (Pogadi chettu) NP 275	*Cordiaceae*	Stem bark (T)	A spoon of stem bark decoction administered orally twice a day for the treatment of snakebites
Flacourtia indica (Burm.f) Merr. (Pulleraka) NP 294	*Flacourtiaceae*	Stem bark (Sh)	A spoon of decoction prepared from stem bark administered orally for 3 days for the treatment of digestive problems
Glosocardia boswellia (L.f.) DC. (Para palanamu) NP 248	Compositae	Leaf (H)	External application of leaf juice prepared along with camphor for the treatment of rheumatic pains
Grewia carpinifolia Juss. (Jana) NP 225	*Tiliaceae*	Root (T)	External application of root juice for the treatment of boils and burns
Habenaria plantaginea Lindl. (Chukka dumpa) NP 228	*Orchidaceae*	Root tuber (H)	A spoon of root tuber powder administered orally twice a day for 3 days for the treatment of fever
Haldina cordifolia (Roxb.) Ridsd. (Rudraganapa) NP 229	*Rubiaceae*	Stem bark (T)	Oral administration of capsules prepared from stem bark powder with gingelly oil given for 3-7 days for the treatment of jaundice
Heliotropium bracteatum R. Br.(Gutta kondi) NP 215	*Boraginaceae*	Leaf (H)	Oral administration of capsule form of leaves twice a day for 2 days and poultice for scorpion sting
Jasminum angustifolium L. (Garuda malli) NP 224	*Oleaaceae*	Root (Sh)	Poultice of fresh form of root with a pinch of turmeric powder applied externally for the treatment of ring worm infection
Jatropha gossypifolia L. (Yerranepalamu) NP 278	*Euphorbiaceae*	Root (Sh)	External application of root paste for 7-10 days for the treatment of leprosy
Lepidagathis cristata Willd. (Nakka pintuka) NP 290	*Acanthaceae*	Root (H)	External application of paste form of whole plant once a day for 3-4 days for the treatment of skin diseases

(*Contd...*)

Table 1: (***Continued***)

Scientific, vernacular name and voucher specimen No.	Family	Part used and life from	Mode of preparation, administration and medicinal use
Maba neilgherrensis Wt. (Pisinki) NP 292	*Ebanaceae*	Stem bark (T)	A spoon of stem bark powder administered orally with glass of hot water for the treatment of fever
Martynia annua L. (Telikondikaya) NP 293	*Pedaliaceae*	Leaf (H)	Oral licking of leaf paste with mixing of jaggery and gingelly oil for the treatment of throat infection
		Fruit	A spoon of fruit powder administered orally once a day for 3 days for the treatment of scorpion sting
Ocimum gratissimum L. (Ramathulasi) NP 285	*Lamiaceae*	Leaf (Sh)	Whole plant powder given orally with glass of milk for the treatment of cough and fever
		Whole plant	Spoonful of whole plant decoction given orally once a day for 3-4 days for the treatment of worm infestations
Opilia amentacea Roxb. (Pacha papiti) NP 264	Opiliaceae	Root (St)	Spoon of root juice given orally once a day for the treatment of snake bites
Pavetta indica Wt. (Papidi chettu) NP 258	*Rubiaceae*	Leaf (T)	Oral licking of leaf paste prepared with mixing of pepper and salt given once a day for 3 days for the treatment of hepatitis
Phyla nodiflora (L.) Greene (Bokkenaku) NP 241	*Verbenaceae*	Leaf (H)	2 to 5 ml of leaf juice given orally for 6-7 days for the treatment of stomach ulcers; it also acts as diuretic
Phyllanthus reticulatus Poir. (Purugudu) NP 227	*Euphorbiaceae*	Stem bark (Sh)	A spoon of stem bark powder given orally twice a day for 3 days for the treatment of dysentery
		Leaf	A spoon of leaf juice administered orally twice a day up to 2 days for the treatment of snake bite
Phyllanthus virgatus Forst. (Gadhasiri) NP 226	*Euphorbiaceae*	Whole plant (H)	A spoon of whole plant powder administered orally twice a day for 7 days for the treatment of jaundice and continued for diabetes for long time
Polycarpaea corymbosa (L.) (Rajuma) NP 262	*Caryophyllaceae*	Leaf (H)	Oral administration of leaf juice for the treatment of jaundice and inflammations in the foot
Polygala chinensis L. (Nelajanumu) NP 267	*Polygalaceae*	Root (H)	Spoonful of root juice given orally for 3 days for the treatment of fever
Premna latifolia Roxb. (Pedda nelli) NP 270	*Verbenaceae*	Stem bark (T)	Oral administration of stem bark powder twice a day for 3-4 days for the treatment of diarrhea
Pterolobium hexapetalum Roth. (Erracheeki) NP 281	*Caesalpiniaceae*	Leaf (Sh)	Oral licking of leaf paste prepared with mixing of jaggery, given orally once a day for 3 days for the treatment of constipation
		Seed	Spoonful of seed powder given orally once a day for 3-4 days for the treatment of piles
Randia dumetorum Lam. (Manga) NP 261	*Rubiaceae*	Fruit (SH)	External application of paste form of fruit for the treatment of dandruff
Rhus mysorensis G. Don. (Sunnarapu chettu) NP 263	*Anacardiaceae*	Leaf (T)	A spoon of leaf powder administered orally along with a glass of hot water for the treatment of hepatitis and diabetes
Sansevieria roxburghiana Schult. (Nela kithalu) NP 242	*Agavaceae*	Rhizome (H)	External application of rhizome paste with the mixing of turmeric powder for the treatment of skin diseases
		Leaf	Pour 2-3 drops of leaf juice in to ear for 3 days for the treatment of earache
Sarcostemma acidum (Roxb.) Viogt (Pullakada) NP 246	*Asclepiadaceae*	Whole plant (St)	Oral administration of spoonful of whole plant powder along with a glass of milk for the treatment of edema
Secamone emetica (Retz.) (Vanthula teega) NP 249	*Asclepiadaceae*	Root (Sh)	A spoon of root juice administered orally once a day up to 3 days at the time of menstrual cycle for the treatment of leukorrhea
		Leaf	Leaf paste applied externally once a day for the treatment of headache
Sida spinosa L. (Nagabala) NP 256	*Malvaceae*	Root (Sh)	A spoon of root powder administered orally along with a glass of water for the treatment of diabetes
			Spoonful of root decoction given orally twice a day for 3 days for the treatment of fever
Striga angustifolia (D.Don.) (Ratibadanika) NP 259	*Scrophulariaceae*	Whole plant (H)	Spoon of whole plant juice administered orally once a day for 3 days for the treatment of snake bite
Thespesia populnea L. (Gangaravi) NP 287	*Malvaceae*	Leaf (T)	External application of leaf paste for the treatment of skin diseases until cure and poultice for the treatment of inflammations
			A spoon of leaf juice administered orally once a day for 3 days for the treatment of stomachache and dysentery
		Flower	External application of flower bud paste up to long periods for the treatment of lesions on the face
		Fruit	External application of fruit paste with the mixing of water for the treatment of cuts
		Stem bark	External application of stem bark paste up to heal for the treatment of scars appeared due to wounds on the skin

(***Contd...***)

Table 1: (***Continued***)

Scientific, vernacular name and voucher specimen No.	Family	Part used and life from	Mode of preparation, administration and medicinal use
Toddalia asiatica L. (Mirapa gandra) NP 282	*Rutaceae*	Leaf (Sh)	Pour 2-3 drops of leaf juice through nostrils once a day until cure for asthma Oral administration of leaf decoction twice a day for 2-3 days for the treatment of fever
Tribulus terrestris L. (Palleru) NP 284	*Zygophyllaceae*	Fruit (H)	Oral administration of spoonful of fruit powder once a day for long time for diabetes and anemia
Triumfetta rhomboidea Jacq. (Dhekki) NP 295	*Tiliaceae*	Flower (H)	Inhalation of flower fumes twice a day for 2 days for the treatment of migraine headache
Tylophora indica Burm. f. (Meka meyaniaku) NP 291	*Asclepiadaceae*	Root (Cl)	Spoon of root decoction given orally twice a day for 3 days for the treatment of cough
Ziziphus oenoplia (L.) Mill. (Pariki chettu) NP 260	*Rhamnaceae*	Fruit (T)	Spoon of fruit paste administered orally once a day for 3 days for the treatment of stomachache and acidity

Cl: Climber, H: Herb, Sh: Shrub, St: Straggler, T: Tree, E: External, I: Inhalation, O: Oral

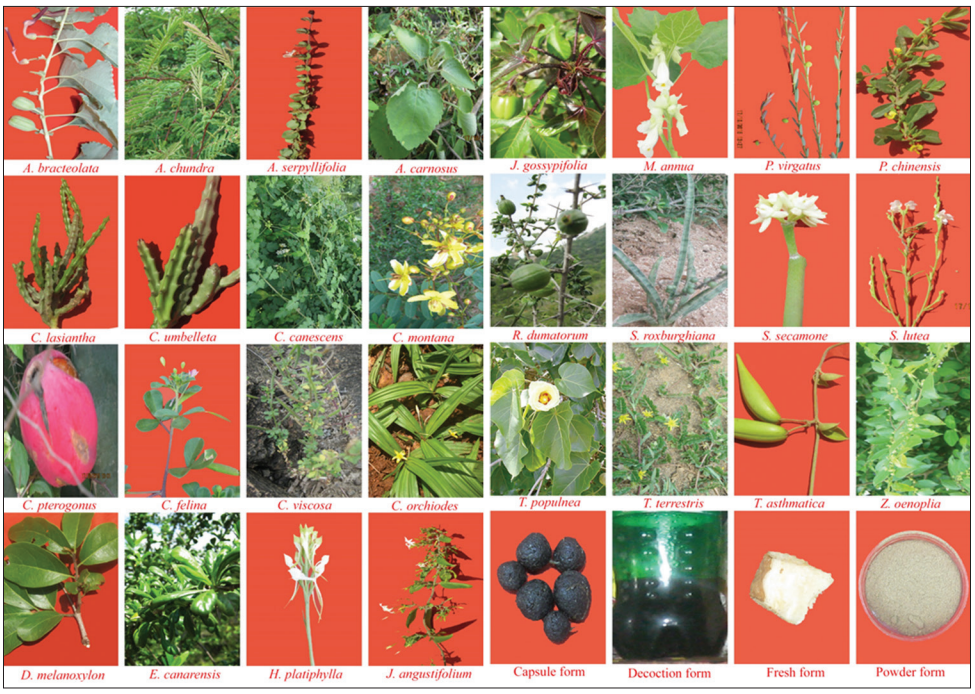


Figure 3: Important medicinal plants documented from Ganugapenta

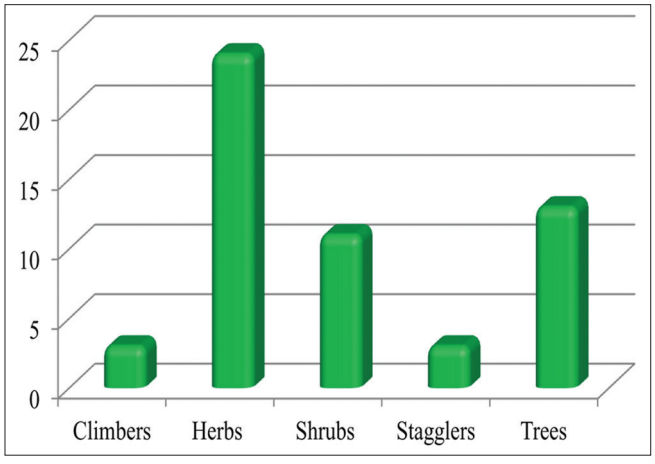


Figure 4: Life form of medicinal plants used by Yanadi tribe of Ganugapenta forest area

Korwa tribe from Chhattisgarh state prepared juice from the leaves to treat neuritis and mad dog bite [34]. Instead of this conventional or traditional knowledge, the work was undergone to exhibit distinctive biological activities of the plant like wound healing [35], memory enhancing [36], hepatoprotective [37], antidiarrheal [38], antioxidant [39], antidiabetic and antihyperlipidemic [40] activities of the plant. Based on the utilization rate by Yanadi tribe, scientific literature on ethnopharmacological data and the prior work on validation of antimicrobial activity of *T. populnea* are vague. Hence, the plant *T. populnea* was chosen and validated its antimicrobial efficacy followed by the method of disc diffusion assay against different microbial pathogens. The antimicrobial activity of *T. populnea* methanol extract showed highest growth inhibitory activity against *E. coli* among bacteria and *F. oxysporum* among fungi [Figure 8 and Table 2; Graph 1].

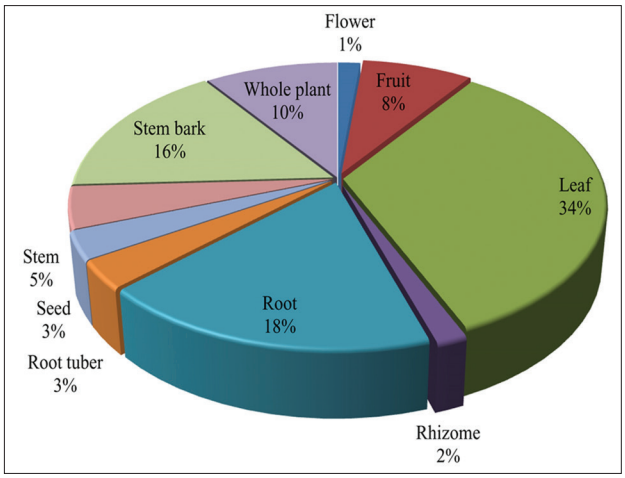


Figure 5: Percentage of different plant parts used by Yanadi tribe for preparation of herbal medicines

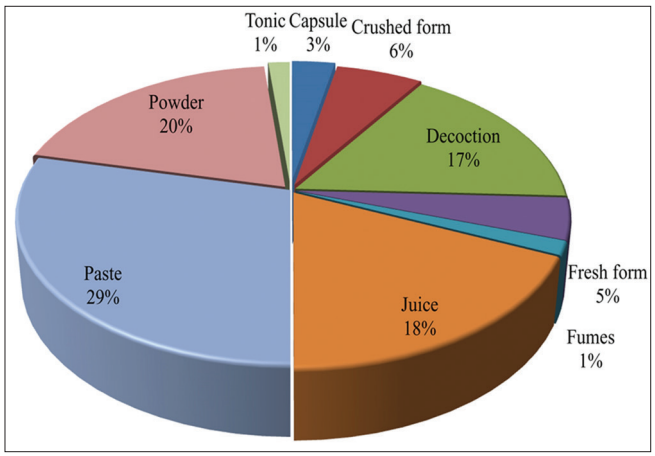


Figure 6: Percentage preparation of different forms of medicines by Yanadi tribe of Ganugapenta forest area

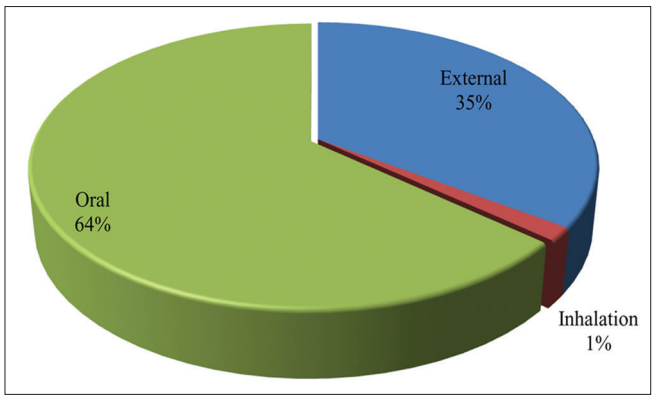


Figure 7: Percentage administration of herbal medicines by Yanadi tribe of Ganugapenta forest area

DISCUSSION

The documented medicinal plants were categorized by diseases wise, the plants such as *Canthium dicoccum*, *Habenaria plantaginea*, *M. neilgherrensis*, *O. gratissimum*, *Polygala chinensis*, *S. spinosa*, and *Toddalia asiatica* recorded as the most utilized plants for the treatment of fever followed by *Andrographis serpyllifolia*, *Aristolochia indica*, *Ehretia laevis*, *Opilia amentacea*, *Phyllanthus reticulatus*, and *Striga angustifolia* for snake bites, *C. sepiaria*, *C. tora*, *Lepidagathis cristata*, *S. roxburghiana* and *T. populnea* for skin diseases, *Allmania nodiflora*, *Cleome felina*, *J. angustifolium* and *O. gratissimum* for worm infections. The remaining plants were utilized for thrice, twice and even for single disease also. Whereas family wise, the *Asclepiadaceae* represents 5 species followed by *Caesalpiniaceae*, *Euphorbiaceae* and *Rubiaceae* with 3 species, *Acanthaceae*, *Lamiaceae*, *Oleaceae*, *Tiliaceae* and *Verbenaceae* with 2 species and the rest of the families represent only 1 species each. More species from *Asclepiadaceae* was used by Yanadi tribes in the Chandragiri reserve forest area. It may be due to the wide distribution of this family and known number of medicinal uses [20]. This tribe selected most of the medicinal plant materials from herbs. Comparative results were accounted from ethnobotanical studies of Japali Hanuman Theertham, a sacred grove of Tirumala hills [41]. The reason could be that the herbs are accessible plenty in the fields and higher distribution within the grasp height. This tribe preferred leaf part of the medicinal plant for their medicine preparation, the common use of leaf in the preparation of remedies could partly due to the relative ease of finding. This observation harmonizes with the ethnomedicinal data documented from Yanadi tribe and local villagers of Veyilingalakona sacred grove [1]. This tribe preferred mostly paste form of the medicine, it may be due to easy intake and rapid action while administration of the medicine. A similar result was reported in medicines prepared by tribal groups of East Godavari district [42]. The oral administration of medicine gives better results when contrasted with topical application of medicines in the case of emergency ailments like scorpion sting and snake bite. The Yanadi tribe of Ganugapenta and the majority of ethnic groups preferred oral administration of medicines, which may be due to rapid healing from an ailment. The Yanadi tribe of Kadapa district also followed similar type of treatment [43].

The documented medicinal plants were cross-checked with Dr. Dukes Phytochemical and Ethnobotanical Database which is only the database available at present. 20 medicinal plants such as *A. indica*, *C. sepiaria*, *C. halicacabum*, *C. tora*, *C. felina*, *C. viscosa*, *C. orchioides*, *D. aegyptium*, *Flacourtia indica*, *J. angustifolium*, *Jatropha gossypifolia*, *L. cristata*, *O. gratissimum*, *Phyla nodiflora*, *Polycarpaea corymbosa*, *P. chinensis*, *S. spinosa*, *T. asiatica*, *Tribulus Terrestris*, and *Ziziphus oenoplia* were matched with this database at least by one medicinal use. Despite the fact that the following medicinal plants were appeared in the database but not correlate with Dukes Database. However, we have gone through the recently published literature for each and every plant documented from the Ganugapenta forest area. The medicinal plants like *A. carnosus* leaves have the ability to mend sores between the foot fingers [44] and the paste form of leaf applied at morning times, once in 2 days to cure ringworm [45], *Aristolochia bracteata*

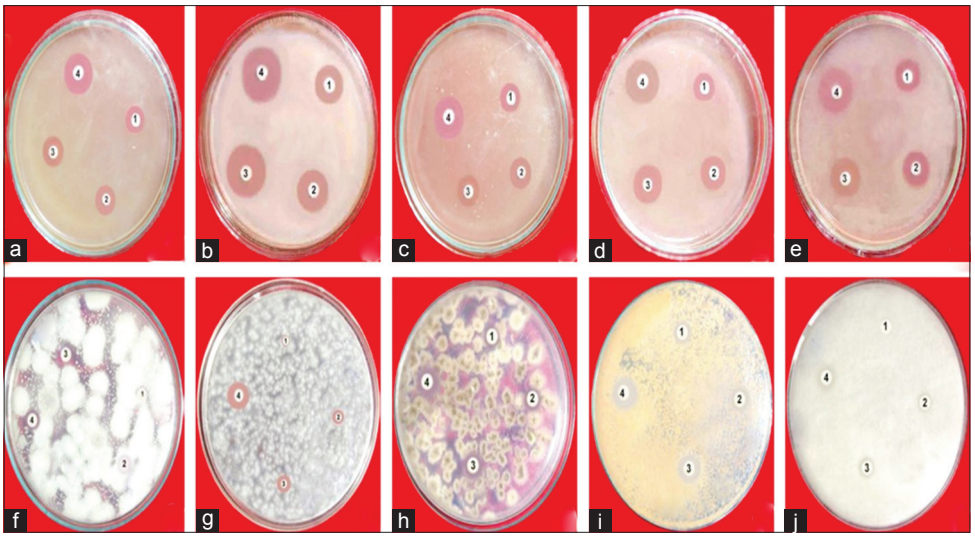
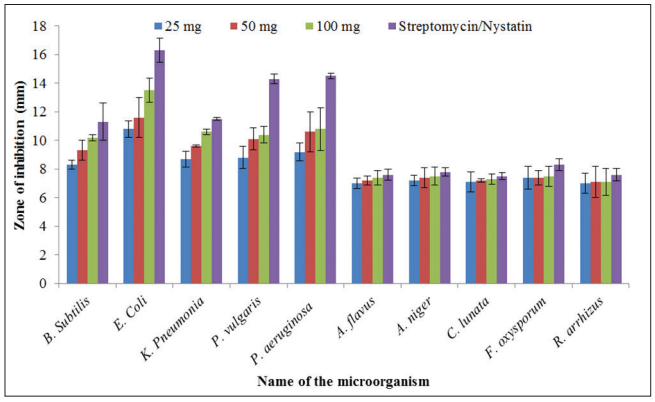


Figure 8: (a-j) Evaluation of growth inhibitory effect of *Thespesia populnea* leaf methanolic extract on different microorganisms



Graph 1: Graphical representation on effect of various concentrations of *Thespesia populnea* methanolic different microorganisms

Table 2: Effect of various concentration of *Thespesia populnea* methanolic leaf extracts

Name of the microorganism	Zone of inhibition (mm)			
	25 mg/ml	50 mg/ml	100 mg/ml	Standard (streptomycin/ nystatin)
Bacillus subtilis	8.3±0.31	9.3±0.70	10.2±0.21	11.3±1.30
Escherichia coli	10.8±0.58	11.6±1.41	13.5±0.84	16.3±0.84
Klebsiella pneumonia	8.7±0.56	9.6±0.07	10.6±0.21	11.5±0.10
Proteus vulgaris	8.8±0.77	10.1±0.77	10.4±0.56	14.3±0.35
Pseudomonas aeruginosa	9.2±0.63	10.6±1.41	10.8±1.48	14.5±0.17
Aspergillus flavus	7.0±0.35	7.2±0.31	7.4±0.49	7.6±0.39
Aspergillus niger	7.2±0.35	7.4±0.70	7.5±0.63	7.8±0.28
Curvularia lunata	7.1±0.70	7.2±0.14	7.3±0.35	7.5±0.24
Fusarium oxysporum	7.4±0.81	7.4±0.49	7.5±0.70	8.3±0.42
Rhizopus arrhizus	7.0±0.70	7.1±1.09	7.1±0.95	7.6±0.42

Values are average of triplicates, ± indicates standard error

leaf was therapeutically familiar in the sake of Tvakroga for the treatment of leprosy [46], *C. dicoccum* was treated for fever by Irula tribes of Nilgiri Biosphere Reserve, India [47], *Diospyros melanoxylon* pounded bark is antiseptic having the capacity to cure wounds and cuts [48], *E. laevis* used as an antidote for snake bites by Koya tribes resided in Warangal District of Telangana, India [49], *M. annua* leaf extract have the capacity to recuperate sore throat [50], *P. reticulatus* leaf paste given orally 2 times per day for the treatment of dysentery by Gingee hills villagers, Villupuram District of Tamil Nadu state [51] and oral administration of juice prepared from equal proportions of *P. reticulatus* leaves and roots from *Borassus flabellifer*, *Tinospora cordifolia* to treat snake bites by Chenchu, Yanadi and Yerukula tribals of Kadapa district, Andhra Pradesh [43], *Randia dumetorum* fruit paste has the ability to cure dandruff and to prevent falling of hairs [52], *S. roxburghiana* leaf paste was prepared with turmeric powder was utilized for the treatment of skin diseases by tribal groups of Nizamabad district, Andhra Pradesh [53] and for earache by Kani tribes of Agasthiyarmalai biosphere reserve, southern Western Ghats, India [54], *Secamone emetica* leaf has the capacity to cure leukorrhea [55] and headache [28], *T. rhomboidea* root is used for the treatment of headache, especially in East Africa [56].

The leaves of *A. nodiflora* given orally for the treatment of stomachache due to worm infection by Thottianaickans community resided in Tiruchirappalli district of Tamil Nadu State [57], *A. serpyllifolia* leaf paste was applied on affected part of the snake bite by local people of Kanjamalai Hills, Salem district of Tamil Nadu [58], *Caralluma umbellata* stem part given orally by the local people of Pachamalai hills of Tiruchirappalli district, Tamil Nadu for the treatment of stomachache [59] and to treat gastric ulcers by Malayali tribes native to Jawadhu hills, Thiruvannamalai district of Tamil Nadu state [60], *Cereus pterogonus* fresh young shoots were used to treat cardiac diseases [61], *G. boswellia* leaves were actually used as a green vegetable and furthermore consumes to cure rheumatic pains by tribal people of Deogarh district of Odisha state, India [62], *H. plantaginea* root tuber acts as an excellent remedy for fever [63], *H. cordifolia* stem bark have the capacity to cure jaundice honed by tribal people of Sitamata wild life sanctuary of Rajasthan, India [64],

O. amentacea root was smoldered with charcoal, pounded with snake teeth and applied to treat snake bite by Kenyan tribals [65], *Phyllanthus virgatus* leaf was given orally for the treatment of jaundice and diabetes by Chenchu and Nakkala tribes of Japali Hanuman Theertham, Chittoor district of Andhra Pradesh state [41], *P. hexapetalum* leaves have the capacity to cure constipation [66], *R. mysorensis* leaves were utilized for the treatment of hepatitis [67] and diabetes [68], *S. emetica* was utilized for the treatment of leukorrhea by Yanadi tribe resided in Sriharikota Island, Andhra Pradesh [28] and finally *S. angustifolia* pulverized form of whole plant was used to treat snake bite by Chenchu and Nakkala tribes of Japali Hanuman Theertham, Chittoor district of Andhra Pradesh State [41]. However the medicinal plants like, *Acacia chundra*, *Caralluma lasiantha*, *Cassia montana*, *Grewia carpinifolia*, *Heliotropium bracteatum* and *Premna latifolia* were does not reflect any appropriate ethnomedicinal values either from Dr. Dukes Phytochemical and Ethnobotanical Database or by any other recent publications. In light of this database and recently published information, we presume that the medicinal knowledge of the Yanadi tribe is highly noteworthy. Coming to the antimicrobial activity of *T. populnea*, it showed higher inhibitory activity on *E. coli* among bacteria and *F. oxysporum* among fungi. The *E. coli* is a Gram-negative bacterium was highly susceptible, whereas the *B. subtilis* is a Gram-positive bacteria showed less susceptibility toward the concentrations prepared from *T. populnea*. The fungal pathogens were less susceptible when compare to bacterial species. It might be due to the Gram-positive bacteria have thick layers of peptidoglycans when compare to Gram-negative bacteria and the fungal cell walls are made up of chitin which is more rigid than peptidoglycan [69]. This is may be the actual reason behind that the *T. populnea* showed maximum growth inhibitory activity against Gram-negative bacteria followed by Gram-positive and fungal pathogens.

As there is no primary health-care center located to nearby village and they are still dependent on traditional medicine system. Most of the medicinal information is possessed by the people of age group between 50 and 70 years. The younger generations are not intrigued to hone their ancestor's knowledge. At the same time, they are attracted and flee to urban cities toward the modern medicine system. In this way, the drastic changes are appeared in way of living will definitely loose the treasure house of medicinal knowledge to the future generations. Hence, we deliberated that this is the right time to document rapidly dwindling traditional knowledge of Yandadi tribe of Ganugapenta village.

CONCLUSION

In the current scenario, the development of resistant pathogens against traditional antibiotics leads to innovation of novel and effective drugs from plant resources. The documented herbal formulations against diseases used by Yanadi tribe will pave the way to investigate efficient alternative antibiotics with high therapeutic potentials to combat the present pathogens. *T. populnea* shows profound inhibitory activity against different microbial pathogens. If isolates therapeutic novel compounds from the plant can be used as a new therapeutic weapon against infectious diseases. We suggested to the research beginners and pharmaceutical companies, these documented medicinal plants will definitely have different biological activities. Because the documented medicinal data of this tribe are mostly correlated with Dr. Dukes Phytochemical and Ethhnobotanical Database and the existing research documentations, the only thing is isolation of novel compounds from the plants are remanent.

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Original Research

Phytochemical screening and study of antioxidant, antimicrobial, antidiabetic, anti-inflammatory and analgesic activities of extracts from stem wood of *Pterocarpus marsupium* Roxburgh

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ABSTRACT

Aims: The main aims of the study were to evaluate the phytochemical constituents and to study the antioxidant, antimicrobial, antidiabetic, anti-inflammatory, and analgesic activities of extracts from stem wood of *Pterocarpus marsupium*. **Materials and Methods:** Ethanol, acetone and isopropyl alcohol (IPA) (1:1) extracts of stem wood of *P. marsupium* were subjected to phytochemical screening and analysis of biological activities from August 2015 to January 2016. The antioxidant assay was carried out using 2, 2-diphenyl-1-picrylhydrazyl scavenging method, antimicrobial activity testing by cup diffusion method, antidiabetic test evaluation by oral glucose tolerance test in mice, anti-inflammatory effect was evaluated by hind paw edema method in mice and analgesic test evaluation by a chemical writhing method in mice. **Results:** The results of the study revealed that *P. marsupium* is a source of various phytoconstituents such as alkaloids, glycosides, saponins, tannins, proteins, carbohydrates, cardiac glycosides, flavonoids, and terpenoids. Both the acetone and IPA extract as well as the ethanol extract of stem wood of *P. marsupium* exhibited a dose-dependent antioxidant activity. Acetone and IPA extract showed antibacterial activity against Gram-positive bacteria, while the ethanolic extract was found to possess antidiabetic activity. The antidiabetic activity of the extract was found to be time and dose-dependent. Similarly, the acetone and IPA extract was found to have anti-inflammatory activity, which was also time and dose-dependent. Furthermore, the ethanolic extract showed analgesic activity, which was dose-dependent. The ethanolic extract was found to be nontoxic. **Conclusions:** Thus, this study laid sufficient background for the further research on extracts from stem wood of *P. marsupium* for identification, subsequent purification and isolation of compounds having antibacterial, antidiabetic, anti-inflammatory, and analgesic activities.

KEY WORDS: Analgesic, antibacterial, antidiabetic, anti-inflammatory, extracts, phytochemicals, *Pterocarpus marsupium*

INTRODUCTION

Medicinal plants are widely used for curing different diseases since ancient time. Among many other curative properties, they have pain relieving, antibacterial, anti-inflammatory, and antidiabetic capabilities [1,2]. According to the World Health Organization, herbal medicine is the major resource for primary health care for people in developing countries [3]. In addition, a large number of modern medicines are either based on or derived from medicinal plants [4].

A medicinal plant is any plant which contains substances those reveal therapeutic effects or which contain substances those can be used as precursors for semi-synthetic drugs [5]. These nonnutrient substances present in plants are known as phytochemicals and serve as protecting agents in the plants from microbial infections or pest infestations [5]. In Nepal, 70-80% of population in the mountain region depends on ethnomedicine for tackling the health problems [6]. Due to the lack of access to modern medicine in rural areas of Nepal, the ethnomedicine has been proved to be an important part of the primary health-care

system of Nepal [7]. A total of 1950 species of medicinal plants are known to be used in Nepal [6]. *Pterocarpus marsupium* is one of the most commonly used medicinal plants. In Nepal, *P. marsupium* Roxburgh is commonly known as Bijayasal and the wooden tumbler prepared from the heartwood of *P. marsupium* is used for drinking water as a traditional remedy for human diseases [8]. Heartwood juice of this plant is known to contain polyphenolic compounds (like flavonoids, diphenylpropane derivatives, and sesquiterpenes), which show strong antioxidant, anti-inflammatory, antidiabetic, antimicrobial, and anticancer activities and is used for the treatment of diabetes, jaundice, ulcer, gastritis, etc. [8]. In the ayurvedic Pharmacopoeia of India, it has been described to be used in the treatment of krmiroga (worm infection), kustha (leprosy), prameha (diabetes), pandu (anemia), and medodosa (obesity) [9]. Further, many previous studies have showed the antioxidant, antimicrobial, antidiabetic, anti-inflammatory, and analgesic activities of different extracts of *P. marsupium* [7,10-12].

In this study, we evaluated the phytochemical constituents of extracts of stem wood of *P. marsupium*, and we determined the antioxidant, antimicrobial, anti-inflammatory, antidiabetic, and analgesic activities of these extracts. In addition, we also studied the toxicity of the extracts.

MATERIALS AND METHODS

Chemicals and Reagents

Water, ferric chloride, acidified alcohol, ammonia, chloroform, acetic acid, Mayer's reagent, Dragendroff's reagent, sulfuric acid, sodium hydroxide, Fehling's solution, Molisch's reagent, hydrochloric acid, zinc dust, magnesium turnings, biuret reagent, Folin–Ciocalteu reagent, sodium carbonate, gallic acid solution, acetone and isopropyl alcohol (IPA), ethanol, methanol, aluminium trichloride, potassium acetate, quercetin solution, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), dimethyl sulfoxide, glucose, glimepiride, diclofenac sodium, etc.

Study Design

An experimental and descriptive study was conducted from August 2015 to January 2016, using the stem wood of *P. marsupium* Roxburgh collected from Kanchanpur, Nepal. Physical evaluation was performed by determination of foreign organic material (FOM), total ash value, acid insoluble ash value, and water soluble ash value [13].

Determination of FOM

The plant material was sorted and shade-dried and then it was placed over the white paper in five parts each weighting 25 g. The FOM was separated and weighted. Then, the percentage of FOM was calculated using the following formula:

$$FOM = [W_2/W_1] \times 100\%$$

W_1 = Weight of sample/plant materials
W_2 = Weight of FOM.

Determination of Total Ash Value

Around 3 g of air-dried material of *P. marsupium* was weighed and was ignited at 550°C until it turned white, showing the absence of carbon. Then, it was weighted after cooling in a desiccator. The total ash value was determined using the following formula:

$$\text{Total ash value} = [(W_2 - W)/W_1] \times 100\%$$

W = Weight of empty crucible
W_1 = Weight of sample taken
W_2 = Weight of crucible.

Determination of Acid Insoluble Ash

Acid insoluble ash value will be calculated as:

$$\text{Acid insoluble ash} = [(W_2 - W)/W_1] \times 100\%$$

W_2 = Weight of crucible and acid insoluble ash
W_1 = Weight of sample taken
W = Weight of empty crucible.

Determination of Water Soluble Ash

Water soluble ash value will be calculated as:

$$\text{Water soluble ash} = [(W_3 - W)/W_1] \times 100\%$$

W_3 = Weight of crucible and acid insoluble ash
W_1 = Weight of sample taken
W = Weight of empty crucible.

Extraction of Phytochemicals

For the extraction of phytochemicals, the stem wood of the *P. marsupium* Roxburgh dried at room temperature was crushed into powder by electric blender and subjected to extraction in Soxhlet apparatus at 55-85°C for 12-24 h using 1:1 acetone and IPA and absolute ethanol. The extracts obtained were then dried using rotavapor drier at 55-85°C, and the solid extracts were preserved in refrigerator for further analysis [7].

Phytochemical Screening

Qualitative screening

The qualitative phytochemical screening of the extracts was performed to identify the main groups of chemical constituents (glycosides, alkaloids, tannins, saponins, terpenoids, carbohydrates, cardiac glycosides, anthraquinones glycosides, flavonoids, and phenols) present in the extracts using the color reactions [14].

Test for tannins

About 0.1 g of the extract boiled in 2 ml of water/dimethyl sulfoxide (DMSO) was filtered and mixed with a few drops of

0.1% of ferric chloride. Then, it was observed for brownish green or a blue black coloration.

Test for alkaloids

10 ml of acidified alcohol was added to 0.1 g of the extract and was boiled and filtered. Then, 0.4 ml of dilute ammonia and 1ml of chloroform was added to 1 ml of filtrate and shaken gently. 2 ml of acetic acid was used to extract chloroform layer. This was then divided into two portions, and Mayer's reagent was added to one portion while Dragendroff's reagent to another. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Dragendroff's reagent) was taken as positive for a test for alkaloids.

Test for glycoside

0.2 g of the test material was extracted with 5 ml of each dilute sulfuric acid and water by boiling/warming on a water bath. Then, the acid extract was filtered and neutralized with 5% solution of sodium hydroxide. Similarly, in the case of water extract the equal amount of water as using of sodium hydroxide in acid extract was added. Fehling's solution A and B were added until the both solutions became alkaline and heated for 2 min using a water bath. If the quantity of red precipitate extracted from acid extract is higher than that extracted from water extract, there may be the presence of glycoside.

Test for carbohydrates (Molisch's test)

The development of purple ring at the interface between the test material and the acid on the addition of Molisch's reagent (α-naphthol dissolved in ethanol) to the extracts followed by addition of a few drops of concentrated sulfuric acid indicates the presence of carbohydrates.

Test for saponins

Development of emulsion on vigorous shaking after addition of 3 drops of olive oil to froth extracted by adding 0.1 g of extract to 1 ml of distilled water indicate the presence of saponins.

Test for cardiac glycosides

Development of a brown ring at the interface after addition of 2 ml of glacial acetic acid that contained one drop of ferric chloride solution followed by further addition of 1 ml of concentrated sulfuric acid to 0.5 mg of extract diluted with 5 ml of water indicate the presence of cardiac glycosides.

Test for flavonoids

- Shinoda test: Development of a few pink scarlet, crimson red or occasionally green to blue color appearance after addition of few magnesium turning followed by dropwise addition of conc. hydrochloric acid was regarded as the presence of flavonoids.
- Alkaline reagent test: Development of intense yellow color on addition of a few drops of sodium hydroxide solution to test solution, which turns to colorless after addition of few drops of dilute acid indicates the presence of flavonoids.
- Zinc hydrochloride test: Development of red color after a few minutes of addition of a mixture of zinc dust and conc. hydrochloric acid to the test solution was taken as positive for flavonoids.

Test for terpenoids (Salkowski's test)

Development of a reddish brown coloration at the interface after addition of 0.4 ml of chloroform followed by concentrated sulfuric acid to 0.1 g of the extract indicates the presence of terpenoids.

Test for proteins

Development of violet color on addition of biuret reagent (2 ml) to the test solution (2 ml), indicates the presence of proteins.

Test for phenol

50 mg of the extract was dissolved in 5 ml of distilled water and a development of a dark green color after addition of a few drops of neutral 5% ferric chloride solution was regarded as positive for phenolic compounds.

Quantitative phytochemical screening

The quantitative phytochemical screening was performed by determining total phenolic content (TPC) and total flavonoid content (TFC) of the extracts [15]. The TPC and TFC of the extracts were expressed as milligrams of gallic acid equivalent per gram (mg GAE/g) of extracts and milligrams quercetin equivalent per gram (mg QE/g) of extracts, respectively.

Total Polyphenolic Content Determination

Folin–Ciocalteu reagent was used for the determination of total polyphenolic content. 0.5 ml of each extract (5 mg/ml), Folin–Ciocalteu reagent (5 ml, 1:10 v/v diluted with distilled water) and aqueous sodium carbonate (4 ml, 1 M) solution were mixed together. The mixture was allowed to stand in the dark for 15 min at room temperature, and the absorbance at 765 nm was measured with the help of ultraviolet (UV-visible) spectrophotometer. Then, the total polyphenolic content was determined in terms of mg GAE/g of dry weight of the extract with the help of a calibration curve prepared with a series of gallic acid standards (10-80 μg/ml).

TFC Determination

0.5 ml of each extract (50 mg/ml) was separately mixed with 1.5 ml methanol and 0.1 ml aluminium trichloride (10%). Then, 0.1 ml of 1 M potassium acetate and 2.8 ml distilled water was added into each test tube. Then, absorbance at 415 nm was measured after it was allowed to stand in the dark for 30 min using a UV-visible spectrophotometer. Finally, a calibration curve was prepared with a series of quercetin standards

(10-50 μg/ml) and the total flavonoid compound concentration was determined in terms of mg QE/g of the extract.

Biological Studies

Antioxidant activity

Antioxidant activity of the extracts was performed using DPPH scavenging method [16].

1 ml of 0.1 mM solution of DPPH in methanol was added to 3 ml of the solution of all the extracts in methanol at different concentrations (5, 20, 40, 60, 80, and 100 μg/ml). The mixtures were shaken and were allowed to stand in dark room for half an hour. Then, the absorbance was measured at 517 nm. Finally, scavenging capability of DPPH radical was determined by the formula:

Scavenging effect = $[(A_0-A_1)/A_0]\times100$

Where, A_0 is the absorbance of the control,

A_1 is the absorbance in the presence of all of the extract samples,

A graph was plotted between scavenging effect and concentration, and a regression equation was obtained to calculate inhibitory concentration 50 (IC_{50}).

Antibacterial activity

The antibacterial activity of the extracts was tested using cup diffusion method on nutrient agar medium [17]. Ofloxacin in the concentration 50 μg/ml was used as positive control and DMSO in the concentration 50 μl served as negative control. 50 μl of the each extract and control were kept into the cups made (using cork borer) on nutrient agar lawn cultured with the bacterial inoculums containing bacteria in the concentration 10^6 CFU/ml. Then, the plates were incubated at 37°C for 24 h and zone of inhibition around the well was measured.

Anti-inflammatory activity

The anti-inflammatory activity of acetone and IPA extract of stem wood of *P. marsupium* was tested using Swiss albino mice [18]. The Swiss albino mice were divided into five groups each consisting of 3 mice.

Group 1: Served as negative control (received distilled water 2 ml/kg/oral)
Group 2: Served as standard (received indomethacin SR 5 mg/kg/oral)
Group 3: Served as test (received acetone and IPA extract 200 mg/kg/oral)
Group 4: Served as test (received acetone and IPA extract 400 mg/kg/oral)
Group 5: Served as positive control (received undiluted egg white in sub-plantar region).

Edema was induced by administration of 0.05 ml of undiluted fresh egg white in the sub-plantar region of Group 2, 3, 4, and 5. The paw volume was measured by marking the point on swell paw by permanent marker at 0, 1, 2, 3, 4, 5, and 6 h after the injection of undiluted fresh egg white using Vernier caliper.

Then, the percentage inhibition of edema is calculated using formula:

Percentage inhibition of edema = [(mean of control-mean of test)/mean of control] × 100

Antidiabetic activity

For testing of antidiabetic activity, oral glucose tolerance test was carried out [19].

In this method 12 h fasted, healthy mice were divided into four groups of 3 animals in each. Blood glucose level (BGL) was measured using Gluco Dr. Auto glucometer.

Group 1: Served as control group (received 10 ml/kg of distilled water)
Group 2: Served as standard group (received standard drug glimepiride (0.43 mg/kg) dissolved in distilled water)
Group 3: Served as test (received ethanol extract of 200 mg/kg dissolved in distilled water)
Group 4: Served as test that received ethanol extract of 400 mg/kg dissolved in distilled water.

Initially, BGL of each mouse was noted. Then, each mouse of Group 1, 2, 3, and 4 was given respective sample intraperitoneally as mentioned above. 30 min after this treatment, glucose (3 g/kg) was given orally through a feeding tube to the each animal of all groups. Blood was drawn from the tail vein of animals at 0, 30, 60, 120, and 180 min.

The percent lowering of BGLs were calculated using formula:

% lowering of BGL = $1-(W_e/W_c)*100$

Where, W_e and W_c represent the blood glucose concentration in glimepiride or ethanolic extract administered mice (Group 2, 3, and 4) and control mice (Group 1), respectively.

Analgesic activity

Acetic acid induced writhing method was used for evaluation of analgesic activity [20].

Mice of either sex with weight between 20 and 25 g were divided into five groups of 3 mice each.

The first group was taken as control. Each of them was given 0.6% acetic acid into the peritoneal cavity in the dose of maximum 1 ml/100 g mouse. Each of them was observed for a number of writhes produced in 20 min.

The second group was taken as standard and each of them was injected with diclofenac sodium (25 mg/kg), 30 min before injection of acetic acid and then observed for number of writhes produced in 20 min.

The remaining three groups were taken as a test, and were given an ethanolic extract of *P. marsupium* in the dose of 250, 125, and 62.5 mg/kg, 30 min before injection of acetic acid and observed for number of writhes produced in 20 min.

The % protection was calculated as follows:

$$\% \text{ protection} = \frac{\text{Number of writhings in control} - \text{Number of writhings in standard / tests}}{\text{Number of writhings in control}} \times 10$$

Acute toxicity test

Acute toxicity of ethanolic extract of *P. marsupium* was tested on Swiss albino mice following Organization for Economic Cooperation and Development guidelines. The animals were subjected to intraperitoneal injection of ethanolic extract of *P. marsupium* in doses 250-1000 mg/kg and kept fasted for overnight providing only water. Then, the animals were given free access to food and water and observed for a period of 48 h. The numbers of death occurred during the period were noted [21].

RESULTS

Physical Evaluation

FOM

The foreign organic value of plant material was found to be 0.92%.

Ash value

The total ash value, acid insoluble ash value, and water soluble ash value were found to be 1.33%, 0.02%, and 0.06%, respectively.

Extractive value

The extractive value of the plant materials with acetone and IPA was 23.70%, while that with ethanol was 28.41%.

Phytochemical Screening

Qualitative phytochemical screening

The qualitative phytochemical screening of stem wood of *P. marsupium* revealed that alkaloids, tannins, terpenoids, carbohydrates, flavonoids, glycosides, cardiac glycosides, protein, and phenol were present in both acetone and IPA extract and ethanol extract, while saponins were present only in ethanol extract.

Quantitative phytochemical screening

TPC of acetone and IPA extract of *P. marsupium* was 38.01 mg GAE/g while that of ethanol extract was 59.42 mg GAE/g. Similarly, TFC of acetone and IPA extract of *P. marsupium* was 82.56 mg QE/gm and that of ethanol extract of *P. marsupium* was 38.56 mg QE/gm.

Antioxidant Activity

Scavenging activity of different extracts of P. marsupium in different concentration is presented in Table 1. The IC_{50} for acetone and IPA extract was lower (36.5 μg/ml) than that of ethanol extract (61.94 μg/ml). This shows that the radical scavenging property is higher in acetone and IPA extract of *P. marsupium*. Correlation of TPC and TFC with antioxidant activity of the extracts was found highly significant ($R^2 = 1.00$).

Antibacterial Activity

The antibacterial activity was measured in terms of the diameter of zone of inhibition. The acetone and IPA extract (50 mg/ml) showed the antibacterial activity against the Gram-positive bacteria, i.e., *Staphylococcus aureus* (zone of inhibition 8 mm) and *Bacillus cereus* (zone of inhibition 8 mm) but did not show antibacterial activity against Gram-negative bacteria, i.e., *Escherichia coli* and *Salmonella Typhi*. Ethanol extract (50 mg/ml) did not show any antibacterial activity. The zone of inhibition for control (50 μg/ml ofloxacin) was 13 mm for *S. aureus*, 12 mm for *B. cereus*, 24 mm for *E. coli*, and 8 mm for *S. Typhi*.

Anti-inflammatory Activity

This study revealed that the acetone and IPA extract showed potential anti-inflammatory activity. The maximum inhibition activity was observed after 5 h, i.e., 52.96% for standard, 45.18% for test (200 mg/kg), and 47.03% for test (400 mg/kg). The extract showed a time and dose-dependent activity [Tables 2 and 3].

Antidiabetic Activity

The ethanolic extract was found to possess antidiabetic activity. The highest blood glucose lowering effect (57.56%) was found in 180 min for standard drug glimepiride as well as for ethanol

Table 1: Antioxidant activity of the acetone and IPA extract and ethanol extract of stem wood of *P. marsupium*

Concentration (μg/ml)	Percentage scavenging activity for DPPH	
	Acetone and IPA extract	Ethanol extract
5	13.70	5.07
20	39.08	15.22
40	58.62	42.89
60	78.93	50.76
80	89.08	67.76
100	90.60	70.05

IPA: Isopropyl alcohol, DPPH: 2, 2-diphenyl-1-picrylhydrazyl

extract in concentrations 200 mg/kg (51.30%) and 400 mg/kg (55.13%). The antidiabetic activity of the extract was found to be time and dose-dependent [Tables 4 and 5].

Analgesic Activity

The ethanolic extract showed potential analgesic activity. At extract dose 250, 125, and 62.5 mg/kg the percent protection effects were 61.11%, 55.00%, and 50.55%, respectively, and the percent protection effect of standard drug diclofenac (25 mg/kg) was found to be 77.96%. The analgesic activity of extract was found to be dose-dependent [Table 6].

Table 2: Percentage inhibition of paw edema in different groups of mice at different time periods

Mouse groups	Percentage inhibition of edema					
	30 min	1 h	2 h	3 h	4 h	5 h
Standard	33.09	39.57	48.76	51.45	52.02	52.96
Test 1 (200 mg/kg)	23.59	33.56	40.98	44.16	45.01	45.18
Test 2 (400 mg/kg)	31.33	34.62	43.46	44.89	46.49	47.03

Table 3: Measurement of paw edema in different groups of mice at different time periods

Mouse groups	Mean diameter of paw in (mm)							
	Normal	0 h	30 min	1 h	2 h	3 h	4 h	5 h
Negative control	1.48	1.48	1.48	1.48	1.48	1.48	1.48	1.48
Positive control	1.56	2.85	2.84	2.83	2.83	2.74	2.71	2.70
Standard	1.54	2.68	1.90	1.71	1.45	1.33	1.30	1.27
Test 1 (200 mg/kg)	1.57	2.48	2.17	1.88	1.67	1.53	1.49	1.48
Test 2 (400 mg/kg)	1.66	2.30	1.95	1.85	1.60	1.51	1.45	1.43

Table 4: BGL in different groups of mice at different time periods

Mouse groups	BGL (mg/dl)					
	Fasting	0 min	30 min	60 min	120 min	180 min
Control	86.00	196.33	194.67	194.67	193.00	191.67
Standard	83.00	188.33	103.00	93.00	87.33	81.33
Test 1 (200 mg/kg)	92.66	171.00	132.66	104.00	95.66	93.33
Test 2 (400 mg/kg)	79.66	182.00	111.66	103.00	93.00	86.00

BGL: Blood glucose level

Table 5: Percentage blood glucose lowering effect in different groups of mice at different time periods

Mouse groups	Percentage blood glucose lowering effect			
	30 min	60 min	120 min	180 min
Standard	47.08	52.22	54.75	57.56
Test 1 (200 mg/kg)	31.85	46.67	50.04	51.30
Test 2 (400 mg/kg)	42.64	47.08	51.81	55.13

Table 6: Number of writhings shown by different groups of mice

Mouse groups	Dose (mg/kg)	Number of writhings
Control	0.0	142
Diclofenac	25.0	39.66
Test 1	250.0	70
Test 2	125.0	81
Test 3	62.5	89

Toxicity

No deaths occurred during the period.

DISCUSSION

Phytochemical screening not only helps to reveal the constituents of the plant extracts and the one that predominates over the others but also is helpful in searching for bioactive agents those can be used in the synthesis of useful drugs [22]. As in our study, the previous study by Maruthupandian and Mohan, on antidiabetic, antihyperlipidemic, and antioxidant activities of ethanol extract of wood and bark reported the presence of the phytochemicals, alkaloids, coumarins, flavonoids, glycosides, terpenoids, tannins, phenols, saponins, and steroids [23]. Due to their vast health benefiting properties, phenolic and flavonoid compounds are considered as the most important classes of phytochemicals [24].

In this study, the acetone and IPA extract and ethanol extract of stem wood of *P. marsupium* showed the dose-dependent antioxidant activity. Similarly, Maruthupandian and Mohan, reported the antioxidant activity of ethanol extract of wood and bark of *P. marsupium* [24]. Phenolic compounds containing free hydrogen are largely responsible for antioxidant activity [25]. Further, the significant antioxidant activity can be due to flavonoids, tannins, polyphenols, and reducing sugars [26]. We reported the IC_{50} value of acetone and IPA extract to be lower than that of ethanolic extract. This showed that the radical scavenging property is higher in acetone and IPA extract of *P. marsupium*. In our study, the IC_{50} value of acetone and IPA extract was found to be 36.50 μg/ml and that of ethanolic extract was found to be 61.94 μg/ml. However, Abirami *et al*. reported the IC_{50} value of methanol, ethyl acetate, and aqueous extracts of *P. marsupium* bark to be 40, 28, and 32μg/ml, respectively [11].

In a previous study by Patil and Gaikwad, the methanolic extract of stem bark of *P. marsupium* showed the highest inhibitory effect against *Pseudomonas aeruginosa* followed by *S. aureus*, *Bacillus subtilis*, *S. Typhi*, *E. coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Micrococcus* sp. [7]. However, in our study, acetone and IPA extract of stem wood of *P. marsupium*, showed antibacterial activity against the Gram-positive bacteria (*S. aureus and B. cereus*) but there was no activity against the Gram-negative bacteria (*E. coli* and *S. Typhi*) and ethanol extract was inactive against all the bacteria tested. This difference may be due to the extracts used from different parts of the plant, different solvents used for extraction and difference in concentration of extract used.

In this study, the acetone and IPA extract of stem wood of *P. marsupium* showed potential anti-inflammatory activity. Accordingly, Mohammed *et al*. reported the anti-inflammatory activities of methanol and aqueous extracts of stem bark of *P. marsupium* in albino rats [27]. Similarly, in our study, ethanol extract exhibited the antidiabetic and analgesic activities. Further, Mishra *et al*. [12] and Maruthupandian and Mohan [23] also noticed the antidiabetic activity of ethanol

extract of *P. marsupium* Roxb., while Sikdar *et al*. reported the analgesic activity of methanol, ethyl acetate, and petroleum ether extracts of *P. marsupium* leaf [28].

This study may be proved to be an important step for the further study for identification, subsequent purification and isolation of compounds with the antioxidant, antibacterial, antidiabetic, anti-inflammatory, and analgesic activities present in the heartwood of *P. marsupium* for clinical use.

CONCLUSIONS

The results of this study revealed that different medically important phytochemicals were present in extracts of stem wood of *P. marsupium*. This research has laid sufficient background for further study for identification, subsequent purification and isolation of compounds with the antioxidant, antibacterial, antidiabetic, anti-inflammatory, and analgesic activities for clinical use. This study has helped in establishing scientific evidences in the rationality of traditional use of plants for curing different human diseases. It has also assisted in exploring the medicinal values and creating a database of medicinal plants available in Nepal.

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Original Research

Effect of Trichilia monadelpha (Meliaceae) extracts on bone histomorphology in complete Freund's adjuvant-induced arthritis

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ABSTRACT

Aim: This study aimed to assess the effect of petroleum ether extract (PEE), ethyl acetate extract (EthE), and ethanol extract (EAE) of *Trichilia monadelpha* stem bark on bone histomorphology in arthritis. **Methods:** Percentage inhibition of edema and arthritic scores in complete Freund's adjuvant-induced (0.1 ml of 5 mg/ml[1] of heat-killed *Mycobacterium tuberculosis* in paraffin oil-injected subplantar into the right hind paw) arthritic Sprague-Dawley rats treated with PEE, EthE, or EAE (10,30, and 100 mg/kg[1], respectively), dexamethasone (0.3-3.0 mg/kg[1]), or methotrexate (0.1-1.0 mg/kg[1]) over a 28-day period were estimated. Rat paws were radiographed and scored. Body weights were taken and paw tissues were harvested for histopathological studies. **Results:** The extracts significantly ($P \leq 0.01$-0.0001) and dose dependently reduced the polyarthritic phase of arthritis. EAE and PEE significantly ($P \leq 0.01$-0.0001) minimized edema spread from acute arthritic phase (days 0-10) to polyarthritic phase (days 10-28). EthE improved which deteriorated body weight in arthritis. All extracts significantly ($P \leq 0.05$-0.01) improved arthritic score; reducing erythema, swelling and joint rigidity, and also significantly ($P \leq 0.05$-0.01) reduced hyperplasia, pannus formation, and exudation of inflammatory cells into synovial spaces. **Conclusion:** The stem bark extracts of *T. monadelpha* reduce bone tissue damage and resorption associated with adjuvant-induced arthritis, hence could be useful in managing arthritis in humans.

KEY WORDS: Arthritic paw radiography, erythema, subchondral erosion, periostitis, osteolysis, *Trichilia monadelpha*

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease of the joints that leads to changes in bone metabolism [1]. The primary target of this inflammatory disease is the synovial membrane of the joints. RA leads to cartilage and bone erosion and joint deformity; manifesting signs and symptoms such as pain, swelling and redness, and fatigue [2]. It can affect other organs of the body resulting in disability and mortality [3]. Globally, RA affects about 1% of adult population [4] and poses a heavy economic burden with disease progression [5].

Non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying antirheumatic drugs (DMARDs), corticosteroids, and cytokine-targeted drugs have been used to manage RA [6,7]. These drugs target specifically at reducing inflammation and relieving pain by blocking cyclooxygenase-mediated prostaglandins release (i.e, the NSAIDs) and control of joint inflammation by suppressing inflammatory-induced bone erosion (glucocorticoids and DMARDs) [8,9]. However, these drugs are associated with undesirable side effects such as gastric ulceration and precipitation of asthma and renal disease and are unaffordable to a lot of individuals [10], hence this search for natural products that are efficacious, but with less

side effects, and affordable to the majority of the populace. *Trichilia monadelpha* (Meliaceae), one such plant, has been used trado-medically to manage chronic inflammatory diseases for decades [11,12]. The stem bark has anti-inflammatory [13] and analgesic effects [13,14]. It improves sperm viability [15] and is relatively non-toxic [13]. Phytochemical analysis conducted revealed the presence of alkaloids, terpenoids, phytosterols, coumarins, tannins, cardiac glycosides, anthraquinones, saponins, flavonoids, and reducing sugars in the petroleum ether, ethyl acetate, and hydroethanolic extracts [13,16].

This study, therefore, assesses the effects of petroleum ether extract (PEE), ethyl acetate extract (EthE), and ethanol extract (EAE) of *T. monadelpha* stem bark in complete Freund's adjuvant (CFA)-induced arthritis in Sprague-Dawley rats.

MATERIALS AND METHODS

Experimental Animals

Male Sprague-Dawley rats (150-200 g) obtained from Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, were kept in the animal house of the Department of Pharmacology, KNUST, Kumasi, Ghana. Animals were housed in aluminum cages and given normal rat diet (Agricare Ltd., Tanoso, Kumasi) and water to consume *ad libitum*. Rats were kept according to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH, Department of Health and Human Services publication no. 85-23, revised 1985). The study was approved by the Institutional Review Board on animal experimentation.

Extract Preparation and Dosing

A voucher specimen of extraction of the stem bark of *T. monadelpha* was kept in the herbarium, Faculty of Pharmacy (No. FP/079/10) as described by Ben *et al*., 2013 [16]. The PEE, EthE, and EAEs were each triturated with Tween-80 (3 drops) in normal saline and administered orally to rats at doses ranging from 10 to 100 mg/kg^{1}.

Drugs and Chemicals used

Heat-killed *Mycobacterium tuberculosis*, mixed strains C, DT, and PN (Ministry of Agriculture, Fisheries and Food, U.K) were used to induce arthritis. Dexamethasone (DEX) (Wuhan Grand, China) and methotrexate (MET) (Dabur Pharma, India) were the reference drugs used for the treatment of arthritis.

CFA-induced Arthritis

Adjuvant arthritis was induced as previously described by Pearson, 1956 [17], with modification according to Woode *et al*., 2008 [18]. In brief, rats were assigned to 17 groups (n=8) and injected with a 0.1 ml suspension of CFA (5 mg/ml^{1} of heat-killed *M. tuberculosis* in paraffin oil) into the right hind paw. Arthritic control group received only subplantar injection of CFA, while non-arthritic control/incomplete Freund's Adjuvant (IFA) group received only subplantar injection of 0.1 ml IFA (sterile paraffin oil). PEE, EthE, or EAE (10, 30, and 100 mg/kg^{1} p.o.), DEX (0.3-3.0 mg/kg^{1} i.p.) or MET (0.1-1.0 mg/kg^{1} i.p.,) were administered to rats in the various groups, respectively, after establishment of arthritis, i.e., on day 10 [Table 1].

Ipsilateral (injected) and contralateral (non-injected) paw volumes were measured using a water displacement plethysmometer (IITC Life Science Equipment, Woodland Hills, USA). This was before subplantar injection of CFA (day 0) and every other day for 28 days after subplantar injection of CFA and IFA. Data obtained for ipsilateral and contralateral paw volumes were individually recorded as percentage of change from their values at day 0 and then averaged for each treatment group. These were presented as the effect of drugs on the time course and the total edema response of adjuvant-induced arthritis for the 28-day period. Total paw volume for each treatment was calculated in arbitrary unit as area under the curve (AUC) to determine the percentage inhibition as per the formula below:

$$\%\,\text{Inhibition of edema} = \left(\frac{\text{AUC}_{\text{control}} - \text{AUC}_{\text{treatment}}}{\text{AUC}_{\text{control}}} \right) \times 100$$

The initial body weight and arthritic score of rats were taken on day 0 after grouping and every other day for 28 days of the experiment, followed by subplantar injection of 0.1 ml CFA. Radiographic images of the paws were taken. Paw tissues were harvested after the 28th day for histopathological assessment.

Body Weight and Arthritic Score

Body weight and arthritic scores were recorded for each hind joint and the tail by a consistent observer blinded to the treatment received by the animals. Scoring was performed on a 0-5 scale [Table 2].

X-ray Radiography

On day 28, the animals were anesthetized by intraperitoneal injection of 20 mg/kg^{1} pentobarbitone. Radiographic images of the hind limbs were taken using a Faxitron X-ray machine (Hewlett-Packard, Buffalo Grove, IL) with a 0.5 mm focal spot,

Table 1: Experimental grouping and treatment in adjuvant-induced arthritis

Group 1	Non-arthritic control/IFA (subplantar injection of 0.1 ml IFA)
Group 2	Arthritic control/CFA (subplantar injection of 0.1 ml of CFA)
Groups 3-5	Treated with DEX (0.3, 1.0, and 3.0 mg/kg^{1} i.p.) from day 9 and administered every other days
Groups 6-8	Treated with MET (0.1, 0.3, 1.0 mg/kg^{1} i.p) from day 9 and administered for every 4 days
Groups 9-17	Treated with extracts (10, 30, and 100 mg/kg^{1} p.o.) from day 9 and administered every day

CFA: Complete Freund's adjuvant

beryllium window, and X OMAT TL (onscreen) film. The focal film distance was 61 cm, and exposures were made over 30 s at 45 kVp and 3 mA. Radiographs were analyzed by a board-certified radiologist who was blinded to the treatment groups. Quantitative scores were generated for radiographic changes in the joints in the following areas: Soft tissue volume, joint space, subchondral erosion, periostitis, osteolysis, subluxation, and degenerative joint changes. The values were based on increasing severity of demineralization [Table 3].

Histopathology

Hind limbs of arthritic rats were removed and fixed in 10% buffered formalin. The limbs were decalcified in 5% formic acid, processed for paraffin embedding, sectioned at 5 μm thickness, and later stained with hematoxylin and eosin for examination under a light microscope. The histopathological change of joints was blindly graded by a pathologist and assigned a score of 0-3 [Table 4].

Statistical Analysis

Statistical analysis of data was done using Sigma Plot version 12.3 (Systat Software Inc. Chicago USA). Significant differences in paw volumes, body weight, arthritic scores, and AUCs for parameters measured were ascertained using 1-way and 2-way analysis of variance and Holm-Sidak's *post hoc* test. Values plotted were mean ± SEM. $P \leq 0.05$ and higher F values ($F \geq 4.0$) were considered statistically significant.

RESULTS

CFA-induced Arthritis

CFA injection into the paws of rats produced a biphasic response observed as paw swelling or edema of the ipsilateral and contralateral paws. The first phase is the acute phase, characterized by unilateral edema of the ipsilateral paws. The subsequent phase, polyarthritic/chronic phase, is characterized by edema of the contralateral paws. All arthritic control animals showed acute inflammatory edema at the ipsilateral (injected paws) paws around days 9-10 followed by subsequent chronic polyarthritic phase which begins around days 10-12. Throughout the 28-day experiment, there was no significant change in the paw volume of the non-inflamed control groups injected with IFA.

Acute-phase Inflammation

PEE, EthE, EAE, DEX, and MET significantly ($P \leq 0.01$-0.0001; $F_{3,28}$= 5.79-60.53) reduced acute-phase inflammation [Figure 1a, c and e; Figure 2a and c]. However, while PEE and EAE ameliorated the edema in the ipsilateral paws by 92.7% and 76.2%, respectively, at 100 mg/kg^{1}, EthE effect was not significant (38.8% at 100 mg/kg^{1}) [Figure 1b, d and f]. DEX and MET significantly ameliorated edema in the ipsilateral paws, i.e., 100% inhibition at 3 mg/kg^{1} and 80.0% inhibition at 1 mg/kg^{1}, respectively [Figure 2b and d].

Table 2: Description and scoring of induced arthritis in Sprague-Dawley rats

Description	Score
• No swelling or erythema	0
• Slight swelling and/or erythema	1
• Low-to-moderate edema	2
• Pronounced edema with limited joint use	3
• Excess edema with joint rigidity	4
• Moderate-to-pronounced edema on tail and/or erythema	5

Table 3: Mode of scoring of radiographs for arthritic paws of Sprague-Dawley rats

Description	Score
No degenerative joint changes	0
Slight soft-tissue volume, joint space, subchondral erosion, periostitis, osteolysis, subluxation, and degenerative joint changes	1
Low-to-moderate soft-tissue volume, joint space, subchondral erosion, periostitis, osteolysis, subluxation, and degenerative joint changes	2
Pronounced soft-tissue volume, joint space, subchondral erosion, periostitis, osteolysis, subluxation, and degenerative joint changes	3
Excess soft-tissue volume, joint space, subchondral erosion, periostitis, osteolysis, subluxation, and degenerative joint changes	4

Table 4: Mode of scoring of photomicrographs of arthritic paws of Sprague-Dawley rats

Description	Score
Absence of synovial hyperplasia, pannus, bone erosion, fibrosis, or presence of inflammatory cells as observed as neutrophils	0
Minimal presence of synovial hyperplasia, pannus, bone erosion, fibrosis, or presence of inflammatory cells as observed as neutrophils	1
Mild/moderate presence of synovial hyperplasia, pannus, bone erosion, fibrosis, or presence of inflammatory cells as observed as neutrophils	2
More intense presence of synovial hyperplasia, pannus, bone erosion, fibrosis, or presence of inflammatory cells as observed as neutrophils	3

Polyarthritic/Chronic-phase Inflammation

PEE, EthE, EAE, DEX, and MET significantly ($P \leq 0.01$-0.0001; $F_{3,28}$= 5.57-57.76) reduced polyarthritic/chronic-phase inflammation [Figure 1a, c and e; Figure 2a and c]. PEE, EAE, DEX, and MET also significantly ($P \leq 0.01$-0.0001; $F_{3,42}$=4.69-10.43) minimized the progression of the inflammation from the acute to the polyarthritic phases, [Figure 1b and f; Figure 2b and d]. PEE, EthE, and EAE ameliorated the edema in the contralateral paws, with inhibitory effects of 98.0, 69.1, and 70.8%, respectively, at 100 mg/kg^{1} [Figure 1b, d and f]. DEX and MET also caused 125.3% inhibition at 3 mg/kg^{1} and 94.7% inhibition at 1 mg/kg^{1}, respectively [Figure 2b and d]. PEE and EAE showed greater potency and efficacy comparable to DEX and MET [Table 5].

Body Weight

The CFA group experienced weight loss with excess swelling, erythema, and joint rigidity in both ipsilateral and contralateral

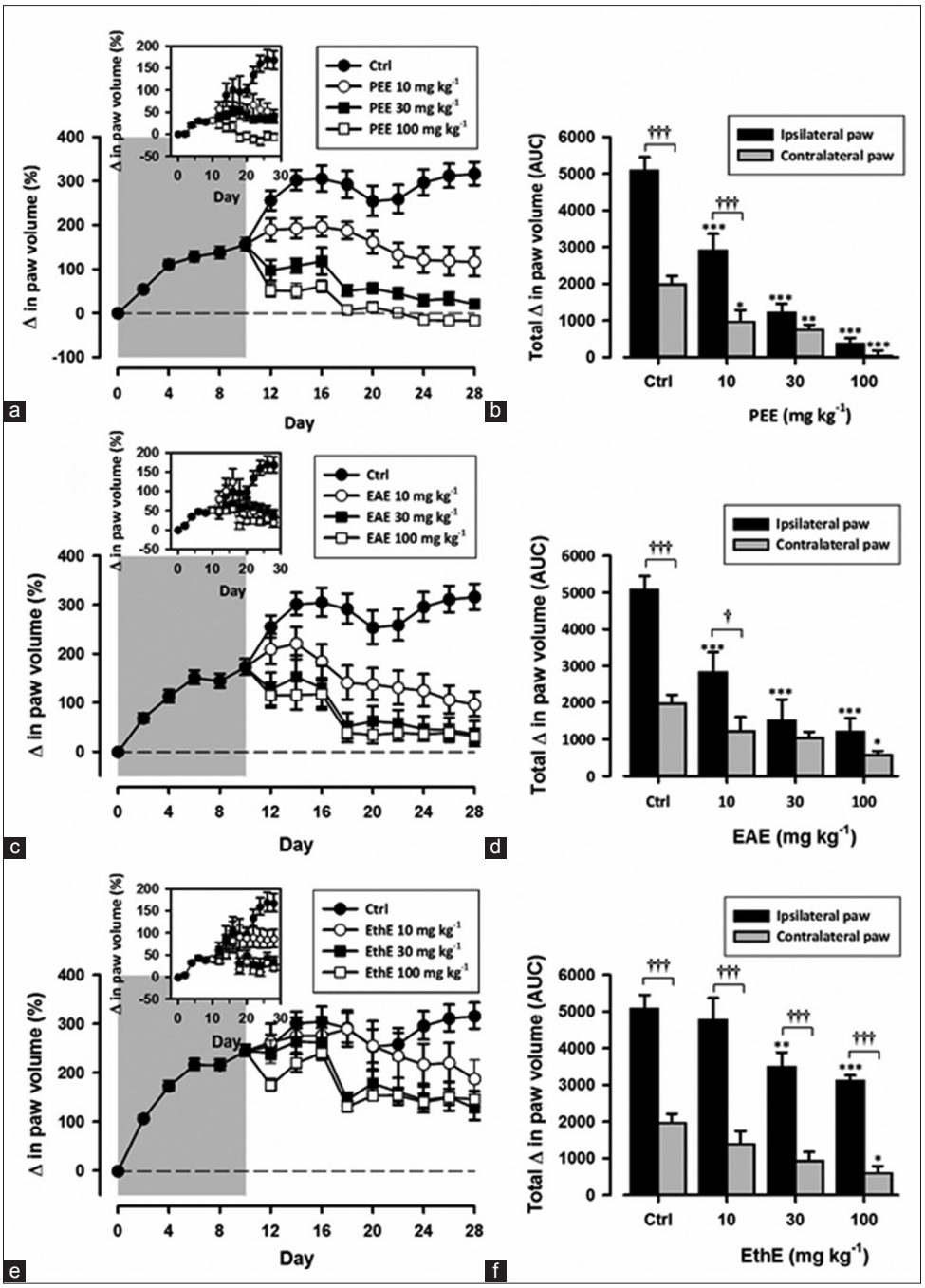

Figure 1: Effects of the petroleum ether extract (PEE), ethyl acetate extract (EthE), and ethanol extract (EAE) of *T. monadelpha* stem bark on complete Freund's adjuvant -induced arthritis. Graphs a, c, and e show the time course curves of PEE, EAE, and EthE treatments (bigger curve depicts acute phase while inserts depict polyarthritic phase). Graphs b, d, and f show the area under the time course curves. Each bar plotted is the mean±SEM ($n = 8$). ***$P \leq 0.001$, **$P \leq 0.01$: *$P \leq 0.05$. †††$P \leq 0.001$, ††$P \leq 0.01$: †$P \leq 0.05$. One-way and two-way analysis of variance followed by Holm-Sidak's *post hoc* test

paws. While EthE, DEX, and MET significantly ($P \leq 0.05$-0.0001; $F_{3,28}$= 4.28-17.32) improved the body weights of the rats [Figure 3c, d, and e], PEE and EAE did not [Figure 3 a and b].

Arthritic Score

PEE, EthE, and EAE significantly reduced ($P < 0.05$-0.01; F= 3.77-5.77) arthritic score. The effect was, however, not comparable to DEX and MET ($P < 0.0001$; $F_{3,28}$=13.34-32.16), which were more potent [Figure 4a-e].

X-ray Radiography

Radiographs of rats from CFA group displayed arthritic changes characterized by soft-tissue swelling with bone demineralization occurring mostly at the tibiotarsal joint which are indications

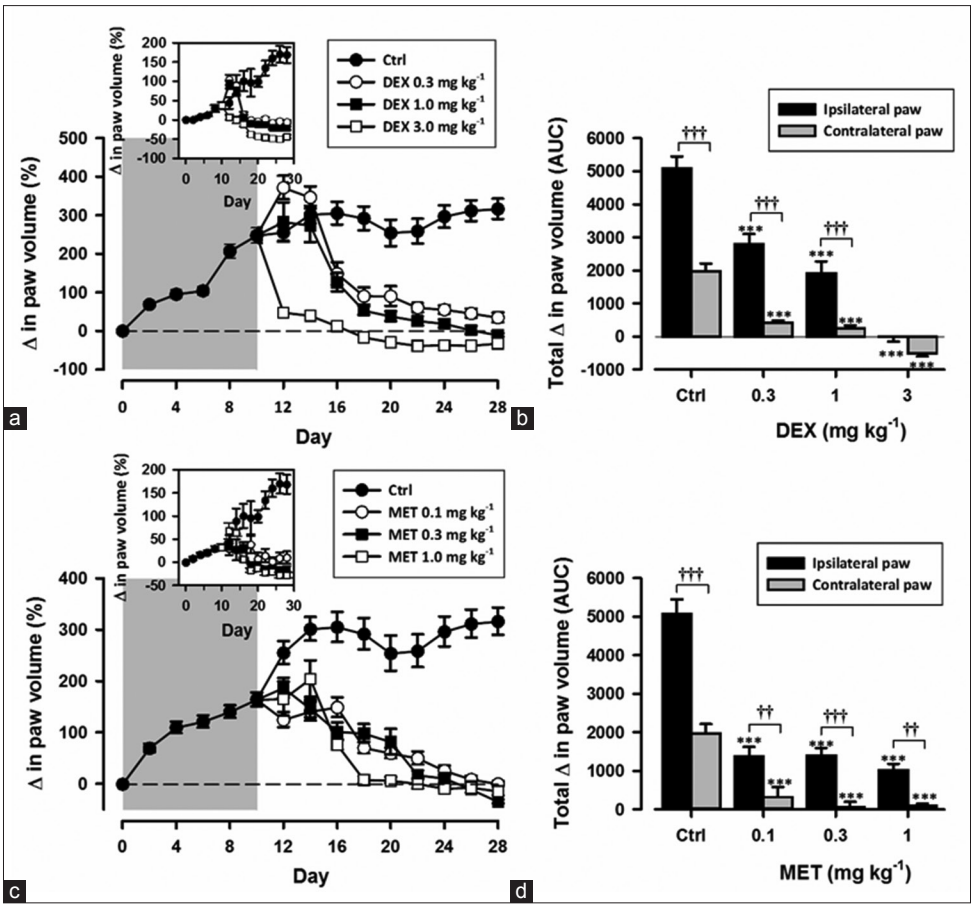

Figure 2: Effects of dexamethasone (DEX) and methotrexate (MET) on complete Freund's adjuvant-induced arthritis. Graphs a and c show the time course curves of DEX and MET treatments (bigger curve depicts acute phase while inserts depict polyarthritic phase). Graphs b and d show the area under the time course curves. Each bar plotted is the mean±SEM (n=8). ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$, †††$P \leq 0.001$, ††$P \leq 0.01$, †$P \leq 0.05$. One-way and two-way analysis of variance followed by Holm-Sidak's *post hoc* test

Table 5: The effective dose (ED50) and percentage maximal effect (E_{max}) of *T. monadelpha* stem bark extracts and reference drugs in CFA-induced arthritis in Sprague-Dawley rats

Drug	Acute phase		Polyarthritic phase	
	ED_{50}	E_{max}	ED_{50}	E_{max}
PEE	12.05±3.48	97.89	11.36±5.74	100
EAE	8.45±3.60	77.05	28.05±11.64	101.4
EthE	18.28±10.52	39.08	15.85±42.40	77.96
DEX	0.14±0.03	100	0.08±0.02	127.1
MET	0.01±0.01	78.46	0.02±0.03	98.59

PEE: Petroleum ether extract, EthE: Ethyl acetate extract, EAE: Ethanol extract, DEX: Dexamethasone, MET: Methotrexate, *T. monadelpha: Trichilia monadelpha*

of bone damage, in both ipsilateral and contralateral paws, compared with IFA group, which had intact bone structure. Radiographs of rats from PEE, EthE, EAE (average score of 2.0 ± 0.9), DEX, and MET-treated (average score of 0.7 ± 0.7) groups showed minimal bone damage. CFA group had the highest arthritic score (4.0 ± 0.3) indicating severe bone enlargement with active osteophytosis in the bone metaphysis, osteolysis, focal areas of excessive bone resorption, subchondral erosion, and subluxation, while the IFA control recorded the lowest score, indicating that the bones were intact [Table 6].

Histopathological Studies

Induced arthritis resulted in synovial hyperplasia, pannus formation, exudation of inflammatory cells (observed as accumulation of abundant monomorphonuclear and polymorphonuclear leukocytes into the joint space), and erosion of bone and cartilage. PEE, EthE, and EAE treatment resulted in a dose-dependent reduction ($P \leq 0.05$-0.001; $F_{4,9}$=6.99-65.14) in inflammation with the morphology of the synovium looking normal [Figure 5a, b and c]. DEX and MET improved the morphology of the tissue significantly ($P \leq 0.01$; $F_{4,9}$=17.75-18.75) [Figure 5d and e].

DISCUSSION

Adjuvant-induced arthritis in rats is an experimental model for therapeutic and pathogenetic studies of chronic forms of arthritis [19-22]. Chronic arthritis is usually associated with bone loss, due in part to systemic or local actions of interleukin (IL)-6 and tumor necrosis factor-α (TNF-α) [20]. These

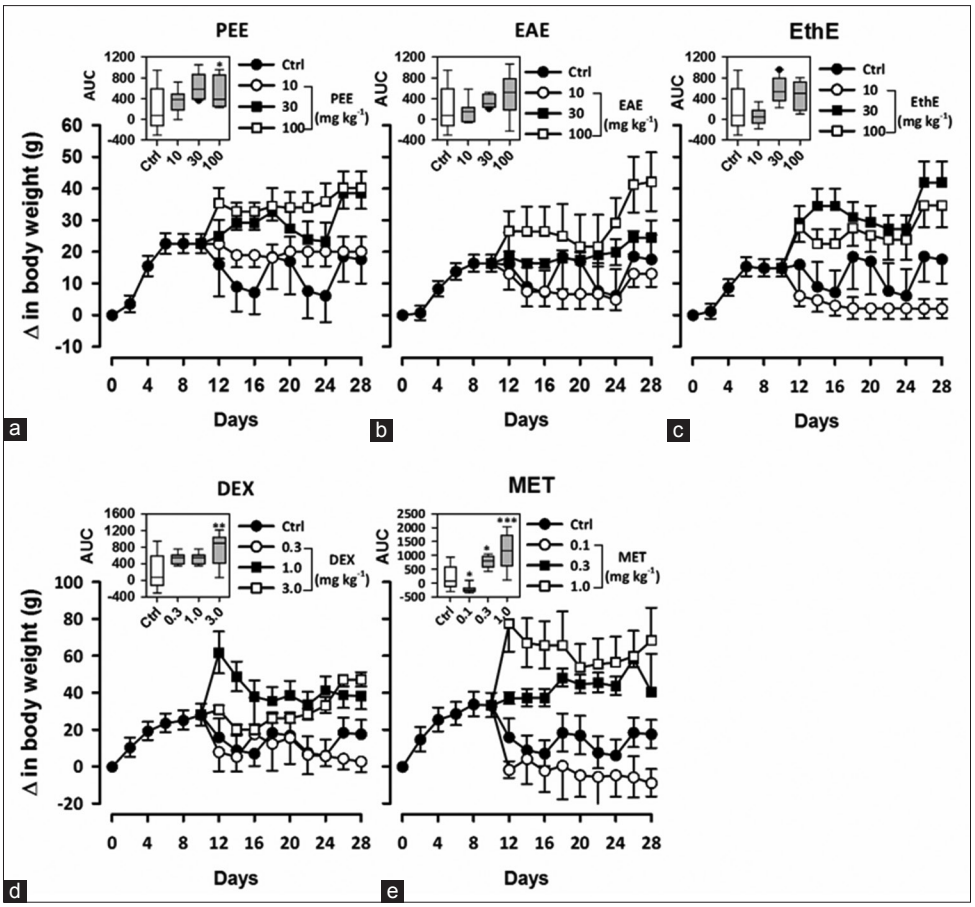

Figure 3: The time course curves of the effects of (a) the petroleum ether extract, (b) the ethyl acetate extract, (c) the ethanol extract of *T. monadelpha* stem bark, (d) dexamethasone and (e) methotrexate treatments on body weight of complete Freund's adjuvant-induced arthritis rats. The insert of the graphs in a boxplot derived from the area under time course curves. Each point and boxplot represents the mean±SEM (n=5). ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$, (one-way analysis of variance followed by Holm-Sidak's *post hoc* test)

cytokines stimulate the release of tissue-destroying matrix metalloproteinases as well as by inhibiting the production of endogenous inhibitors of these metalloproteinases, the net result being joint damage. Pathological features of this disorder include edema, infiltration of mononuclear and polymorphonuclear cells into the joint (synovial spaces), pannus formation, periostitis, and erosion of cartilage and bone [21].

In this study, adjuvant-injected paw was typified by a rapid onset of inflammation evident within 24 h of adjuvant injection, which continued to increase up to day 21 post-induction. This allowed for the study of acute inflammatory reactions locally (at the site of injection), i.e., in the ipsilateral paw, as well as the immunological reaction that develops later in the contralateral paw and various organs [22]. The arthritic rats showed soft-tissue swelling around the ankle joints during arthritis, and it was considered edema of those particular tissues. As the disease progressed, a more diffused demineralization developed in the extremities [23]. This was observed from the X-ray of the control group. Secondary lesions of adjuvant arthritis occurred after a delay of approximately 10 days and were characterized by inflammation of non-injected sites (right hind legs, ears, and tail) and further increases in the volume of the injected hind leg.

A therapeutic treatment regimen was followed in this research by initiating treatment from day 10 to day 28. All treatments with extracts, especially PEE and EAE, were effective in reducing the primary edema by day 18. The non-injected paw developed secondary lesion by day 14 post-adjuvant injection as a result of immune response to the bacterial adjuvant [17]. Treatment of adjuvant-injected rats with the extracts showed a significant reduction of secondary paw inflammation (compared with arthritic controls). These observations suggest that the extracts have very significant anti-inflammatory activity, comparable to DEX and/or MET. These drugs (DEX and MET) target specifically two major aspects, namely reducing inflammation and relieving pain by blocking cyclooxygenase (COX)-mediated prostaglandins release and control of joint inflammation by suppressing inflammatory-induced bone erosion. This finding is in line with earlier publications which indicate that preparations of the stem bark of *T. monadelpha* have been used in Ghanaian traditional medicine to treat pain and inflammation for many years and their efficacies are widely acclaimed in different communities in Ghana [13,14,24-26].

Earlier phytochemical screening conducted revealed the presence of alkaloids, terpenoids, phytosterols, and reducing sugars in all the extracts studied. EAE and EthE also contained

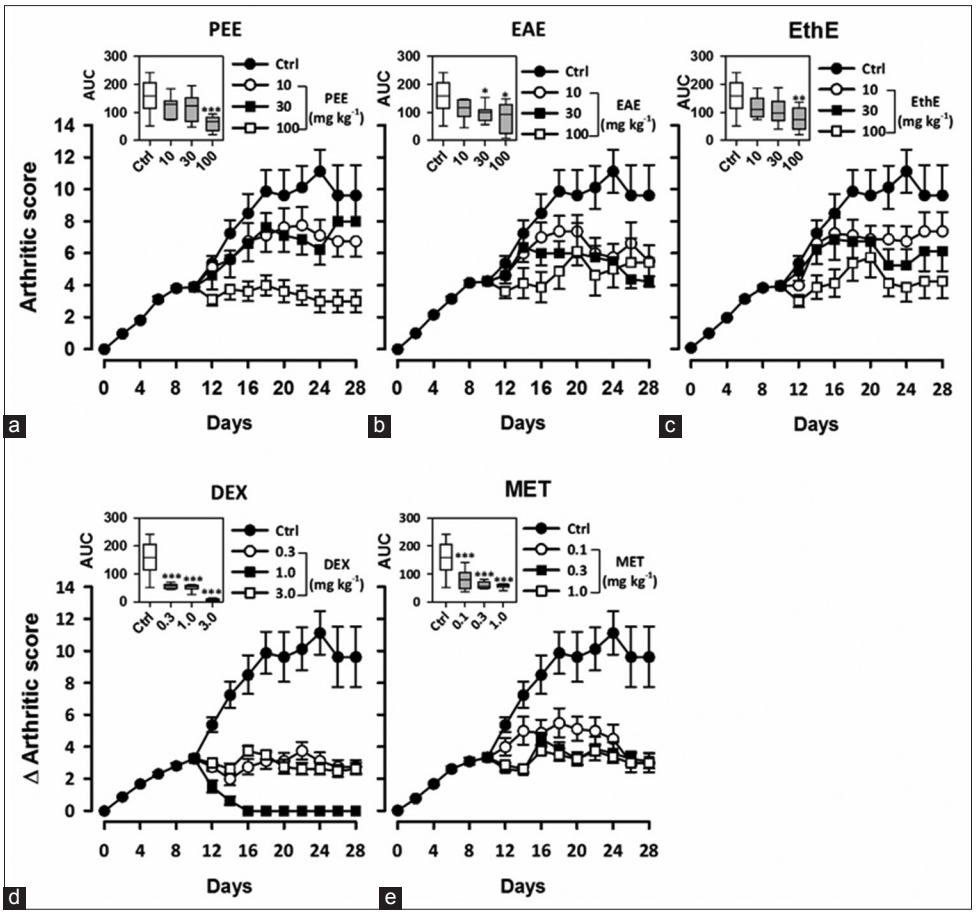

Figure 4: The time course curves of the effects of (a) the petroleum ether extract, (b) the ethyl acetate extract, (c) the ethanol extract of *T. monadelpha* stem bark, (d) dexamethasone and (e) methotrexate treatments on arthritic score in complete Freund's adjuvant -induced arthritis rats. The insert of the graphs in a boxplot derived from the area under time course curves. Each point and boxplot represents the mean±SEM (n=5). ***$P \leq 0.001$, **$P \leq 0.01$: *$P \leq 0.05$, (one-way analysis of variance followed by Holm-Sidak's *post hoc* test)

tannins, cardiac glycosides, anthraquinones, and saponins [16]. The anti-inflammatory activity exhibited by the extracts could be attributed to the combined effects of some of these phytochemicals. Many alkaloids have been ascertained to have anti-inflammatory activity using *in vivo* models such as carrageenan-induced pedal edema, 5-HT-induced pedal edema, xylene-induced ear edema, among others, and *in vitro* model including inhibitory activity on COX-1 and COX-2 and inhibitory activity on prostaglandin E_2 (PGE_2) and NO production [27]. This indicates diverse mechanisms by which alkaloids exert anti-inflammatory effect. Terpenoids and cardiac glycosides have the ability to modulate critical cell signaling pathways involved in the inflammatory response of the body such as nuclear transcription factor-kappa B activation [28,29], significant inhibition of cytokine production, and inhibition of T-cell immune responses among others [30]. A mixture of tannins (hydrolysable and non-hydrolysable) have been demonstrated to have apparent anti-inflammatory activity in carrageenan- and dextran-induced rat paw edema, cotton pellet granuloma test, and adjuvant-induced polyarthritis in rats. It is thought to be due to antagonism of the permeability-increasing effects of some inflammatory mediators, thus inhibiting the migration of leukocytes to an inflammatory site [31]. Anthraquinones were also found to possess anti-inflammatory activity after their inhibitory activities on NO production, COX-2, and PGE_2 which was determined in a lipopolysaccharide-induced inflammation model and in carrageenan-induced paw edema [32]. Reduction of paw swelling from the 3rd week onward may have been due to immunological protection rendered by the plant extracts, preventing systemic spread and ultimately reducing the destruction of joints as seen in the arthritic scores for the photographs and the radiographs. The phytochemicals in the extracts could have contributed to the immunological protection due to their significant anti-inflammatory properties which suppresses the generation and spread of pro-inflammatory agents [27].

Reduced bone structure and increased re-absorption cause bone loss in adjuvant-induced arthritis in rats [33,34]. Results of radiographic scores clearly showed increased bone loss in arthritic groups. The extracts, especially PEE, and reference drug treatment decreased bone loss due to arthritis. This suggests a suppression of synovitis and protection of bone structure resulting in joint protection [9,35,36]. This effect conforms to one of the therapeutic strategies of managing arthritis. The major target for inflammatory process in adjuvant-induced arthritis is the synovium which results in tissue inflammation as a result of infiltration of the tissue with multiple immune cells and cytokines [37]. The tissue inflammation is observed

as expansion of the synovial tissue and pannus formation that invades the bone and cartilage, destroying the tissue as it proceeds [38]. This process promotes osteoclastogenesis that leads to focal articular bone erosion at the site of pannus formation, as well as systemic bone loss similar to osteoporosis [38,39]. Inflammatory tissue invasion, into the subchondral bone, results in involvement of many cell types such as fibroblasts, lymphocytes, and monocytes [37]. Monocytes are the precursors of osteoclasts which bring about reabsorption of bone through the acidic disillusion of bone mineral and enzymatic destruction of bone matrix. This reabsorption of the bone by osteoclasts is due to the synthesis of proteases by the synovial fibroblasts, neutrophils, and the chondrocytes. Studies have shown that at the sites of bone erosion, large multinucleated osteoclastic cells resorb subchondral bone. Osteoclast formation is as a result of exposure of inflammatory cytokines present in synovial tissue [37]. The extracts were able to reduce cell infiltration, thereby ameliorating tissue inflammation in the rat synovial tissue, in a similar manner as DEX and MET, due to their potent anti-inflammatory activity.

Table 6: Arthritic scores of arthritic paws (ipsilateral and contralateral) obtained by X-ray radiography after CFA-induced arthritis and treatment with *T. monadelpha* stem bark extracts and reference drugs

Groups	Doses	Arthritic score	
		Ipsilateral paws	Contralateral paws
IFA		0±0.0**	0±0.0**
CFA		4±0.3	4±0.3
PEE	10 mg/kg^1	2±0.9	2±0.3
	30 mg/kg^1	2±0.7	0.7±0.7
	100 mg/kg^1	1±0.6	1±0.3
EthE	10 mg/kg^1	3±0.6	2±0.3
	30 mg/kg^1	2±0.9	2±0.9
	100 mg/kg^1	4±0.3	2±0.9
EAE	10 mg/kg^1	3±0.3	2±1.0
	30 mg/kg^1	3±0.6	1±0.7
	100 mg/kg^1	2±0.9	2±0.9
DEX	0.3 mg/kg^1	1±0.9	0.7±0.7
	1.0 mg/kg^1	0±0.0**	0.3±0.3*
	3.0 mg/kg^1	0±0.0**	0±0.0**
MET	0.1 mg/kg^1	0.7±0.3	0.3±0.3*
	0.3 mg/kg^1	1±0.6	0.7±0.7
	1.0 mg/kg^1	0.3±0.3*	0±0.0**

Values are mean±SEM. ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$ ANOVA followed by Holm-Sidak's *post hoc* test. PEE: Petroleum ether extract, EthE: Ethyl acetate extract, EAE: Ethanol extract, DEX: Dexamethasone, MET: Methotrexate, IFA: Incomplete Freund's adjuvant, CFA: Complete Freund's adjuvant, *T. monadelpha: Trichilia monadelpha*, ANOVA: Analysis of variance

Chronic arthritis is usually associated with weight loss. This may be due to the systemic or local action of inflammatory cytokines such as TNF-α and IL-1ß [20,40] produced primarily by monocytes and macrophages [41]. The high concentrations of TNF-α and IL-1ß exert a powerful influence on whole-body protein and energy metabolism. Although the specific mechanism(s) is not known, TNF-α is thought to stimulate muscle catabolism [42]. The increased catabolism raises resting energy expenditure, which leads to weight loss and reduced lean body mass, especially if energy and protein requirements are not met; a phenomenon recognized as "rheumatoid cachexia." Changes in body weight, therefore, have also been used to assess the course of disease and response to therapy of anti-inflammatory drugs [43]. The extracts significantly improved body weight of arthritic rats, indicating a reduction of catabolism caused by the inflammatory cytokines and hence their therapeutic potential in the management of RA.

CONCLUSION

This study has demonstrated that PEE, EthE, and EAEs of the stem bark of *T. monadelpha* have interesting antiarthritic property by reducing bone tissue damage and resorption in Freund's complete adjuvant-induced arthritis and hence the extracts are worth further investigating as they could be very useful to humans.

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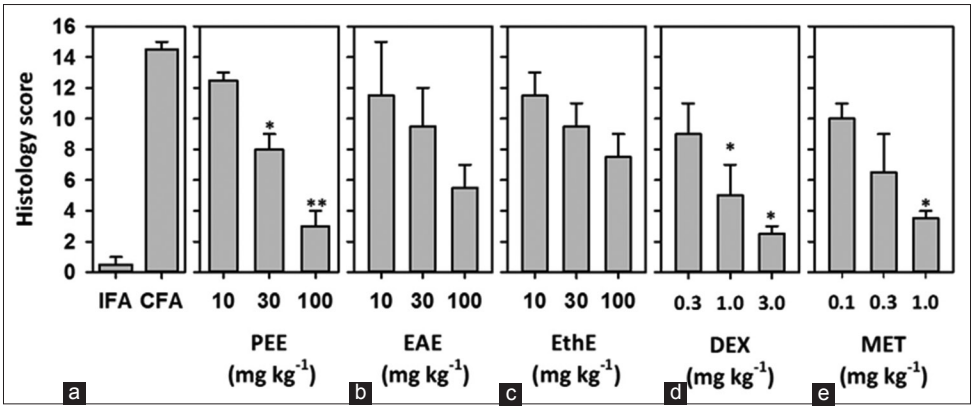

Figure 5: Histopathological score for bone erosion, inflammatory cell infiltration, pannus formation, synovial hyperplasia, and fibrosis of arthritic rats treated with (a) the petroleum ether extract, (b) the ethyl acetate extract, (c) the ethanol extract of *T. monadelpha* stem bark, (d) dexamethasone and (e) methotrexate incomplete Freund's adjuvant, complete Freund's adjuvant. Bars plotted represent mean score±SEM ($n = 8$). ***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$ compared to complete Freund's adjuvant group. (one-way analysis of variance followed by Holm-Sidak's *post hoc* test)

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Original Research

A synergistic effect of artocarpanone from *Artocarpus heterophyllus* L. (Moraceae) on the antibacterial activity of selected antibiotics and cell membrane permeability

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ABSTRACT

Aim/Backgrounds: Artocarpanone isolated from *Artocarpus heterophyllus* L. (Moraceae) exhibits antibacterial activity. The present study investigated synergistic activity between artocarpanone and tetracycline, ampicillin, and norfloxacin, respectively, against methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, and *Escherichia coli*. **Materials and Methods:** A broth microdilution method was used for evaluating antibacterial susceptibility. Synergistic effects were identified using a checkerboard method, and a bacterial cell membrane disruption was investigated by assay of released 260 nm absorbing materials following bacteriolysis. **Results and Discussion:** Artocarpanone exhibited weak antibacterial activity against MRSA and *P. aeruginosa* with minimum inhibitory concentrations values of 125 and 500 μg/mL, respectively. However, the compound showed strong antibacterial activity against *E. coli* (7.8 μg/mL). The interaction between artocarpanone and all tested antibiotics revealed indifference and additive effects against *P. aeruginosa* and *E. coli* (fractional inhibitory concentration index [FICI] values of 0.75-1.25). The combination of artocarpanone (31.2 μg/mL) and norfloxacin (3.9 μg/mL) resulted in synergistic antibacterial activity against MRSA, with an FICI of 0.28, while the interaction between artocarpanone and tetracycline, and ampicillin showed an additive effect, with an FICI value of 0.5. A time-kill assay also indicated that artocarpanone had a synergistic effect on the antibacterial activity of norfloxacin. In addition, the combination of artocarpanone and norfloxacin altered the membrane permeability of MRSA. **Conclusion:** These findings suggest that artocarpanone may be used to enhance the antibacterial activity of norfloxacin against MRSA.

KEY WORDS: Ampicillin, artocarpanone, norfloxacin, synergistic, tetracycline

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INTRODUCTION

The emergence of multidrug-resistant bacteria has led to global concerns about failure to cure certain highly dangerous infectious diseases. *Staphylococcus aureus* is an opportunistic Gram-positive bacterium that may cause dangerous infections, due to its ability to carry the resistance genes for many antibiotics [1]. Currently, the most prevalent resistant bacterium, particularly in a hospital environment is the methicillin-resistant *S. aureus* (MRSA). Acquisition of the *mec*A gene and its ability to over-express efflux pumps as well as to produce a β-lactamase enzyme are the underlining causes for the resistance of MRSA toward many antibiotics, especially β-lactam antibiotics [2]. A high prevalence of nosocomial infections caused by MRSA has been reported from many countries worldwide [3]. The increasing drug resistance of Gram-negative bacteria, including *Pseudomonas aeruginosa*, mainly due to mutation in target enzymes [4] has also raised concerns. Consequently, the identification and development of new antibiotics with new targets and modes of action are urgently needed, but major time

and cost factors are involved to ensure that the new compound is safe and effective and will not induce resistance when used clinically. The combination of conventional antibiotics with an agent that can enhance antibacterial activity has been suggested as an alternative strategy to overcome these problems [5].

Plant-derived compounds are recognized as an important source of new antibacterials. Many flavonoids have been reported to have antimicrobial activity [6], and some have been demonstrated to exert a synergistic effect on the activity of commercial products against resistant bacteria including MRSA [7,8]. Artocarpanone (Figure 1) is a flavonoid isolated from *Artocarpus heterophyllus*, which exhibits a range of pharmacological properties including antibacterial, anti-tyrosinase, and cytotoxic activity [9-11]. However, we have found no reports describing the synergistic effect of artocarpanone on the activity of antibiotics. The aim of the present study was to determine whether artocarpanone could enhance the antibacterial activity of the conventional antibiotics tetracycline, ampicillin, and norfloxacin that are normally used against *S. aureus* but are not effective against MRSA. The study as also extended to encompass the Gram-negative bacteria, *P. aeruginosa* and *Escherichia coli*. We also investigated the ability of artocarpanone/antibiotic combinations to disrupt bacterial cell membranes in a synergistic manner.

MATERIALS AND METHODS

Chemicals

Artocarpanone was purified from the crude ethyl acetate extract of A. *heterophyllus* heartwoods as described previously [9]. The antibiotics ampicillin, tetracycline, and norfloxacin were purchased from Sigma (Sigma-Aldrich, UK). Crystal violet was obtained from LabChem Inc. (Laboratory Chemical, Australia). Brain–heart infusion (BHI) was from the Becton, Dickinson and Company (Franklin Lakes, New Jersey, USA).

Bacterial Strains

MRSA (DMST 20654), *P. aeruginosa* (DMST 15442), and *E. coli* (ATCC 25922) were obtained from the Department of Medical Sciences, Ministry of Public Health, Thailand.

Determination of Minimum Inhibitory Concentrations (MICs)

A microdilution assay was used to determine the MIC of each antibiotic system against the selected bacteria strains [12]. Two-fold dilutions of each sample in BHI were prepared in a sterile 96-well plate. Bacterial suspensions were prepared in 0.85% NaCl, and the turbidity was adjusted to 0.5 McFarland standard (equivalent to 1×10^8 CFU/mL). The suspension was diluted with normal saline to contain 1×10^6 CFU/mL and added into each well. The final cell concentration was 5×10^5 CFU/mL. The plate was incubated at 37°C for 24 h, and the MIC was recorded as the lowest concentration of the sample that produced suppression of visible growth.

Checkerboard Assay for Antibacterial Activity

The antibacterial activity of combination antibiotics was evaluated against the selected bacteria as described by Chang *et al.*, with a slight modification [13]. The assay was performed using artocarpanone in combination with ampicillin, tetracycline, and norfloxacin, respectively, in 96-well plates. Two-fold dilutions of artocarpanone were prepared in BHI along the X-axis, while 2-fold dilutions of the antibiotics were prepared along the Y-axis. Subsequently, each well was inoculated with bacteria suspension of 1×10^6 CFU/mL and the plates were incubated at 37°C for 24 h. The fractional inhibitory concentration index (FICI) was quantified as the FIC for artocarpanone and the FIC for antibiotic, where the FIC for artocarpanone was the MIC of artocarpanone in combination divided by MIC for artocarpanone alone, while the FIC for antibiotic was the MIC of antibiotic in combination divided by the MIC of antibiotic alone.

FICI = FIC of artocarpanone + FIC of the antibiotics

$$\text{FIC} = \frac{\text{MIC of artocarpanone or antibiotics in combination}}{\text{MIC of artocarpin or antibiotics alone}}$$

The results were interpreted as synergistic (FICI ≤ 0.5), additive (0.5 ≤ FICI ≤ 1), indifferent (1 ≤ FICI ≤ 4), or antagonistic (FICI > 4) [14].

Time-kill Assay

Bacterial suspension containing 1×10^6 CFU/mL was added to BHI broth containing various combinations of antibiotics to reach a final cell concentration of 5×10^5 CFU/mL, then incubated at 37°C. A time-kill assay was performed at eight time intervals (0, 1, 2, 4, 6, 8, 12, and 24 h). Aliquots (50 μL) of the cultures were diluted (1:10) with 450 μL of normal saline, and 20 μL of each dilution was cultured on BHI agar. The numbers of viable colonies were recorded after a 24-h incubation [15].

Bacteriolysis Assay

The alteration of cell membrane permeability was investigated by measuring uptake of crystal violet [16]. Briefly, a suspension of MRSA in normal saline was prepared from an overnight culture on BHI agar. A single dose of artocarpanone and norfloxacin as well as artocarpanone in combination with norfloxacin was added to the cell suspension and incubated at 37°C for 1 h. The final cell concentration was 5×10^7 CFU/mL. Untreated suspensions of MRSA were used as a negative control. The cells were harvested at 13,400 × g for 5 min and resuspended in a crystal violet solution (10 μg/mL in normal saline). The cells were incubated at 37°C for 10 min and harvested by centrifugation at 25°C for 15 min. The optical density (OD of the supernatant) at 590 nm was measured using an ultraviolet-visible (UV-Vis) spectrophotometer (Genesis-6, Becthai, Bangkok). The OD reading of the crystal violet solution used for the assay was considered to represent the value of 100%.

The percentage crystal violet uptake was calculated as the OD value of the sample supernatant/OD value of the crystal violet solution ×100. This experiment was performed in triplicate.

Loss of 260 nm Absorbing Material

The concentration of released UV-absorbing material from bacteria exposed to antibiotics is a measure of metabolites, nucleic acid, and ion that were detected at 260 nm [16-18]. Overnight cultures of MRSA were washed with normal saline and resuspended in normal saline. Artocarpanone and norfloxacin alone as well as combinations of artocarpanone and norfloxacin were added to the cell suspensions to give a final cell concentration of 5×10^7 CFU/mL. Untreated cell suspensions were used as the control. Test samples were incubated at 37°C for 1 h and then centrifuged at 25°C at 13,400 × g for 15 min. The OD_{260} of the supernatant was measured using a UV-Vis spectrophotometer to determine the quantity of intracellular UV-absorbing material released by the cells. The assay was performed in triplicate.

Statistical Analysis

All experiments were carried out in triplicate with the average value and standard deviations reported. The data were analyzed using ANOVA followed by the Tukey's honestly significant difference *post-hoc* test to identify significant difference between group means. Statistical significance was accepted at the level $P < 0.01$.

RESULTS

MICs

Artocarpanone exhibited strong antibacterial activity against *E. coli* with an MIC of 7.8 μg/mL, but it had a weak antibacterial activity against *P. aeruginosa* and MRSA with MICs of 500 and 125 μg/mL, respectively [Table 1]. Norfloxacin showed the strongest antibacterial activity against *E. coli* and *P. aeruginosa* with MICs of 1.9 μg/mL while tetracycline and ampicillin also demonstrated strong antibacterial activity with MIC values of 7.81-15.62 μg/mL. However, all tested antibiotics only revealed moderate-weak antibacterial activity against MRSA (MIC of 62.5-125 μg/mL).

Checkerboard Analysis

Interaction between artocarpanone (125 μg/mL) and norfloxacin (0.9 μg/mL) showed an additive effect against *P. aeruginosa* (FICI of 0.75), while combination of artocarpanone (250 μg/mL) and tetracycline (7.8 μg/mL) as well as artocarpanone (125 μg/mL) and ampicillin (15.6 μg/mL) gave the indifference effects with FICIs of 1 and 1.25, respectively [Table 2]. On the one hand, artocarpanone (3.9 μg/mL) exhibited an additive effect on the antibacterial activity of tetracycline (1.9 μg/mL) against *E. coli* with FICI of 0.75 and showed indifference effect in combination artocarpanone (0.9 μg/mL) and ampicillin (15.6 μg/mL) as well as artocarpanone (3.9 μg/mL) and norfloxacin (0.5 μg/mL) with FICIs of 1.1 and 1, respectively [Table 3]. In case of MRSA, artocarpanone (31.2 μg/mL) also performed additive effects when combined with tetracycline (31.2 μg/mL) and ampicillin (15.6 μg/mL) with FICIs of 0.5. Interestingly, in combination with norfloxacin, artocarpanone (31.2 μg/mL) enhanced the antibacterial activity of norfloxacin (3.9 μg/mL) with a synergistic effect (FICI value of 0.28) [Table 4].

Time-kill Assay

The combination of 31.2 μg/mL artocarpanone and 3.9 μg/mL norfloxacin completely inhibited bacterial growth at the limit of quantification (10^2) within 12 h, while artocarpanone and norfloxacin alone at the concentration of 62.5 μg/mL did not completely inhibit bacterial growth until 24 h [Figure 2].

Bacteriolysis

The percentage of uptake of crystal violet indicated the bacteriolytic activity of the compounds against MRSA

Table 1: Antibacterial activity of artocarpanone and antibiotics against three tested bacteria

Bacteria	MIC (μg/mL)			
	Artocarpanone	Tetracycline	Ampicillin	Norfloxacin
E. coli	7.8	7.8	15.6	1.9
P. aeruginosa	500	15.6	15.6	1.9
MRSA	125	125	62.5	125

MRSA (methicillin-resistant *Staphylococcus aureus*)

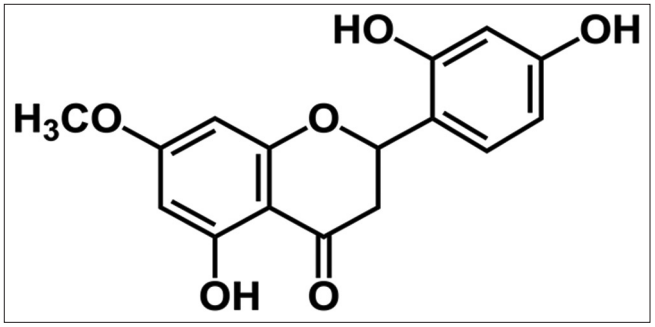

Figure 1: Chemical structure of artocarpanone

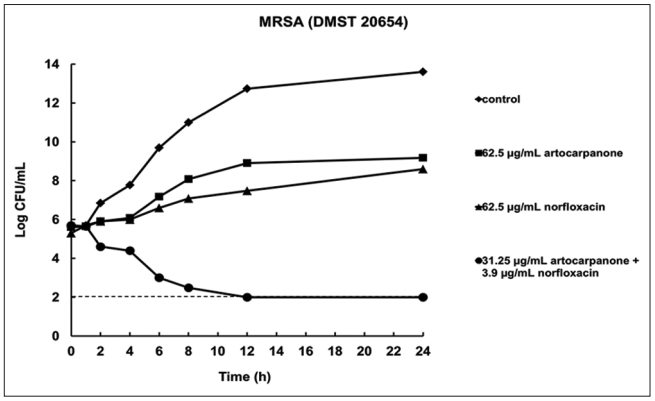

Figure 2: Time-kill curves of artocarpanone, norfloxacin, and their combination against methicillin-resistant *Staphylococcus aureus*

[Figure 3]. Artocarpanone significantly increased the uptake of crystal violet when compared to the control ($P < 0.01$), while norfloxacin did not have any significant effect. It was of interest that the crystal violet uptake of artocarpanone in combination with norfloxacin was significantly higher than the other groups, including the control as well as a single dose of artocarpanone and norfloxacin ($P < 0.01$).

Loss of 260 nm Absorbing Material

The result indicated that the absorbance of the combined artocarpanone and norfloxacin was significantly higher than for the control group as well as those of the single compounds, artocarpanone and norfloxacin ($P < 0.01$) [Figure 4].

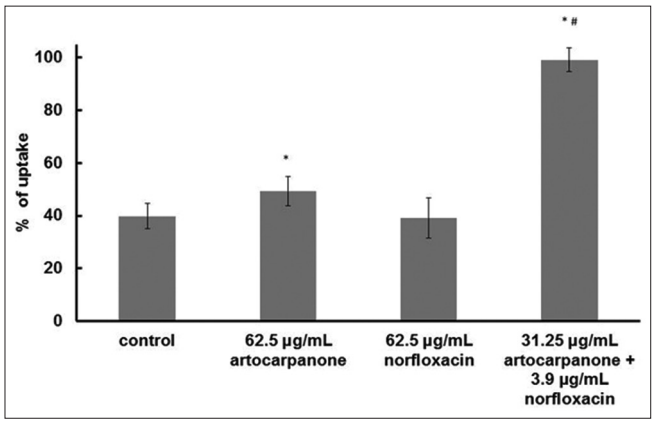

Figure 3: Crystal violet uptake of artocarpanone, norfloxacin, and their combination treated methicillin-resistant *Staphylococcus aureus*. The mean ± standard deviation for three replicates is illustrated. *Samples demonstrate significant differences compared to control ($P < 0.01$), #combination of artocarpanone and norfloxacin demonstrates significant difference compared to drugs alone ($P < 0.01$)

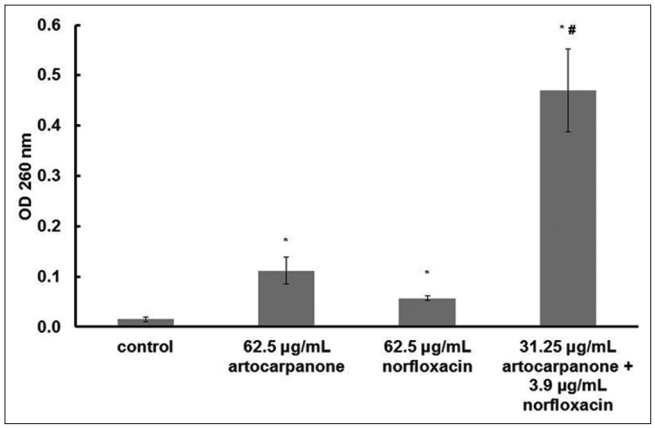

Figure 4: Presence of 260 nm absorbing material in the supernatant of methicillin-resistant *Staphylococcus aureus* treated with artocarpanone, norfloxacin, and their combination. The mean ± standard deviation for three replicates is illustrated. *Samples demonstrate significant differences compared to control ($P < 0.01$), #combination of artocarpanone and norfloxacin demonstrates significant difference compared to drugs alone ($P < 0.01$)

Table 2: Effect of artocarpanone on the antibacterial activity of antibiotics against *P. aeruginosa*

	MIC[a] (µg/mL)	MIC[c] (µg/mL)	FIC	FICI	Interaction
Artocarpanone-Tetracycline					
Artocarpanone	500	250	0.5	1	Indifference
Tetracycline	15.6	7.8	0.5		
Artocarpanone-Ampicillin					
Artocarpanone	500	125	0.25	1.25	Indifference
Ampicillin	15.6	15.6	1		
Artocarpanon-Norfloxacin					
Artocarpanone	500	125	0.25	0.75	Additive
Norfloxacin	1.9	0.9	0.5		

[a]MIC of one sample alone, [c]MIC of samples in combination)
FIC (fractional inhibitory concentration), FICI (fractional inhibitory concentration index)

Table 3: Effect of artocarpanone on the antibacterial activity of antibiotics against *E. coli*

	MIC[a] (µg/mL)	MIC[c] (µg/mL)	FIC	FICI	Interaction
Artocarpanone-Tetracycline					
Artocarpanone	7.8	3.9	0.5	0.75	Additive
Tetracycline	7.8	1.9	0.25		
Artocarpanone-Ampicillin					
Artocarpanone	7.8	0.9	0.1	1.1	Indifference
Ampicillin	15.6	15.6	1.0		
Artocarpanone-Norfloxacin					
Artocarpanone	7.8	3.9	0.5	1.0	Indifference
Norfloxacin	1.9	0.5	0.5		

[a]MIC of one sample alone, [c]MIC of samples in combination)
FIC (fractional inhibitory concentration), FICI (fractional inhibitory concentration index)

Table 4: Effect of artocarpanone on the antibacterial activity of antibiotics against MRSA

	MIC[a] (µg/mL)	MIC[c] (µg/mL)	FIC	FICI	Interaction
Artocarpanone-Tetracycline					
Artocarpanone	125	31.2	0.25	0.5	Additive
Tetracycline	125	31.2	0.25		
Artocarpanone-Ampicillin					
Artocarpanone	125	31.2	0.25	0.5	Additive
Ampicillin	62.5	15.6	0.25		
Artocarpanone-Norfloxacin					
Artocarpanone	125	31.2	0.25	0.28	Synergistic
Norfloxacin	125	3.9	0.03		

[a]MIC of one sample alone, [c]MIC of samples in combination)
FIC (fractional inhibitory concentration), FICI (fractional inhibitory concentration index)

DISCUSSION

On the basis of the broth microdilution method, artocarpanone has demonstrated variable antibacterial activity against tested bacteria. Against Gram-negative bacteria, norfloxacin was the strongest agent. However, all tested antibiotics as well as artocarpanone only showed a weak antibacterial activity against MRSA. It has been known that many antibiotics in sublethal

concentration cannot significantly exhibit any activities against MRSA due to its resistant mechanism. One appealing strategy to overcome resistant problem is the use of drug in combination. This strategy may increase their biological activities due to the interaction of each compound. Checkerboard method was used to determine the interaction of combination between artocarpanone and antibiotics. Interestingly, against resistant bacteria, artocarpanone only had a synergistic interaction when combined with norfloxacin. By this combination, artocarpanone could decrease the dose of norfloxacin by 32-fold. The time-kill assay was conducted to confirm the synergistic effect of artocarpanone on the anti-MRSA activity of norfloxacin. These results indicated that artocarpanone may overcome the problems associated with MRSA when used in combination with the conventional antibiotic, i.e., norfloxacin.

A use of drug in combination may increase their biological activities due to the interaction of each compound. Different compounds may have different target sites and influence each site to achieve the same response that leads to enhanced biological activities in the cells. On the other hand, the different compounds might affect the same target site and that could result in an agonistic activity [19]. Over-expression of the efflux pump is one of the resistance mechanisms of MRSA toward antibiotics. It has been suggested that the efflux pump can be inhibited by altering the membrane permeability as well as by inhibiting the metabolic pathway [20]. Cell membrane disruption is one of the antibacterial mechanisms of flavonoids [6,21]. This study therefore also focused on investigation any cell membrane disruption by artocarpanone, norfloxacin, and their synergistic mixtures. Based on the bacteriolysis assay, artocarpanone in combination with norfloxacin had a bacteriolytic activity by increasing the uptake of crystal violet. A further study was performed to determine the release of UV-absorbing material at 260 nm that indicated the leakage of the intracellular components of MRSA as an indicator for membrane damage [22]. This result corresponded well with the synergistic bacteriolytic effect of the mixture of artocarpanone and norfloxacin. It implied that the mixture of artocarpanone and norfloxacin enabled the alteration of the membrane permeability and caused a release of intracellular components.

This finding indicated that the synergistic activity of artocarpanone and norfloxacin against MRSA may be operated through different targets sites. It has been shown that the incorporation of flavonoids, especially a flavanone at the lipophilic side of the cell membrane, can cause a reduction of membrane fluidity [23]. For example, sophoraflavanone G isolated from *Sophora exigua* exhibited antibacterial activity against MRSA by reducing the fluidity of the cellular membrane as well as by reducing the cytoplasmic contents [20,24]. Therefore, such membrane alteration may allow norfloxacin to enter the cells more easily and occupy its site of action for inhibiting the DNA gyrase that resulted in interfering with cell division and induced the cells death [25]. Investigation of this synergistic activity between artocarpanone and norfloxacin may provide opportunities for understanding their mechanism of actions against MRSA and provide a new prospect for the discovery an alternative strategy to overcome resistance problems. Nevertheless, further experiments are required to elucidate other mechanisms of action including any inhibitory activity on the efflux pumps.

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Original Research

Anti-nociceptive and anti-inflammatory potentials of *Vernonia amygdalina* leaf extract via reductions of leucocyte migration and lipid peroxidation

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ABSTRACT

Background: *Vernonia amygdalina* is well known as a medicinal plant in folk medicine as antidiabetic, anthelmintic, antimalarial, laxative/purgative, and expectorant among others. **Aim:** This study was conducted to investigate the antinociceptive and anti-inflammatory effects of *V. amygdalina.* **Materials and Methods:** Methanol extract of *V. amygdalina* leaf (MEVA) was evaluated for antinociceptive effect and possible mechanisms of action in the presence of naloxone (1 mg/kg), atropine (2 mg/kg), and prazosin (1 mg/kg) using acetic acid writhing test in mice. The anti-inflammatory effect was evaluated in carrageenan hind paw edema and carrageenan air pouch models. Protein concentration, malondialdehyde (MDA), glutathione (GSH), and superoxide dismutase (SOD) assays were carried out for its antioxidative activities in inflammation. Hematoxylin and eosin staining was used to assess the level of inflammation. **Results:** From the acetic acid writhing test results, MEVA (50,100 mg/kg) showed significant antinociceptive effect. Naloxone, atropine and prazosin did not significantly reverse the antinociceptive effect of MEVA (50 mg/kg). MEVA (50, 100, and 200 mg/kg) showed dose-dependent inhibition of edema (41.4, 63.0, and 68.6%) at 4 h post-carrageenan injection. In the carrageenan air pouch model, MEVA (200 mg/kg) significantly ($P < 0.05$) reduced infiltrating leukocytes, protein concentration and MDA levels, while GSH and SOD were unaffected. The histological study showed a reduction in the infiltration of inflammatory cells in MEVA-treated groups. **Conclusion:** *V. amygdalina* showed antinociceptive activity and anti-inflammatory effect via reductions of leukocyte migration and lipid peroxidation.

KEY WORDS: Anti-inflammatory, antinociceptive, malondialdehyde, *Vernonia amygdalina*

INTRODUCTION

Pain is one of the general manifestations of inflammatory disorders and it is of paramount importance that it is handled with proper and specific treatments [1]. To understand the concept of pain, there is need to recognize that the pain that occurs after most types of noxious stimuli is usually protective and quite distinct from the pain resulting from overt damage to tissues or nerves [2]. A primary cardinal feature of inflammatory states is that normally innocuous stimuli produce pain [3,4].

At present, most anti-inflammatory drugs are effective for suppressing inflammation but with considerable negative side effects such as gastrointestinal bleeding and cardiovascular effects. These limit their therapeutic usage and thus have encouraged most drug development researchers in the development of new therapies [5-7]. Natural products have been one of the most successful sources for the discovery of new therapeutic agents, and novel therapies for treatment are essential to overcome the adverse effects of existing therapies for pain treatment [8].

Vernonia amygdalina leaf is commonly called bitter leaf in English because of its bitter taste. Leaves of this plant are used in Nigeria as a green vegetable or as spice in soups, especially in the popular "bitter leaf soup." The leaves can be taken as an

appetizer and the water extract as a digestive tonic. It is well known as a medicinal plant in folk medicine as antidiabetic, anthelmintic, antimalarial, laxative/purgative, expectorant, worm expeller and fertility inducer in subfertile women, antipyretic, and recently for a non-pharmacological solution to persistent fever, headache, and joints pain associated with AIDS [9,10]. Studies have also been conducted to establish its antinociceptive, anti-inflammatory, and antioxidant properties of V. *amygdalina* among others [9].

However, the exact mechanisms underlying the therapeutic actions of V. *amygdalina* on pain and inflammation are yet to be elucidated. Hence, this experiment was performed to elucidate the mechanisms of action of the antinociceptive and anti-inflammatory potentials of V. *amygdalina*.

MATERIALS AND METHODS

Reagents and Drugs

The chemicals and drugs include: Formalin, acetic acid, naloxone hydrochloride dehydrate (Sigma-Aldrich, USA), atropine, prazosin, carrageenan, indomethacin (Sigma-Aldrich, USA), morphine, and distilled water.

Experimental Animals

Swiss mice weighing (25-30 g) and Wistar rats weighing (180-200 g) of both sexes were used for this study. They were purchased, housed and bred at the Pre-clinical Animal House, College of Medicine, University of Ibadan, Ibadan, Nigeria where this study was conducted. Animals were housed in 5 cages and in at a temperature of 22±2°C and 45-65% relative humidity environment under a 12 h light/12 h dark cycle (8:00 a.m. - 8:00 p.m). The animals were acclimatized for 2 weeks with unrestricted access to food and water before the experiment. All experimental procedures on rodents were conducted in accordance with established protocols under the guidelines of the Principle of Laboratory Animal Care (NIH publication No. 85-23) [11] and ethical guidelines for investigation of experimental pain in conscious animals by Zimmerman [12].

Collection and Extraction of *V. amygdalina* Leaves

Fresh leaves of V. *amygdalina* were collected from the Aroro-Makinde area, Arulogun, Ojoo, Ibadan, Nigeria, which was authenticated at the Forestry Research Institute of Nigeria, Ibadan Oyo state. The voucher number: FHI - 110415 was assigned. Fresh leaves of V. *amygdalina* were collected and air dried after which they were blended into a powdery form, and then 2.26 kg of V. *amygdalina* was macerated in 10 L of methanol at room temperature for 48 h. It was then decanted with a filter paper. The process was repeated 3 times for exhaustive extraction. The extract was concentrated with a rotary vacuum evaporator at 40°C to produce a methanol extract of V. *amygdalina* (MEVA). The extract was further concentrated in a vacuum oven at a temperature of 40°C.

In vivo Antinociceptive Studies in Acetic Acid-induced Abdominal Writhing Test in Mice

This test was carried out using the modified method [13]. The mice were pre-treated, orally, with the vehicle, MEVA of 50 mg/kg, 100 mg/kg, and 200 mg/kg for 3 days and indomethacin (10 mg/kg), once. Mice were injected with 0.2 ml (i.p.) of 3% acetic acid solution, 1 h after treatment with the extract, which induced the characteristic writhing.

Mechanisms of Action: Evaluation of the Mode of Action of *V. amydalina* Extract for Antinociceptive Activity

This was designed to assess the possible participation of different systems in the antinociceptive effect of MEVA, (50 mg/kg), mice were pre-treated with naloxone (1 mg/kg, i.p.), a non-selective opioid receptor antagonist; atropine (2 mg/kg, i.p.), a non-selective muscarinic receptors antagonist; and prazosin (1 mg/kg i.p.), an alpha-1- adrenoreceptor antagonist.

Carrageenan-induced Hind Paw Edema Model in Rat

A total of 30 rats were divided into five groups, and they were pre-treated with MEVA for 3 days before the experiment. The doses given include 50 mg/kg, 100 mg/kg, and 200 mg/kg. Control animals received 1% tween 80 (10 ml/kg) and indomethacin (5 mg/kg) was used as a reference drug. Carrageenan was injected 1 h after the past treatment. Paw edema was induced by right subplantar injection of 0.1 ml/paw of 1% freshly prepared carrageenan suspension in distilled water into the right hind paw of each rat. The paw edema volume was measured using the Ugo basile plethysmometer before and as well as at 1, 2, 3, and 4 h after the injection of carrageenan [14].

Carrageenan-induced Air Pouch Model in Rats

Air pouch was induced in rats as described [15]. Briefly, rats were anesthetized with ketamine (100 mg/kg, i.p.) and air cavities were produced by subcutaneous injection of 20 ml of sterile air into the intrascapular area of the back (1st day). An additional 10 ml of air was injected into the cavity on the 4th day [16]. Rats were divided into three groups (n = 6); carrageenan (1% tween 80; 10 ml/kg), MEVA 200 mg/kg, and indomethacin (5 mg/kg) and orally pre-treated for 3 days before induction of inflammation. On the 6th day, 2 ml of 2% carrageenan solution dissolved in sterile saline was injected into the pouch cavity to induce inflammatory responses. 24 h after the carrageenan injections, rats were anesthetized with deep ether anesthesia and the pouch was carefully opened by a small incision. 2 ml of 0.9% normal saline was given to wash out the cavity.

Determination of Exudates Volume and Leukocyte Infiltration

The pouch cavity was opened with a small incision; the exudates were harvested and their volumes were measured [17]. The leukocytes in the fluid were counted using a hemocytometer. The exudates were transferred to ice-cold tubes. Cell-free

exudate was achieved by centrifugation at 3000 revolutions per minute (rpm) at 4°C for 15 min, and was stored at 4°C to prevent degeneration of cells. The supernatant was collected and stored at 4°C.

Determination of Protein Concentration

The protein concentrations of the exudates from the air pouch experiment were determined by the means of Biuret method [18]. 50 μl of the supernatant was added to 1.950 ml of distilled water. 3 ml of Biuret reagent was also added to the content in a test tube. Then, the whole sample was incubated at room temperature for 30 min. The absorbance was read at 540 nm and the concentration was determined from the standard curve.

Determination of Reduced Glutathione (GSH) Level

The method of Beutler and co-workers [19] was used in estimating the level of reduced glutathione (GSH) in air pouch exudate. 0.1 ml of test sample (supernatant) was diluted in 0.9 ml of phosphate (PO_4) buffer. 1 ml of 20% trichloroacetic acid (TCA) was added and allowed to stand for 20 min before centrifugation at 10,000 rpm for 10 min. 0.25 ml of the supernatant was removed and added to 0.75 ml of phosphate buffer. 2 ml of 0.0006 M of 5, 5^1-Dithiobis (2-nitrobenzoic acid) was added and incubated for 10 min. Absorbance was read at 412 nm.

Determination of Malondialdehyde (MDA)

MDA is a presumptive biomarker for lipid peroxidation in plasma, live organisms, and cultured cells. 0.1 ml of test sample was dissolved in 1.9 ml of Tris-potassium chloride buffer. 0.5 ml of 30% TCA was added and 0.5 ml of 0.75% of thiobarbituric acid was also added. The whole mixture was incubated at 80°C for 45 min. It was allowed to cool on ice and then centrifuged at 4000 rpm for 10 min. Absorbance was read at 532 nm [20].

Determination of Superoxide Dismutase (SOD)

The level of SOD activity was determined by the method of Misra and Fridovich [21]. Superoxide (O_2) radical generated by the xanthine oxidase reaction caused the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per O_2 introduced increased with increasing pH and also increased with increasing concentration of epinephrine. 10.8 mg of adrenaline was dissolved in 20 ml of 0.1 M of hydrochloric acid to yield 0.3 mm. 0.1 ml of test sample (supernatant) was dissolved in 2.6 ml of carbonate buffer. Then, it was allowed to equilibrate for about 5-10 min. The content was poured into cuvettes and the 0.3 mm of prepared adrenaline was added. Increase in absorbance for 60 s, 120 s, and 180 s (0, 1, 2, and 3 min) was read.

Histological Analysis

The pouch area was excised and fixed in 10% formalin in 0.01 M phosphate buffer (pH 7.4) and embedded into paraffin wax blocks. Sections were stained with hematoxylin and eosin.

RESULTS

Effects of MEVA on Acetic Acid-induced Writhing Test in Mice

Figure 1 shows acetic acid-induced writhing response in mice which serve as an indication of antinociceptive activities of MEVA. Intraperitoneal injection of acetic acid produced 40.4 ± 4.7 mean number of writhes in the group administered with the vehicle. MEVA at 50, 100, and 200 mg/kg significantly inhibits writhes response by 63.9, 50.9, and 32.5%, respectively. Positive control drug (Indomethacin, 10 mg/kg) significantly inhibits writhing response by 84.7%.

Mechanism of Action of *V. amygdalina* Involving Opioidergic, Adrenergic, Cholinergic Receptors in Acetic Acid-induced Writhing Test in Mice

Figure 2 shows the results of the effect of pretreatment of mice with various antagonists of the antinociceptive activity MEVA (50 mg/kg). The results showed that pretreatment with naloxone (1 mg/kg) insignificantly ($P > 0.05$) reversed the antinociceptive activity of MEVA (50 mg/kg) by increasing the number of writhes in mice. Pretreatment of mice with atropine (2 mg/kg) or prazosin (1 mg/kg) did not prevent the antinociception caused by MEVA in mice.

Effects of MEVA on Carrageenan-induced Hind Paw Edema in Rats

Hind paw edema was induced by injecting carrageenan (1%) in the left hind paw after administration. The result of the inhibitory effect of MEVA on increase in hind paw volume (edema) is shown in Figure 3. The increase in paw volumes in animal pretreated with MEVA (50, 100, and 200 mg/kg) for 3 days before injection with carrageenan was significantly ($P < 0.001$) less than animals pretreated with vehicle. MEVA (50, 100, and 200 mg/kg) showed dose-dependent inhibition of edema (41.4%, 63.0%, and 68.6%) at 4 h post carrageenan injection. At the same time, rats pretreated with indomethacin

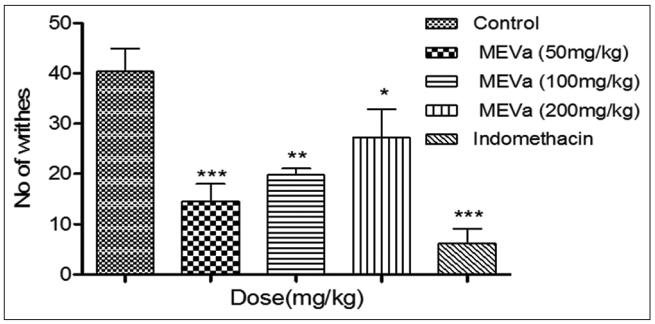

Figure 1: Effects of pretreatment of methanol extract of *Vernonia amygdalina* in acetic acid-induced writhing test in mice. Data are expressed as mean ± SEM (n=6). Comparisons were made using one-way ANOVA followed by *post-hoc* Newman-Keuls test. The symbol denotes significant levels: *P<0.05; **P<0.01; ***P<0.001 compared with the control group

(5 mg/kg) showed a more significant inhibition (78.7%; $P < 0.001$) on the increase in paw volume at the 4 h.

Anti-inflammatory Effects of *V. amygdalina* in Carrageenan-induced Air Pouch Model of Inflammation

The volume of exudates recovered from the carrageenan-induced air pouch revealed that pretreatment with MEVA caused a reduced but insignificant ($P > 0.05$) fluid exudation compared to carrageenan alone [Figure 4a]. Indomethacin (5 mg/kg) however caused a significant reduction in fluid exudates. Leukocytes migration into the air pouch was increased 24 h post carrageenan injection. The results revealed that pretreatment with MEVA (200 mg/kg) and indomethacin (5 mg/kg) caused a significant ($P < 0.001$) reduction in total leukocytes accumulation in the air pouch compared to carrageenan alone [Figure 4b]. The protein concentration in fluid exudates was significantly ($P < 0.05$) reduced by MEVA (200 mg/kg) and indomethacin (5 mg/kg) [Figure 4c].

Antioxidant Effects of MEVA in Carrageenan-induced Air Pouch in Rats

Carrageenan injection into the pouch induces rapid recruitment of leukocytes with concormittant elevation in the lipid peroxidation reactions as measured by the MDA level as well as the reduction of GSH and SOD. Pretreatment with MEVA (200 mg/kg) and indomethacin (5 mg/kg) for 3 days before induction significantly prevented the carrageenan-induced lipid peroxidation by 48.01% and 73.0%, respectively, when compared to vehicle-treated controls [Table 1]. While MEVA inhibited MDA significantly ($P < 0.05$), it prevents depletion of GSH and z ($P > 0.05$). Meanwhile indomethacin (5 mg/kg) significantly increase GSH levels (23.3%; $P < 0.05$) and SOD activities (47.9%; $P < 0.001$) when compared to vehicle treated controls.

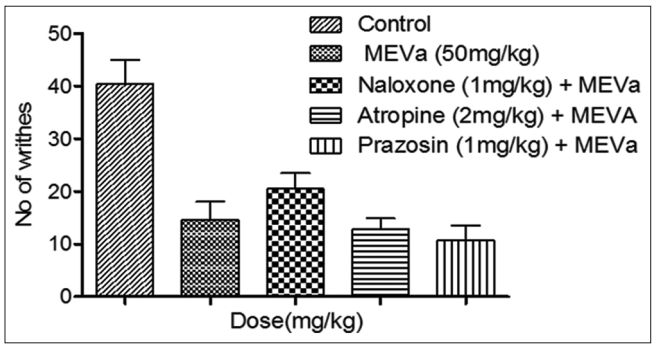

Figure 2: Effects of administration of opioidergic, adrenergic, and cholinergic blocker on the antinociceptive effects of methanol extract of *Vernonia amygdalina* against acetic acid-induced writhing test in mice. Data are expressed as mean ± SEM (n=6). Comparisons were made using one-way ANOVA followed by *post-hoc* Newman-Keuls test

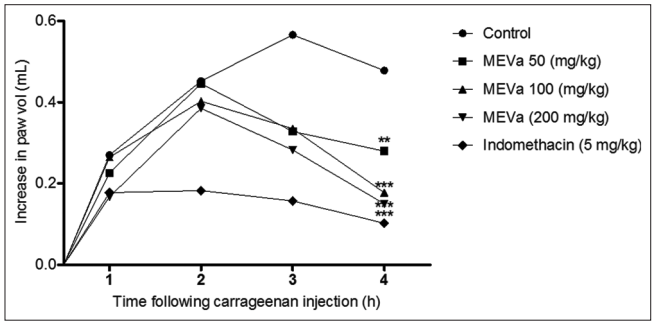

Figure 3: Effects of pretreatment of methanol extract of *Vernonia amygdalina* on carrageenan-induced hind paw edema in rat. Data are expressed as Mean ± SEM (n=5). Comparisons were made using one-way ANOVA followed by *post-hoc* Newman-Keuls test. The symbol denotes significant levels: ***P<0.001 compared with the control group

Histological Findings-effect of MEVA on Carrageenan-induced Air Pouch in Rat

The anti-inflammatory effects of MEVA were further assessed by examining the pouch tissue histologically [Figure 5]. The pouch tissue appeared thickened (characteristics of edema) due to infiltration of leukocytes after carrageenan injection in the air pouch [Figure 5a]. The treated groups which received of MEVA (200 mg/kg) and indomethacin (5 mg/kg) in Figure 5b and Figure 5c, respectively, showed a reduced in infiltration of inflammatory cells when compared with the vehicle-treated group.

DISCUSSION

This study investigated the antinociceptive and anti-inflammatory effects of the MEVA leaf and its possible mechanisms of action through the investigation of the involvement of the adrenergic, cholinergic, and opioidergic systems. To investigate its antinociceptive effect, the acetic acid-induced writhing in mice considered as a model for visceral pain was selected.

The acetic acid-induced writhing model has been known to produce tissue necrosis by a chemical irritant in the peritoneal region in laboratory rodents. It has been suggested that acetic acid acts by releasing endogenous inflammatory mediators of the nociceptive neurons [22,23], such as bradykinin, prostaglandins, and pro-inflammatory cytokines, when injected intraperitoneally [24,25]. The main cytokines involved in nociception, induced by acetic acid, are TNF-α, interleukin-1β, and interleukin 8, and they are released from resident peritoneal macrophages and mast cells [23,25]. From the acetic acid writhing test results, the doses of 50 mg/kg and 100 mg/kg of MEVA were observed to be more statistically significant in bringing about antinociception. These results suggest that 50 mg/kg may be the effective dose of MEVA in carrying out its antinociceptive property. The inhibitory effect carried out by MEVA against the nociceptive activity of acetic acid suggests the presence of active antinociceptive phytochemical present in *V. amygdalina* leaf.

Furthermore, from the acetic acid writhing tests, naloxone (a non-selective opioid antagonist) co-administered with MEVA was observed to have a more reversible effect on its antinociceptive property in comparison with atropine (a

Table 1: Effect of MEVA on MDA, GSH, and SOD in carrageenan-induced air pouch model of inflammation

Treatment	MDA (ηM of MDA/mg protein)	GSH (μM GSH/ml exudate)	SOD (mIU/mg protein)
Carrageenan	58.56±9.68	0.89±0.04	22.2±1.41
MEVA (200 mg/kg)	30.44±5.81**	0.98±0.06	28.52±2.71
Indo (5 mg/kg)	15.82±2.71***	1.16±0.08*	42.62±3.80***

MDA: Malondialdehyde, GSH: Glutathione, SOD: Superoxide dismutase, MEVA: Methanol extract of *V. amygdalina* leaf, Data are expressed as mean±SEM (n=5). Comparisons were made using one-way ANOVA followed by *post-hoc* Newman-Keuls test. *P<0.05, **P<0.01, ***P<0.001 compared with the carrageenan group

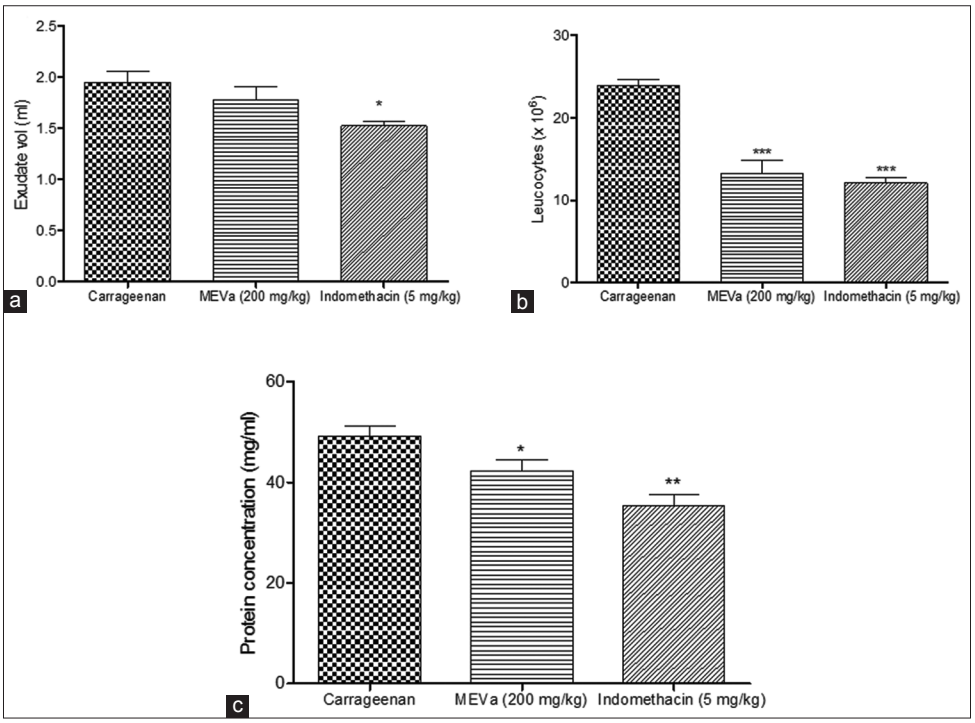

Figure 4: Anti-inflammatory effects of methanol extract of *Vernonia amygdalina* (200 mg/kg) in carrageenan-induced air pouch in rats. (a) Exudate volume, (b) leukocytes count, and (c) protein concentration. Data are expressed as mean ± SEM (n=5). Comparisons were made using one-way ANOVA followed by *post-hoc* Newman-Keuls test. *P<0.05; **P<0.01; ***P<0.001 compared with the carrageenan group

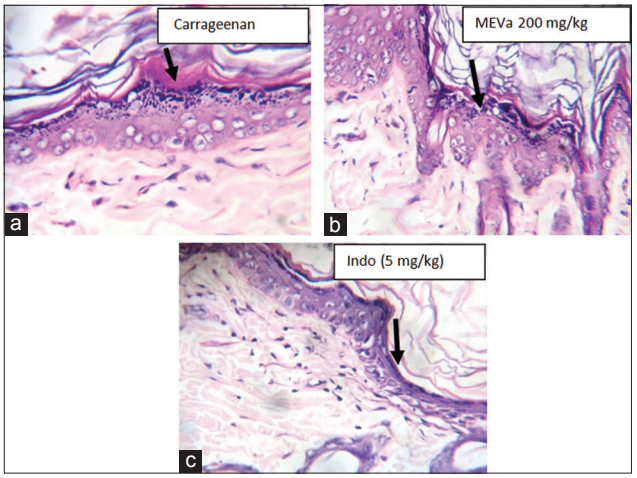

Figure 5: (a-c) Representative photomicrographs of H and E stained section of pouch tissue in the carrageenan-induced air pouch (magnification ×100)

cholinergic receptor antagonist) and prazosin (an adrenergic receptor antagonist). This result suggests that the basis of our findings on the antinociceptive property might involve the opioidergic system with little or no contribution of the adrenergic and cholinergic systems.

Carrageenan-induced rat paw edema is also a suitable experimental animal model for evaluating the anti-edematous effect of natural products and is believed to be biphasic [9]. The first phase (1 h) involves the release of serotonin and histamine while the second phase (over 1 h) is mediated by prostaglandins, the cyclooxygenase products, and the continuity between the two phases is provided by kinins [9,26]. Carrageenan - induced paw edema has been increasingly used to test new anti-inflammatory drugs as well as to study the mechanisms involved in inflammation [27]. From the results of this study, it was observed that MEVA (50-200 mg/kg) significantly inhibited the carrageenan-induced rat paw edema at the 4th h. Indomethacin, used as the reference drug, is shown to have a statistically significant inhibition at the 4th h post carrageenan-induction. Based on the results of this study, it could be suggested that the anti-inflammatory effect of this extract may be attributed to the inhibition of prostaglandin release or synthesis. This is

also in tandem with the mechanism of action of nonsteroidal anti-inflammatory drugs in the inhibition of inflammatory processes. Agents that suppressed carrageenan-induced paw edema particularly in the second phase are good candidates for further anti-inflammatory screening.

We further evaluated the anti-inflammatory mechanism of MEVA in carrageenan-induced air pouch model in rats. Carrageenan air pouch model was used because of the flexibility in its usage for other assessments and biochemical assays. The carrageenan air pouch model has been extensively used for the study of various types of inflammation and inflammatory processes. This model has distinct advantages over other models of inflammation because of the ability to perform biochemical analysis of both exudates and inflammatory cells together with the histological and angiogenesis analysis of the air pouch lining [17]. The results of this study indicated that after injection of the carrageenan solution into the air pouch, the pouch fluid volume, the total number of infiltrating leukocytes in the pouch fluid and distinct granulation of the pouch wall lining increased significantly.

From the results obtained, it was observed that there was a significant increase in the amount of exudate collected from the pouches of animals of the carrageenan only when compared with indomethacin-treated animals. MEVA (200 mg/kg) slightly reduced fluid exudation in the air pouch. We also observed a significant reduction in the infiltrating leukocytes, this was evidenced in the total leukocytes number in the exudates as well as the histological analysis of the pouch tissues. The protein concentration estimation of the exudates collected from the air pouches of the animals was carried out to ascertain the extent of increased vascular permeability that causes increased exudation of plasma protein into the pouch. MEVA reduced protein concentration in the air pouch. Carrageenan-induced inflammation is associated with protein leakage in the air pouch, mediated by inflammatory factors such as bradykinin, serotonin, histamine, prostaglandins [28]. Furthermore, leukocytes recruitment is characterized by infiltrating neutrophils which account for further release of potent pro-inflammatory molecules [29]. Since indomethacin (standard nonsteroidal anti-inflammatory drugs) demonstrated a similar profile, it is possible that both MEVA and indomethacin are acting through common pathways.

The main cells involved in the inflammatory response are monocytes/macrophages, polymorphonuclear leukocytes, and endothelial cells. When these cells become activated, they aggregate and infiltrate tissues where they undergo a respiratory burst, increasing their oxygen use and production of cytokines, reactive oxygen species (ROS), and other mediators of inflammation. These events can initiate and also perpetuate inflammatory cascades and cause subsequent tissue damage [30]. In the MDA content estimation of the exudates from the pouches of the animals, it was observed that the MDA level was significantly increased in the animals' group that received carrageenan only. MDA is a biomarker for lipid peroxidation. Lipid peroxidation is the oxidative degeneration of membrane lipids of endoplasmic reticulum. These lipids are rich in polyunsaturated fatty acids. These leads to the formation of lipid peroxides which in turn gives product such as MDA that can cause damage to the membranes.

Carrageenan injection into air pouch was hypothesized to cause increase in lipid peroxidation mediated via the increase in NO radical production [31]. The increased NO might be accompanied with the superoxide radical which induce a strong oxidant and peroxynitrite that results in acute endothelial dysfunction and thus activation of inflammation [32]. In our study, the increased level of MDA in the group of animals that received no treatment suggests that the antioxidant defense mechanism was compromised. MEVA and indomethacin, however, protected the animals against carrageenan-induced lipid peroxidation, hence reducing the oxidative stress levels in the animals.

GSH is a water-soluble tripeptide composed of the amino acids glutamine, cysteine, and glycine. GSH plays such an important role in the detoxification of a variety of electrophilic compounds and peroxides through catalysis by GSH S-transferases and GSH peroxidases [33]. Being a major endogenous non-enzymic antioxidant produced by the cells, it participates in neutralization of free radicals and ROS. From the results obtained, it was observed that the groups that received no treatment after carrageenan injection had a reduction in GSH level when compared with the group of animals that received normal saline only. Furthermore, the group of animals that received treatment of 200 mg/kg dose of MEVA had a slight increase in GSH level compared with the positive control group. The indomethacin group of animals also had a slight increase in GSH level. SOD is an important antioxidant defense in nearly all living cells exposed to oxygen. From the results, it was observed that the MEVA slightly increased SOD activities but significantly increased in indomethacin - treated animals. This suggests the fact that the generation of oxidative stress was slightly reduced by the antioxidant potentials of the MEVA.

Taken together, these findings showed that MEVA prevented the alteration on oxidative stress markers, it significantly decreased MDA level in exudate and prevented the depletion in reduced GSH levels and SOD activities induced by carrageenan. These results demonstrated the antioxidant action of MEVA in this model and suggest the effect as a possible mechanism of MEVA anti-inflammatory action.

Histological study showed that carrageenan-induced air pouch inflammation is characterized with infiltration of inflammatory cells, production of prominent artifactual granules, granuloma formation of the epidermal and dermal layer, and keratinization of the epidermal layer. From the results, it was observed that there was a reduction in the infiltration of inflammatory cells into the dermis of skin tissues of animals pre-treated with MEVA. This suggests that MEVA was able to reduce the migration of pro-inflammatory cells such as neutrophils into the pouches of the animal's skin. The *Stratum basale* appears normal, but the *Stratum corneum* and *Granulosum* appear mildly distorted unlike those which received no treatment.

CONCLUSION

V. amygdalina leaf possesses antinociceptive and anti-inflammatory properties. This justifies its use as a medicinal plant in some parts of the world. However, the mechanism through which it carries out its antinociceptive property is unlikely to involve the opioidergic, cholinergic, and adrenergic system. Its anti-inflammatory mechanism is mediated in part through reducing inflammatory leukocytes migration to inflammatory focal point and lipid peroxidation.

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Original Research

Geraniin attenuates naloxone-precipitated morphine withdrawal and morphine-induced tolerance in mice

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ABSTRACT

Background and Aim: Potentially life-threatening and unpleasant side effects associated with some analgesics have fueled the drive for the search for more analgesics with better side effect profiles. Geraniin, the most dominant secondary metabolite in the aqueous extract of the aerial parts of *Phyllanthus muellerianus*, has been shown to possess antinociceptive properties mediated partly by opioidergic mechanisms. The purpose of this study is to determine whether geraniin exhibits tolerance and if it is able to ameliorate withdrawal signs in naloxone-precipitated morphine withdrawal. **Materials and Methods:** After chronic treatment of mice with geraniin orally, the formalin test was used to ascertain whether tolerance will develop to its antinociceptive effects and if there is morphine-induced tolerance cross-generalization with geraniin. The effect of geraniin on naloxone-precipitated morphine withdrawal signs in morphine-dependent mice was also investigated. **Results:** Geraniin (3-30 mg/kg) did not produce any tolerant effects after chronic administration and there was also no cross-generalization with the tolerant effects of morphine. Geraniin did not induce withdrawal signs but significantly reduced the number of jumps in morphine-dependent mice. **Conclusion:** Geraniin does not produce any tolerant effects like morphine and also reduced the signs associated with naloxone-precipitated morphine withdrawal in mice.

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KEY WORDS: Dependence, geraniin, muellerianus, naloxone, withdrawal

INTRODUCTION

Pain forms part of most diseases and it is usually the major factor of the disease that alerts the patient to seek medical treatment [1]. Despite the frequency of pain symptoms, individuals often do not obtain satisfactory relief of pain possibly because of inappropriate or insufficient use of existing therapies [2,3]. Insufficient use of existing therapies can be due to the numerous and life-threatening side effects associated with the use of most of these agents [4,5]. The opioids are extensively used in moderate to severe pain management. However, physical dependence and tolerance to opioids occur to some degree whenever opioids are administered for more than a few days [6]. Dependence can result in various physical and psychological signs which in humans may include restlessness, irritability, increased salivation, lacrimation and sweating, muscle cramps, vomiting, and diarrhea [7]. These drawbacks limit the use of opioids in the management of pain and thus fueling the search for novel analgesics that do not have these side effects.

Although the mechanisms of the development of tolerance to and dependence on opioids have been vigorously investigated, it still remains unclear. Several cellular models including blockade of glutamate action at the N-methyl-D-aspartate (NMDA) receptors, phosphorylation and receptor conformation changes, decoupling of receptors from G-proteins and the receptor desensitization, μ-opioid receptor internalization and/or receptor downregulation and upregulation of the cyclic adenosine monophosphate pathway have been proposed to play important roles in the development of opioid tolerance and dependence [8,9]. Some medicinal plants and isolates, especially those with significant antioxidant activity, have proven beneficial in the attenuation of dependence and tolerance to opioid agents [10,11].

Geraniin ($C_{41}H_{28}O_{27}$) is a dehydroellagitannin which occurs as two isomers in solution [Figure 1]. It is pale amorphous compound with a molecular weight of 952.64 g/mol and density of 2.26 g/mL [12]. Geraniin has been shown to possess strong cellular proliferation effects using primary dermal fibroblasts and human adult high calcium low temperature (HaCaT) keratinocytes [12]. It has also been shown to possess antiviral [13] and anti-inflammatory properties [14]. Geraniin has been reported previously as a very potent antioxidant [15].

The antinociceptive properties and possible mechanism of action of geraniin in animal models have been recently reported [16] to have some opioidergic involvement. Considering the drawbacks of opioid analgesics, this study, therefore, seeks to determine if geraniin exhibits tolerance like the conventional opioids and if geraniin can have a potential use in the management of naloxone-precipitated morphine withdrawal.

MATERIALS AND METHODS

Source of Geraniin

Geraniin (96% w/w HPLC grade) (CAS number: 60976-49-0) [Figure 1], isolated from the aqueous extract of the aerial parts of *Phyllanthus muellerianus* (Kuntze) Exell., was a kind donation by Prof. Andreas Hensel, Institute of Pharmaceutical Biology and Phytochemistry, University of Muenster, Muenster, Germany. This study was conducted in June 2015.

Animals

ICR mice (30 ± 5 g) were obtained from the Noguchi Memorial Institute for Medical Research, Ghana and housed in the vivarium of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology, KNUST. They were housed in stainless steel cages in groups of 5 animals per cage with soft wood shavings as bedding. Food (normal mice chow: Agricare Ltd., Kumasi, Ghana) and tap water were *ad libitum*. Mice were also maintained under standard laboratory conditions (temperature 24-25°C, relative humidity 60-70%, and 12 h light-dark cycle). All procedures and techniques used in these studies were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (Animal Care and Use Committee, 1998). All protocols used were approved by the Departmental Ethics Committee (No.: FPPS/PCOL/0115/2015).

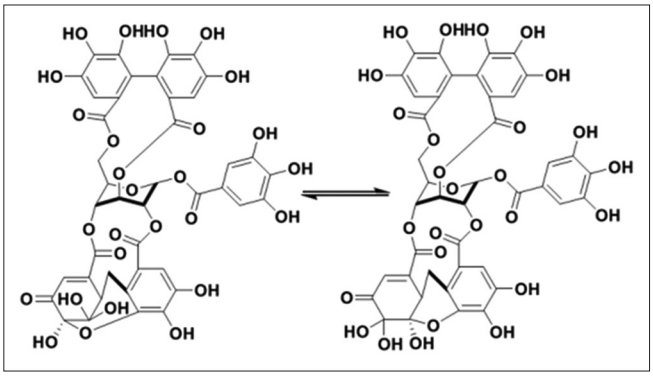

Figure 1: Chemical structures of the two isomers of geraniin in solution (adapted from Agyare *et al.,* 2011)

Drugs and Chemicals

Morphine hydrochloride and naloxone hydrochloride were obtained from Bodene (PTY) Limited Trading; Port Elizabeth, South Africa; formalin was purchased from British Drug Houses, Poole, England and diazepam (DZP) from Kilitch Drugs, Maharashtra, India. Doses of drugs used in this study are based on previous preliminary works carried on in the laboratory. Normal saline was used as vehicle throughout this study.

Assessment of Tolerance Liability

The formalin test [16] was used to ascertain whether, after chronic treatment, tolerance develops to the antinociceptive effects of geraniin and morphine as well as whether morphine-induced tolerance cross-generalize with geraniin. Mice were divided randomly into six groups ($n = 5$) and for the first 8 days were treated as follows:

- Groups I-III - Vehicle (10 ml/kg, p.o.)
- Groups IV - geraniin (20 mg/kg, p.o.)
- Groups V-VI - Morphine (6 mg/kg, i.p.).

On day 9, animals in Group I were given vehicle (10 ml/kg, p.o.), Groups II and IV received geraniin (10 mg/kg), and Groups III and V were also given morphine (3 mg/kg). To ascertain the possibility of morphine-induced tolerance cross-generalizing with geraniin, Group VI also received geraniin (10 mg/kg). Summary treatments on day 9 were as follows:

- Group I - Vehicle treatment for 8 days and vehicle again on day 9
- Group II - Vehicle treatment for 8 days and geraniin (10 mg/kg) on day 9
- Group III - Vehicle treatment for 8 days and morphine (3 mg/kg) on day 9
- Group IV - Geraniin (20 mg/kg) treatment for 8 days and geraniin (10 mg/kg) on day 9
- Group V - Morphine (6 mg/kg) treatment for 8 days and morphine (3 mg/kg) on day 9
- Group VI - Morphine (6 mg/kg) treatment for 8 days and geraniin (10 mg/kg) on day 9.

60 min after geraniin and 30 min after morphine administration, formalin was injected into the right paw and the behavior of the mice was recorded for 60 min. A nociceptive score was determined for each 5 min time block by measuring the amount of time spent in the biting/licking of the injected paw. Tracking of the behavior was done using public domain software JWatcher™, Version 1.0. The average nociceptive score for each animal per time block was calculated by multiplying the frequency and time spent in biting/licking and data were expressed as the mean ± standard error of the mean (SEM) of scores between 0 and 10 min (first phase) and 10 and 60 min (second phase) after formalin injection.

Ability of Geraniin to Induce Withdrawal Syndromes of Dependence

A method previously described by [17] was used to determine whether geraniin induces withdrawal signs similar to that produced by morphine administration. Mice were grouped ($n = 5$) and received the following drug treatments.

For the first 3 days,

- Group I - morphine (50, 50 and 75 mg/kg s.c. at 1100, 1400 and 1700 h, respectively)
- Group II - vehicle (10 ml/kg s.c. at 1100, 1400 and 1700 h, respectively)
- Group III - geraniin (150, 150 and 225 mg/kg p.o. at 1100, 1400 and 1700 h, respectively).

On the 4th day, Group I received morphine (50 mg/kg, s.c.), Group II received vehicle (10 ml/kg, s.c.), and Group III received geraniin (150 mg/kg, p.o.). 2 h later, naloxone (5 mg/kg, s.c.) was administered to all the animals and mice were immediately placed in a glass cylinder (30 cm high, 20 cm in diameter). The number of jumping episodes (withdrawal symptoms) was recorded for 30 min.

Effect of Geraniin on Naloxone-precipitated Morphine Withdrawal Signs

To determine whether geraniin can ameliorate withdrawal signs produced by naloxone in morphine-dependent animals [17], mice were randomly assigned to 5 groups (I-V) ($n = 5$). Groups I-V received morphine (50, 50 and 75 mg/kg s.c. at 1100, 1400 and 1700 h, respectively) for 3 days.

On the 4th day, group I received vehicle (10 ml/kg, p.o.), Groups II-IV received geraniin (3-30 mg/kg, p.o.), and Group V were treated with DZP (5 mg/kg, i.p.). 30 min (for i.p.) or 1 h (for p.o.) later, animals in all the groups received morphine (50 mg/kg, s.c.). 2 h later they were all treated with naloxone (5 mg/kg, s.c.) to precipitate opioid-induced withdrawal. Animal behavior was recorded for 30 min and the number of jumps counted using the public domain software JWatcher™, version 1.0.

Effects of Geraniin on Motor Co-ordination

To rule out the possible effect of geraniin affecting motor function, the rota-rod test was performed. Naive mice were trained on 3 successive days on the rota-rod (Ugo Basile, model 7600, Comerio, Varese, Italy) at a speed of 25 rpm. On the test day (day 4), five groups of mice ($n = 5$) were administered geraniin (3-30 mg/kg, p.o.), D-tubocurarine (0.1 mg/kg, i.p.) or vehicle. The animals were then repeatedly tested for their motor co-ordination performance on the rota-rod (cut-off time 120 s) at 0.5, 1, 1.5, 2, 2.5, and 3 h after drug administration. The maximum time that the animals were able to spend on the rota-rod was recorded [18].

Data Analysis

All data are presented as mean ± SEM ($n = 5$). The time-course curves were subjected to two-way (treatment × time) repeated measures analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. Total nociceptive score for each treatment was calculated in arbitrary unit as the area under the curve (AUC). Differences in AUCs were analyzed using one-way ANOVA for normally distributed data or Kruskal–Wallis test for data that was not normally distributed with drug treatment as a between subjects factor. Further comparisons between vehicle and drug-treated groups were performed using the Tukey's multiple comparisons test. $P \leq 0.05$ was considered statistically significant. Data analysis was done in September 2015.

RESULTS

The Assessment of Tolerance Liabilities

A two-way ANOVA showed a significant difference in the observed effects ($F_{5,24} = 104.5$, $P < 0.0001$) [Figure 2]. Morphine (3 mg/kg, i.p.) significantly attenuated nociceptive responses in both phases ($F_{2,12} = 51.15$, $P < 0.0001$ Phase I; $F_{2,12} = 82.99$, $P < 0.0001$ Phase II) of the formalin test in chronic vehicle-treated animals as seen in the AUC graphs. However, the same dose of morphine administered at day 9 in animals chronically treated with morphine (6 mg/kg, i.p.) failed to show such effect indicating the development of tolerance [Figures 2 and 3]. In contrast, oral administration of geraniin (10 mg/kg) showed a comparable antinociceptive activity ($F_{3,16} = 53.72$, $P < 0.0001$ Phase I; $F_{3,14} = 137.2$, $P < 0.0001$ Phase II) in mice treated chronically with either geraniin (20 mg/kg, p.o.) or normal saline, indicating lack of tolerance development [Figures 2 and 3]. Furthermore, geraniin (10 mg/kg, p.o.) still demonstrated appreciable antinociceptive activity in mice chronically treated with morphine (6 mg/kg, i.p.), indicating that no cross-tolerance exists with morphine [Figures 2 and 3].

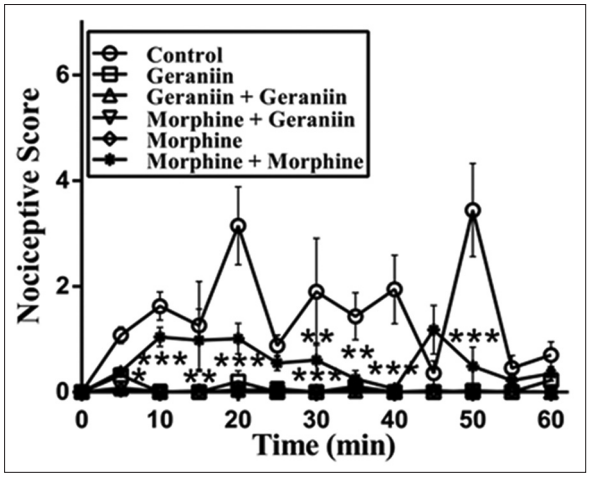

Figure 2: Effect of geraniin (10 mg/kg) and morphine (3 mg/kg) challenge on the time course effect of the total nociceptive score of formalin-induced licking of mice chronically treated with vehicle, geraniin (20 mg/kg) or morphine (6 mg/kg) for 8 days. Each point represents the mean ± standard error of the mean. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ compared to respective controls (two-way ANOVA followed by Dunnett's multiple comparisons test)

Evaluation of Dependence Liability

Withdrawal signs notably jumping, writhing, and diarrhea were observed after naloxone was administered to the morphine-treated animals. However, these effects were not observed in the geraniin or normal saline treated animals as shown in Figure 4. The incidence of jumping episodes was quantified and used to assess the extent of withdrawal [Figure 4]. Two-way ANOVA revealed a significant incidence of jumping episodes for morphine $F_{2,12} = 48.39$, $P < 0.0001$ [Figure 4a]; $F_{2,12} = 48.83$, $P < 0.0001$ [Figure 4b] compared to the normal saline treated animals. There was, however, no significant difference between the numbers of jumps for the geraniin treated and normal saline treated animals as depicted in the AUC graph [Figure 4].

Two-way ANOVA of the effect of geraniin showed a significant suppression in the jumping behavior of the mice after it was administered 1 h before the last dose of morphine ($F_{4,15} = 62.34$, $P < 0.0001$) [Figure 5a]. From the AUC, geraniin at the highest dose used blocked the morphine-dependent withdrawal effect by 96.52% [Figure 5b]. DZP, used as a positive control also caused a significant reduction in the total number of jumps by 97.90% [Figure 5].

Effects on Motor Co-ordination

Geraniin (3-30 mg/kg, p.o.) at all doses tested did not cause any significant change in the time spent on the rota-rod as shown in Figure 6a and b. The nondepolarizing neuromuscular blocker D-Tubocurarine (D-TC, 0.1 mg/kg), however, caused a significant reduction in the time spent on the rota-rod [Figure 6a and b].

DISCUSSION

This study has been able to show that tolerance does not develop to the antinociceptive effects of geraniin. Furthermore, geraniin

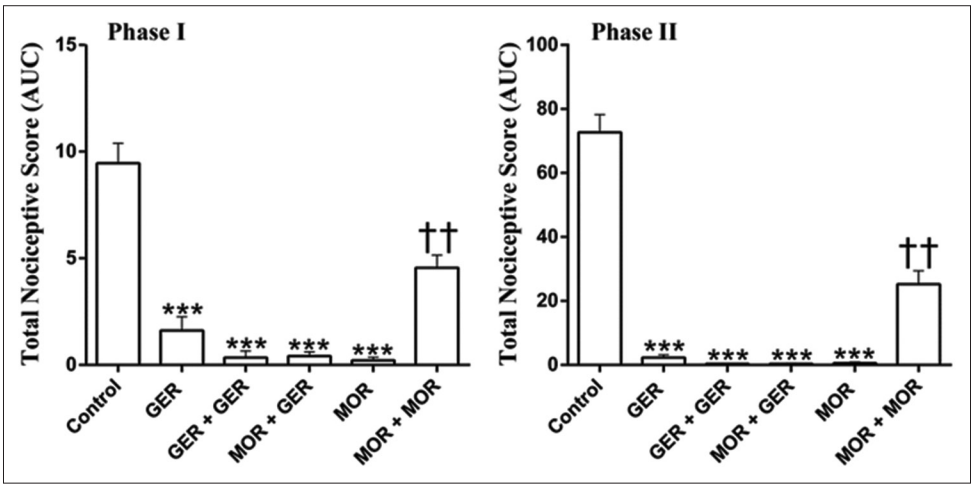

Figure 3: Effect of geraniin (10 mg/kg) and morphine (3 mg/kg) challenge on mice chronically treated with vehicle, geraniin (20 mg/kg) or morphine (6 mg/kg) for 8 days on the total nociceptive score of formalin-induced licking test in mice area under the curve for (a) phase I and (b) phase II of formalin-induced pain, respectively. Each column represents the mean ± standard error of the mean. ***$P \leq 0.001$ compared to vehicle control, ††$P \leq 0.01$ compared to MOR (one-way ANOVA followed by Tukey's multiple comparisons test). Control = animals treated with vehicle for 8 days and vehicle again on day 9, GER = animals treated with vehicle for 8 days and geraniin (10 mg/kg) on day 9, MOR = animals treated with vehicle for 8 days and morphine (3 mg/kg) on day 9, GER+GER = animals treated with geraniin (20 mg/kg) for 8 days and geraniin (10 mg/kg) on day 9, MOR+MOR = animals treated with morphine (6 mg/kg) for 8 days and morphine (3 mg/kg) on day 9, MOR+GER = animals treated with morphine (6 mg/kg) for 8 days and geraniin (10 mg/kg) on day 9

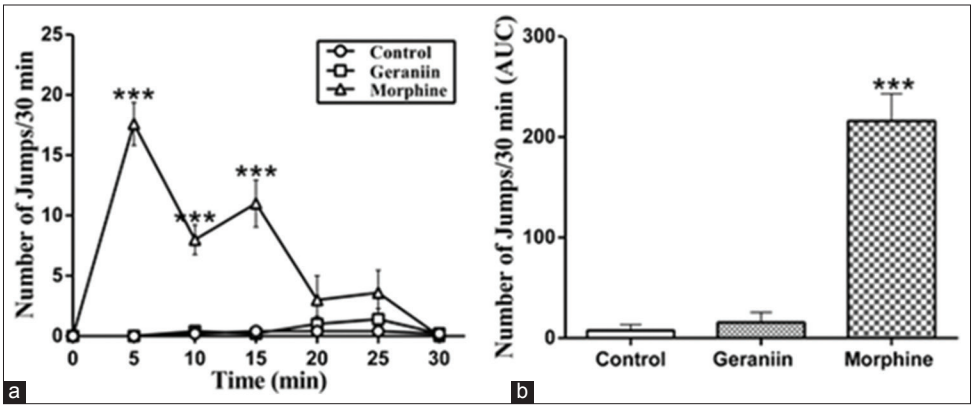

Figure 4: Effect of geraniin, vehicle and morphine in inducing withdrawal signs in mice depicted as (a) a time course curve and (b) an area under the curve graph, respectively. Data represented as mean ± standard error of the mean. ***$P \leq 0.001$ compared to the control

did not cause dependence in mice after multiple administrations like morphine and was, however, effective in reducing signs of withdrawal (jumping episodes) in morphine-dependent mice.

The usefulness of opioid analgesics in clinical settings is often hampered by the development of tolerance that necessitates dose increase regardless of disease progression [19]. Tolerance produced by sustained morphine treatment has been associated with regulation of Transient Receptor Potential Vanilloid 1 (TRPV1) receptor, which is induced by chronic morphine application via mitogen activated protein kinases (MAPK) signaling pathways [19]. Other mechanisms that have also been suggested to contribute to morphine induced-tolerance include activation of the NMDA receptors and calcium regulated intracellular protein kinase C mechanisms [20,21]. Results from this study have shown that, unlike morphine, geraniin, a plant isolate with antinociceptive effect involving opioidergic mechanisms [16], at doses tested, does not induce tolerance after chronic administration in the formalin test of nociception. Moreover, in animals that showed significant tolerance to the analgesic effects of morphine, geraniin was still able to exhibit analgesic effects. These observed effects of geraniin could be due to interactions or blockade of the TRPV1 receptor or the MAPK signaling pathway and or blockade of the NMDA receptor or alterations in glutamate levels.

Development of physical dependence which is manifested by a unique withdrawal syndrome with diverse behavioral and physiological signs is also a limiting factor to the usefulness of most of the opioid analgesics including morphine [22]. Withdrawal is typically observed following abrupt termination of morphine intake or precipitated by administration of a narcotic antagonist such as naloxone [23]. Several withdrawal behaviors have been reported in rodents including jumping, exploratory rearing, body shakes, ptosis, weight loss, and diarrhea [22]. However, jumping has been widely considered the most sensitive and reliable index of withdrawal intensity and thus commonly used to assess the extent of morphine withdrawal [24]. Jumping is the most suitable sign of measuring withdrawal as jumps are easily counted and jumping rate increases when dependence increases or dose of antagonist increased [25].

Physical dependence signs associated with opioid withdrawal have been attributed to a rebound hyperactivity of both the dopaminergic and adrenergic systems in the central nervous system (CNS) [11]. Adenylate cyclase supersensitivity has also been observed in different brain areas including the nucleus accumbens, amygdala, and the locus coeruleus of morphine-dependent animals [26].

Benzodiazepines, via Gamma aminobutyric acid type A receptors, have been shown to have an inhibitory effect on the

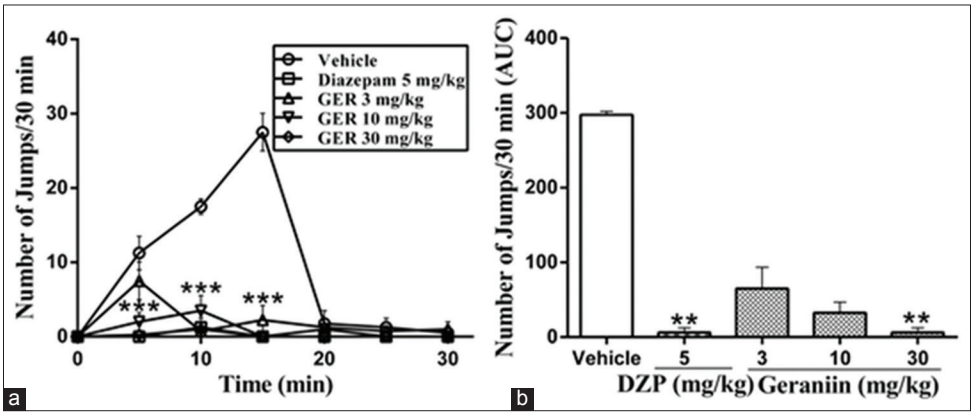

Figure 5: Effect of geraniin, diazepam and vehicle on the naloxone-precipitated withdrawal syndrome of morphine dependence in mice depicted as (a) the time course curve and (b) area under the curve, respectively. Data represented as mean ± standard error of the mean. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ compared to the control

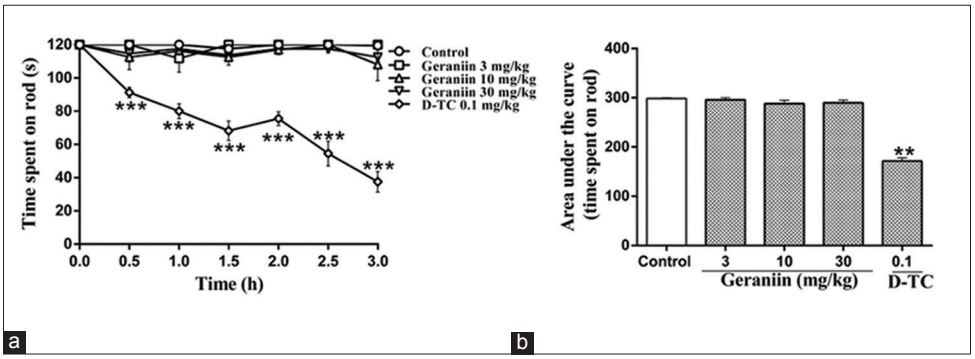

Figure 6: Effects of geraniin (3-100 mg/kg), D-Tubocurarine (D-TC, 0.1 mg/kg) and vehicle on time spent on rods shown as (a) time course curves and (b) area under the curve. Time course curve analyzed by a two-way ANOVA followed by Dunnett's multiple comparison test while area under the curve was analyzed by Kruskal–Wallis test followed by Dunn's multiple comparison test. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$

dependence of morphine [22] and this was illustrated in this study when DZP at a dose of 5 mg/kg significantly reduced the jumping episodes in morphine-dependent mice. Proposed reasons for the effectiveness of benzodiazepine in suppressing the development of physical dependence on morphine include their continuous activation of benzodiazepine binding sites during chronic morphine treatment and the inhibition of the increase in Ca^{2+} level which results from chronic morphine treatment [22].

In this study, geraniin showed inhibitory effect against withdrawal signs of morphine dependence without impairing motor function. It is possible that geraniin was able to interfere with dopaminergic and/or adrenergic activities in the CNS. With geraniin also being a polyphenolic compound, these results are in agreement with previous reports citing the inhibitory effects of polyphenolic compounds on naloxone precipitated morphine withdrawal [27,28]. Even though, the extent of its inhibitory effect was comparable to that of DZP at the highest dose, the mechanism of action cannot be emphatically stated to be via the same pathway as DZP.

Some oxidant species including nitric oxide, superoxide anions, and malondialdehyde have been implicated in the development of opiate abstinence symptoms [29,30]. As such, some agents with significant antioxidant activity have proven effective in reducing opioid withdrawal signs [11]. Geraniin has been reported previously as a very potent antioxidant [15] which could also contribute to the effectiveness of geraniin in attenuating naloxone-precipitated morphine withdrawal signs in mice.

In conclusion, results presented from this study indicate that geraniin does not appear to have any tolerant effect, it does not induce withdrawal signs and also alleviates signs associated with naloxone-precipitated morphine withdrawal.

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Original Research

Bio-efficacy of medicinal plants used for the management of diabetes mellitus in Gabon: An ethnopharmacological approach

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ABSTRACT

Background/Aim: People suffering of diabetes increased significantly worldwide. Population, in Sub-Saharan Africa and mainly in Gabon, rely on medicinal plants to manage diabetes, as well in rural as in urban areas. This study aimed to survey a wide range of Gabonese plants for their antidiabetic activity. **Materials and Methods:** This study focused on the identification of medicinal plants used in the local treatment of diabetes mellitus. Ethnobotanical investigations were carried out in rural and urban areas of three provinces of Gabon using a semi-structured interview. **Results:** About 50 plant species belonging to 31 families and 50 genera were recorded, a majority of which have been documented previously to have medicinal properties. Most have documented antidiabetic properties with characterized therapeutic chemical compounds. Of the plant parts used for treatment, stem barks were employed most frequently (50%), followed by leaves (26%); the remaining 24% comprised roots, fibers, fruit, bulbs, flowers, rhizom, skin, and stem. Regarding the mode of preparation, decoction was the most widely used (58%), followed by maceration (18%) and infusion (14%). Almost all the plant products were administered orally (98%). **Conclusions:** Taken in concert, this study highlights the possibility of exploiting traditional knowledge of specific medicinal plants for the inexpensive treatment and management of diabetes.

KEY WORDS: Medical plants, Bio-efficacy, diabetes mellitus, ethnopharmacology, Gabon

INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by disruption of carbohydrate, fat, and protein metabolism. The disorder is associated with severe complications, including retinopathy, microangiopathy, and peripheral neuropathy [1]. Diabetes causes major economic losses worldwide and impedes country development [2,3].

The number of persons affected by diabetes is expected to reach 438.4 million worldwide in 2030 [4]. Only a fraction (49.3%) of the population in Africa has been tested for the disease [5] but, in sub-Saharan Africa alone, an estimated 10.4 million people lived with diabetes in 2007 [6]. In the central African country of Gabon, which has a population of ~1.7 million people [7], 10.71% of the population has been diagnosed with this disease [8]. Since, pharmaceutical products used for the management of diabetes are expensive for rural populations and may induce serious side effects [9], medicinal plants are used predominately to treat this disease. According to George *et al.* [10], medicinal plants contain biologically active compounds with diverse therapeutic applications. For example, saponins and alkaloids in *Alstonia boonei* De Wild. have a diuretic effect and are utilized in the treatment of urinary edema and hypertension [11]. The fungicidal action of saponins in (*Piptadeniastrum africanum* Hoof. f.) Brenan provides another example [12] used in traditional medicine. In Gabon, 78.2% of the species of plants in forests are used medicinally by pygmies [13], which exemplifies this country's botanical medicinal heritage. It is important to improve understanding of plants used by local people in the treatment of diabetes in Gabon and which may have beneficial applications for the world at large. The aim of this study is to survey a wide range of Gabonese plants for their antidiabetic activity. Studies were performed in villages and towns across three provinces in Gabon that represent different types of rainforest.

MATERIALS AND METHODS

Study Area

Gabon is a small francophone country located in Central Africa bordering the Atlantic Ocean at the Equator between the Republic of the Congo and Equatorial Guinea. The climate

is always hot and humid. Gabon houses some of Africa's most biodiverse rainforests, which comprise approximately 80% of the country and stretch to the coast. Research in the Northwest and South Central/East of Gabon was done in the following three provinces: Estuaire (N.W. coastal region), Ogooué-Lolo (south-central forest region), and Haut-Ogooué (southeast mosaic of forest-savanna) [Figure 1]. The sampling was conducted in both rural areas and urban regions, including is even towns and six departments of the three provinces [Table 1].

Investigation Method

The ethnobotanical survey was conducted between October 2014 and March 2015, which spans periods of sparse but heavy rainfall (October-November), a short dry season (December-January), and part of the long wet season with heavy rainfall (February-April). The investigation was carried out using a semi-structured questionnaire in French or in the native language of the informant. Interviewees included diabetic patients, traditional healers, traditional health practitioners, herbalists, and other knowledgeable people. The recorded parameters were locality, sociodemographic data (age and gender), vernacular or local plant names, plant parts used, method of preparation, method of administration, quantity consumed, and type of material, samples collected for botanical identification were dried, preserved and identified by an expert botanist, ISSEMBE Yves, at National Herbarium of Libreville, Gabon. The Latino names of some plant species have been updated using the plant list database [14].

Data Analysis

The frequency of citation (FC) of a plant species was evaluated using the following formula: FC = (Number of times a particular species was mentioned/Total number of times that all species were mentioned) × 100 [15,16].

Table 1: Demographic data of key informants

Informants group	Number of persons (urban/rural)	Age (years)	Professional experience (years)
Diabetic patients	8/6	50-65	-
Traditional healers	0/29	27-69	10-30
Traditional health practitioners	10/0	40-65	10-30
Herbalists	15/0	25-45	3-10

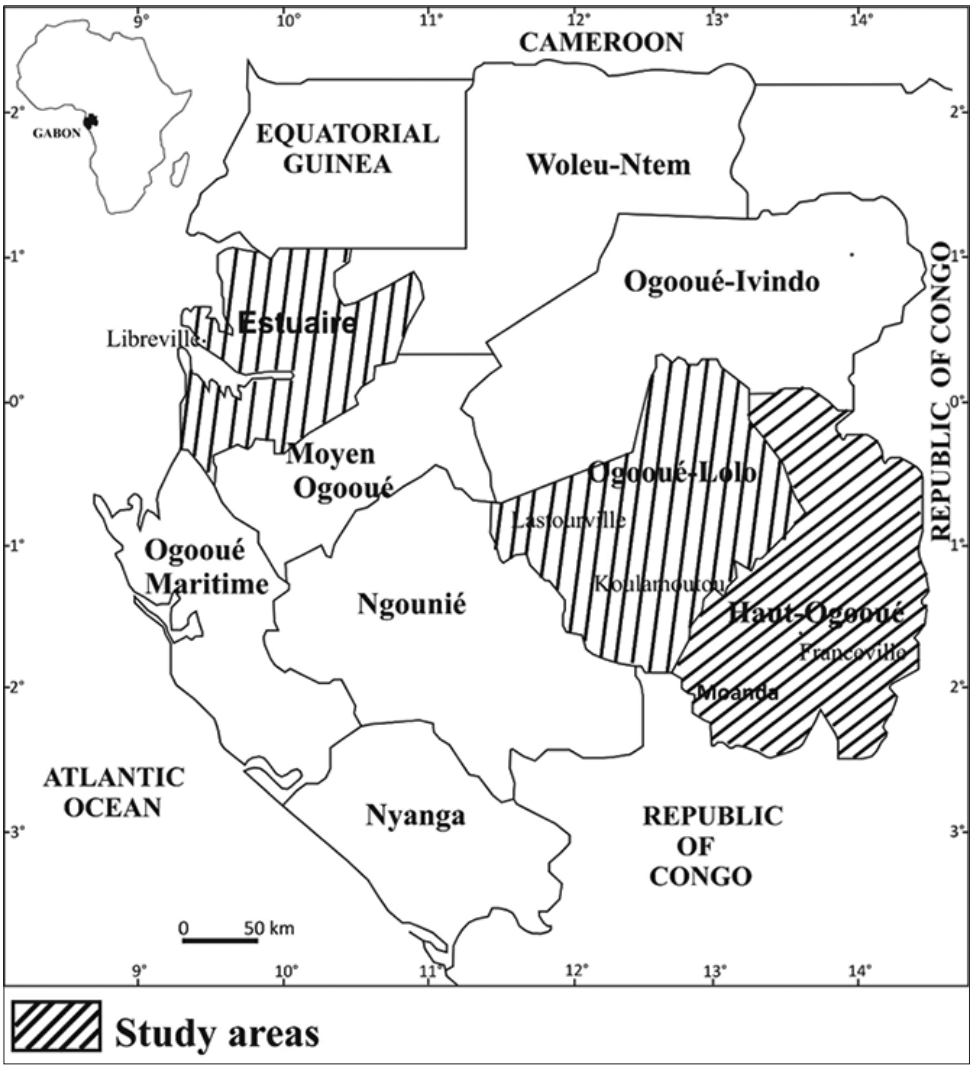

Figure 1: Map of study areas

RESULTS

Demographic Characteristics

A total of 80 people were investigated, of which 68 informants had a rich knowledge of herbal medicine [Table 2]. The balance did not report knowledge of medicinal plants and was excluded from further study. Of those that reported informations; 14 were patients with physician-diagnosed a diabetes mellitus or people were relatives of people suffering from diabetes, 29 were traditional healers, 10 were traditional health practitioners, and 15 were herbalists. More than half (65%) of the interviewees were male, and the average age of both sexes was approximatively 53 years with informants ranging in age until 70 years. More than half of all respondents (51.5%) were from rural areas, traditional healers who were the most numerous informants were mainly represented areas rural while herbalists and traditional health practitioners were only recorded that in urban areas.

Ethnobotanical Characteristics and Associated Knowledge

The species cited by respondents in this study were listed in alphabetical order by scientific name, local or vernacular name, family, genus, plants parts used, mode of preparation, mode of administration, and FC [Table 2]. 50 species belonging to 31 families and 50 genus were used for the treatment of diabetes. The Annonaceae was the most commonly represented of all families [Figure 2], with particular use of soursop *Annona muricata* L. Nine plant species were most cited by interviewers as a remedy for diabetes, of which *Guibourtia tessmannii* (Harms) J. Leonard (*Caesalpinioideae*) was the most frequent (7.14%) followed by A. *boonei* (Apocyanceae), *Carica papaya* L. (*Caricaceae*), *Persea americana* Mill. (*Lauraceae*), *Allium sativum* L. (*Amaryllidaceae*), *A. muricata* (*Annonaceae*), *Ceiba pentandra* (L.) Gaertn. (*Malvaceae*), *Cocos nucifera* L. (*Arecaceae*), *Picralima nitida* (Stapf) T. Durand and H. Durand (*Apocynaceae*) (4.29%). The others species were least cited, it is the case of *Annickia chlorantha* (Oliv.) Setten and Maas (*Annonaceae*), *Cymbopogon citratus* (DC.) Stapf (*Poaceae*), *Eurypetalum tessmannii* Harms (*Caesalpinioideae*), *Lantana camara* L. (*Verbenaceae*), *Musa* × *paradisiaca* L. (*Musaceae*), *Psidium guajava* L. (*Myrtaceae*), *Vernonia amygdalina* Delile (*Asteraceae*), *Xylopia aethiopica* (Dunal) A. Rich. (*Annonaceae*), and the gymnosperm *Gnetum africanum* Welw. (*Gnetaceae*) [Table 2]. Bibliographic research showed that about 94% of plants were well-documented in literature [Table 3]. All 50 plants are used to prepare medicinal drugs individually or in various combinations.

The result shows that the most frequently used plant parts were stem barks (50%) followed by leaves (26%) and other plant parts (24%), including roots (6%), fibers (4%), bulbs, fruit, flower, rhizom, skin, and stem (2% each) [Figure 3]. Most components were prepared by decoction (58%). Maceration (18%) and infusion (14%) were other modes of preparation and use, as was chewing (4%), burning and cooking (2%) [Figure 4]. Three modes of administration were used. Herbal products were primarily administered orally (98% of cases), mostly in liquid form (88%). Administration by mastication was also recorded (10% of cases) as was treatment by vapor bath (2% of cases) [Figure 5].

DISCUSSION

The results of demographic data showed that most knowledgeable interviewees were male (65%) of average age >50 years. A previous study found that women (69%) frequently used more medicinal plants than men (31%) [145]. Uniyal *et al.* [146] also found that men knew comparatively more about plant-based medicines than females because women were occupied by household working pressure. In Gabon, women tender house gardens and are more ready than men to bring out the first health care.

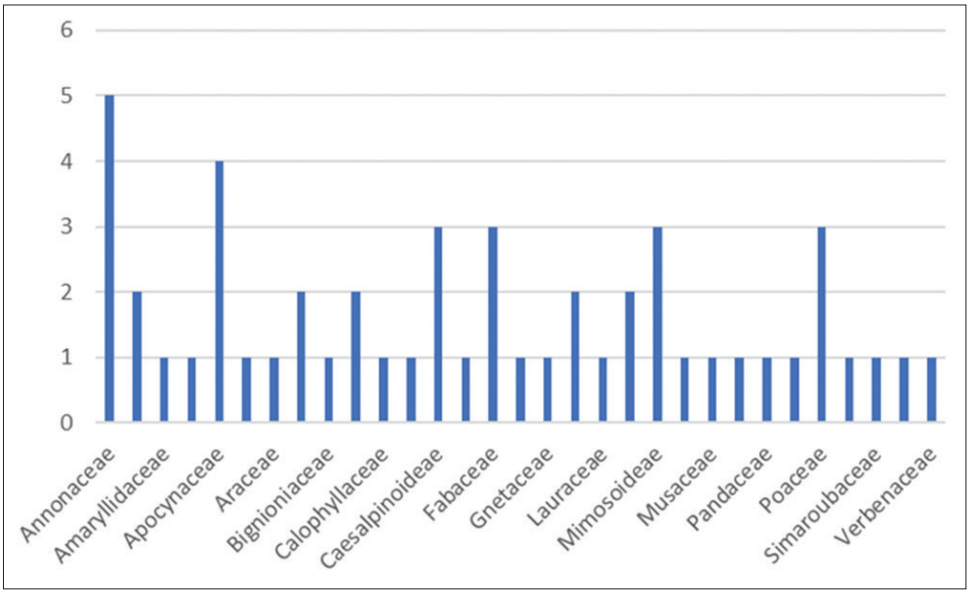

Figure 2: Repartition of plants families

Table 2: Data of medicinal plants traditionally used for the management diabetes mellitus

Botanicals names	Local names/ venacular	Families	Genus	Parts used	Mode of preparation	Mode of administration	Numbers of citations	Frequencies of citations
Acacia auriculiformis Benth.	Akasmani	*Fabaceae*	Acacia	Leaves	Infusion	Steam bath	1	1,428,571
Allium sativum L.	Garlic	*Amaryllidaceae*	Allium	Bulb	Decoction	Drink	3	4,285,714
Alstonia boonei De Wild.	Emien	*Apocynaceae*	Alstonia	Stem barks	Maceration	Drink	3	4,285,714
Anchomanes difformis (Blume) Engl.	Nkwe-ndôjgu (Galoa)	*Araceae*	Anchomanes	Rhizom	Maceration	Drink	1	1,428,571
Annickia chlorantha (Oliv.) Setten & Maas	Mwamba jaune	*Annonaceae*	Annickia	Stem barks	Decoction	Drink	1	1,428,571
Annona muricata L.	Soursop	*Annonaceae*	Annona	Stem barks	Decoction	Drink	3	4,285,714
Anonidium mannii (Oliv.) Engl. and Diels	Ebom	*Annonaceae*	Anonidium	Stem barks	Decoction	Drink	1	1,428,571
Antrocaryon klaineanum Pierre	Onzabili	*Anacardiaceae*	Antrocaryon	Stem barks	Infusion	Drink	1	1,428,571
Aucoumea klaineana Pierre	Okoumé	Burseraceae	Aucoumea	Stem barks	Maceration	Drink	1	1,428,571
Carica papaya L.	Papaya	*Caricaceae*	Carica	Root	Decoction	Drink	3	4,285,714
Ceiba pentandra (L.) Gaertn.	Fromage	*Malvaceae*	Ceiba	Stem barks	Decoction	Drink	3	4,285,714
Celtis tessmannii Rendle	Diania	*Cannabaceae*	Celtis	Stem barks	Decoction	Drink	1	1,428,571
Cleistopholis glauca Pierre ex Engl. and Diels	Unknown	*Annonaceae*	Cleistopholis	Stem barks	Decoction	Drink	1	1,428,571
Cocos nucifera L.	Coconut	*Aracaceae*	Cocos	Fiber	Decoction	Drink	3	4,285,714
Combretum micranthum G. Don	Kinkêliba	*Combretaceae*	Combretum	Leaves	Infusion	Drink	1	1,428,571
Copaifera mildbraedii Harms	Murei (Punu)	*Caesalpinioideae*	Copaifera	Stem barks	Decoction	Drink	1	1,428,571
Cylicodiscus gabunensis Harms	Okan	*Mimosoideae*	Cylicodiscus	Stem barks	Decoction	Drink	1	1,428,571
Cymbopogon citratus (DC.) Stapf	Lemongrass	*Poaceae*	Cymbopogon	Leaves	Infusion	Drink	1	1,428,571
Duboscia macrocarpa Bocq.	Akak	*Malvaceae*	Duboscia	Stem barks	Decoction	Drink	1	1,428,571
Entada gigas (L.) Fawcett and Rendle	Cœur de mer	*Mimosoideae*	Entada	Stem barks	Decoction	Drink	1	1,428,571
Eurypetalum tessmannii Harms	Anzilim	*Caesalpinioideae*	Eurypetalum	Stem barks	Decoction	Drink	1	1,428,571
Gnetum africanum Welw.	Nkumu	*Gnetaceae*	Gnetum	Leaves	Cooking	Eat	1	1,428,571
Guibourtia tessmannii (Harms) J. Leonard	kévazigo	*Caesalpinioideae*	Guibuortia	Stem barks	Decoction	Drink	5	7,142,857
Harungana madagascariensis Lam. ex Poir.	Atsui	Hyperaceae	Harungana	Leaves	Chewing	Eat	1	1,428,571
Lantana camara L.	Lantanier	*Verbenaceae*	Lantana	Leaves	Infusion	Drink	1	1,428,571
Mammea africana Sabine	Oboto	*Calophyllaceae*	Mammea	Stem barks	Decoction	Drink	1	1,428,571
Microdesmis puberula Hook.f. ex Planch.	Inko	*Pandaceae*	Microdesmis	Stem barks	Infusion	Drink	1	1,428,571
Milicia excelsa (Welw.) C. C. Berg	Obiga (Akélé)	*Moraceae*	Milicia	Stem barks	Decoction	Drink	1	1,428,571
Mimosa pudica L.	Bodji (Punu)	*Fabaceae*	Mimosa	Leaves	Decoction	Drink	1	1,428,571
Musa×paradisiaca L.	Plantain	*Musaceae*	Musa	Skin	Burning	Eat	1	1,428,571
Musanga cecropioides R.Br. ex Tedlie	Parassolier	*Urticaceae*	Musanga	Leaves	Decoction	Drink	1	1,428,571
Nauclea diderrichii (De Wild.) Merr.	Bilinga	Rubiaceae	Nauclea	Stem barks	Decoction	Drink	1	1,428,571
Newbouldia laevis (P. Beauv.) Seem.	Ossomedzo (Ndoumu)	*Bignioniaceae*	Newbouldia	Stem barks	Decoction	Drink	1	1,428,571
Pennisetum purpureum Schumach.	Mikuku (bakota)	*Poaceae*	Pennisetum	Stem	Maceration	Drink	1	1,428,571
Peperomia pellucida (L.) Kunth	Pepper -elder	*Piperaceae*	Peperomia	Leaves	Infusion	Drink	1	1,428,571
Persea americana Mill.	Avocado	*Lauraceae*	Persea	Leaves	Maceration	Drink	3	4,285,714
Petroselinum crispum (Mill.) Fuss	Parsley	*Apiaceae*	Petroselinum	Leaves	Chewing	Eat	1	1,428,571
Phaseolus vulgaris L.	Bean	*Fabaceae*	Phaseolus	Fruit	Decoction	Drink	1	1,428,571
Picralima nitida (Stapf) T. Durand and H. Durand	Ebam	*Apocynaceae*	Picralima	Stem barks	Maceration	Drink	3	4,285,714
Piptadeniastrum africanum (Hook.f.) Brenan	Dabéma	*Mimosoideae*	Piptadeniastrum	Stem barks	Decoction	Drink	1	1,428,571
Pseudospondias longifolia Engl.	Ofoss	*Anacardiaceae*	Pseudospondias	Stem barks	Decoction	Drink	1	1,428,571
Psidium guajava L.	Guava	*Myrtaceae*	Psidium	Leaves	Decoction	Drink	1	1,428,571
Quassia africana (Baill.) Baill.	Mukèdji (Punu)	*Simaroubaceae*	Quassia	Stem barks	Maceration	Drink	1	1,428,571

(Contd...)

Table 2: *(Continued)*

Botanicals names	Local names/ venacular	Families	Genus	Parts used	Mode of preparation	Mode of administration	Numbers of citations	Frequencies of citations
Santiria trimera (Oliv.) Aubrév.	Ebo	*Burseraceae*	Santiria	Root	Decoction	Drink	1	1,428,571
Tabernanthe iboga Baill.	Iboga	*Apocynaceae*	Tabernanthe	Stem barks	Maceration	Drink	1	1,428,571
Tithonia diversifolia (Hemsl.) A. Gray	Daisy	*Asteraceae*	Tithonia	Flowers	Decoction	Drink	1	1,428,571
Vernonia amygdalina Delile	Ndolé	*Asteraceae*	Vernonia	Leaves	Chewing	Eat	1	1,428,571
Voacanga africana Stapf ex Scott-Elliot	Ondou or Ontueles (Téké)	*Apocynaceae*	Voacanga	Root	Maceration	Drink	1	1,428,571
Xylopia aethiopica (Dunal) A. Rich.	Mugana (Punu)	*Annonaceae*	Xylopia	Fruit	Decoction	Drink	1	1,428,571
Zea mays L.	Maize	*Poaceae*	Zea	Fiber	Decoction	Drink	1	1,428,571

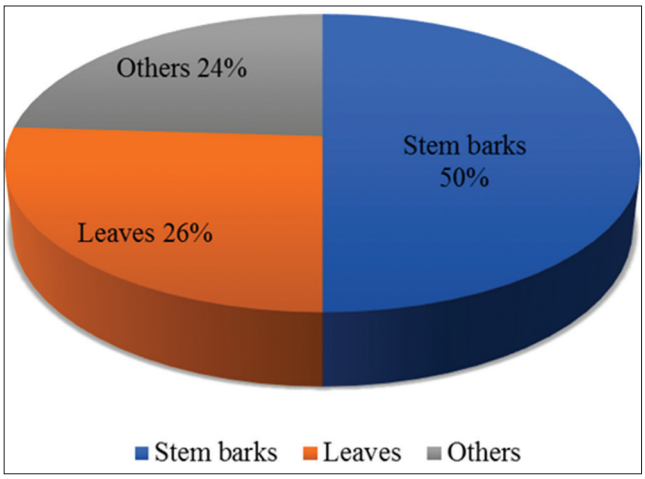

Figure 3: Plant parts cited for treating diabetes in the same areas of Gabon

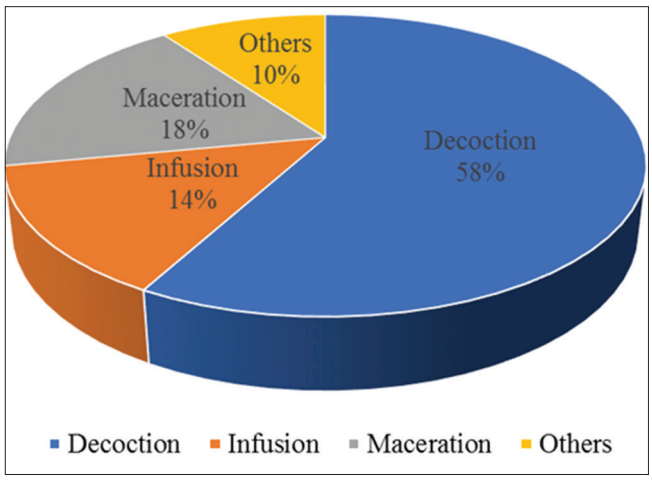

Figure 4: Pharmaceutical forms used to treat diabetes in some Gabonese regions

Respondents were dominated by aged people (>50 years). This experience is consistent with the study of Etuk *et al.* [147], in which showed the estimated age range of respondents was 40-70 years. Others have documented a profound and growing knowledge gap regarding medicinal plants between old and young people [148]. According to Uniyal *et al.* [146], the younger generations are ignorant of the vast medicinal resources available in their surroundings and are occupied in the search for money through market resources. Transmission of traditional medicinal knowledge from one generation to the next is thereby under threat [13,16].

It was also found that plant-based medicinal knowledge was more prevalent among people living in rural rather than urban area as described earlier by Vashistha [149]. Indeed, in a rural area, endogenous knowledges being more preserved [150], people resort, culturally, to the use of traditional medicine and herbal drugs are socioeconomically acceptable [151,152].

50 medicinal plants were exploited by both rural and urban people for the treatment of diabetes. Annonaceae was the most represented family. Members of the Annonaceae contain natural products with varied therapeutic properties, such as the anti flavonol taxifolin [153], which is known to possess antidiabetic, antitumor, and anti-inflammatory properties [154]. In addition, *Annonaceae* acetogenins are potent mitochondrial toxins with anticancer and anti-HIV activities [154]. However, excessive use of A. *muricata* has been associated with atypical parkinsonism on the island of Guadeloupe [155].

Among plant components used for medicinal purposes, stem barks were most often used followed by leaves in accord with the findings of other investigators [13,16,147]. Bark is easily collected and contains concentrated bioactive [58,60]. However, leaves which also accumulate pharmacologically active principles reportedly are often used to manage diabetes [15,156]. Whereas the collection of leaves does not induce plant damage, collection of bark, roots or the whole plant is destructive and may lead to species depletion [157]. Some respondents recognized and addressed this problem with a traditional ritual in which a coin was placed at the base of the tree and while the injured part was wiped with dead leaves. This practice reportedly was undertaken to facilitate a rapid regeneration of the excised part of the plant.

Herbal drugs were most commonly used as oral decoctions. This observed was in accordance with the work of Madingou *et al.*, [68] who observed that healing plants are generally boiled in medicinal recipes and then taken orally by many healers in Gabon and also many other reports worldwide [158-160].

Evaluating the bio-efficacy of the medicinal plants recorded, it was observed that each plant was mentioned at least twice by

Table 3: Phytochemical and pharmacological properties of plants

Botanicals names	Biological properties	Phytochemicals compounds	References
Acacia auriculiformis Benth.	Antifilarial effect. Antioxidant activity	Triterpenoid saponins. Proacaciaside and acacia mini. Tetrahydroxyflavanone, teracacidin, and trihydroxyflavanone, phenols, and tannins. Proanthocyanidins.	[17-20]
Allium sativum L.	Antioxidant activity. Antidiabetic and hypolipidaemic properties. Antihypertensive effect	Phenylpropanoids. Saponins, steroids, tannins, carbohydrates and cardiac glycosides. Propenyl cysteine and allyl cysteine	[21-24]
Alstonia boonei De Wild.	Diuretic activity. Hypoglycemic properties. Antioxidant	Saponins and indole alkaloids. Alkaloids, tannins, steroids, glycosides, flavonoids, and terpenoids. Triterpenes	[9,11,25,26]
Anchomanes difformis (Blume) Engl.	Antimicrobial activity. Anti-inflammation and anti-nociception activities	Cardiac glycosides, terpenoids, steroids, phlebotannins, and flavonoids.	[27,28]
Annickia chlorantha (Oliv.) Setten and Maas	Antibacterial activity. Noteworthy biological activity	Phenolics, flavonoids, alkaloids, glycosides, saponins. Isoquinoline, acetogenins, and sesquiterpenes	[29,30]
Annona muricata L.	Hypoglycemic effects. Antineoplastic potential. Antioxidant and anticancer agent	5-(3-hydroxybutyl) furanone, chloranthalactone E, dimethyl-6-hydroxycoumarin, triazole nucleosides, L-tryptophan, L-phenylalanine. Tannins, cardiac glycosides, terpenoids, and reducing sugars. Alkaloids, saponins, anthraquinones, phenols and phytosterols	[10,31-33]
Anonidium mannii (Oliv.) Engl. and Diels	Antibacterial activities. Cytotoxic agent	Alkaloids, phenols, polyphenols, saponins, tannins, sterols and triterpenes	[34,35]
Antrocaryon klaineanum Pierre	Antioxidant activity. Antiplasmodial	Phenolic, total flavonoids, total tannins total proanthocyanidins, coumarins, anthracenosids, saponosids, and triterpenoids. Antrocarine A-F. Ergostane-type antrocarine E	[36-38]
Aucoumea klaineana Pierre	Antioxidant activity. Antimicrobial activity	Monoterpenoids	[39,40]
Carica papaya L.	Antimicrobial activity, Antihyperglycemic and hypolipidemic activities. Antithrombocytopenic activity. Useful antioxidant. Antifungal agent	Saponins, cardiac glycoside, anthraquinone, flavonoids, steriods, tannins, and triterpenoids. Phenolics, carpaine. Benzylglucosinolate. Benzyl isothiocyanate	[41-45]
Ceiba pentandra L. Gaertn.	Hypoglycemic and antihyperglycemic effects. Antioxidant activity	Phenolic, flavonoid, alkaloid and tannins	[46,47]
Cleistopholis glauca Pierre ex Engl. and Diels	Antibacterial activity	Cleistriosides-2. Patchoulenone, cyperene and germacrene D	[48,49]
Cocos nucifera L.	Cytoprotective and antihyperglycemic properties. Antimalarial activity	Phenolic compounds, flavonoids, resins, alkaloids, carbohydrate, proteins, and fibers. Tannins, saponins, glycosides, steroids and anthraquinones	[50,51]
Combretum micranthum G. Don	Antihyperglycaemic activities. Antibacterial agent	Gallic acid, rutin trihydrate, (+)-catechin and benzoic acid. Alkaloids, saponins, tannins, anthraquinones, cardiac glycosides, flavonoids, and steroids	[52-54]
Cylicodiscus gabunensis Harms	Antiplasmodial activity. Antimicrobial activity. Antimalarial activity	Alkaloids and terpenes. Leucoanthocyanins, saponins, tannins, polyphenols, coumarins, cardiac glycosides, reducing sugars, steroids, flavonoids, sterols and or triterpenes. Gallic acid, oligosaccharides	[55-57]
Cymbopogon citratus (DC.) Stapf	Anti-inflammatory and sedative. Hypoglycemic and hypolipidemic effects. Antibacterial activity. Anti-inflammatory activity	Citral and terpenes. Alkaloids, saponins, tannins, anthraquinones, steroids, phenols. Carlinoside, isoorientin, cynaroside, luteolin 7-O-neohesperidoside, kurilesin A and cassiaoccidentalin B	[58-61]
Duboscia macrocarpa Bocq.		Dubosane. Dubosciasides	[62,63]
Entada gigas (L.) Fawcett and Rendle	Used for diarrhea. Microbial infection	Alkaloids, phenols, and tannins	[64,65]
Gnetum africanum Welw.	Potential chemopreventive agents. Antimicrobial activity	Phenolic compounds, flavonoids, phytosterols, alkaloids, tannins, saponins, chlorophyll, and glycosides. β-caryophyllene, (E)-phytol and trimethyl-2-pentadecanone	[66,67]
Guibourtia tessmannii (Harms) J. Leonard	Hypotensive activity. Antioxidant activity. Hypoglycemic effect	Triterpenes, sterols, alkaloids, tannins, polyphenols, sugars and saponosides	[68-70]
Harungana madagascariensis Lam. ex Poir.	Anti-inflammatory, antioxidant and antidiabetic activities	Polyphenols, tannins, and triterpenes. Alkaloids, saponins, and flavonoids	[71-73]

(Contd...)

Table 3: *(Continued)*

Botanicals names	Biological properties	Phytochemicals compounds	References
Lantana camara L.	Hypoglycemic and wound healing properties. Antihyperglycaemic agent. Antimicrobial and cytotoxic activities	Carbohydrates, flavonoids, phytosterols, saponins. β-caryophyllene, ar-curcumene/zingiberene, γ-curcumen-15-al/ epi-β-bisabolol, (E)-nerolidol, davanone, eugenol/alloaromadendrene, and carvone	[74-76]
Mammea africana Sabine	Cytotoxic and antimicrobial activities. Hypoglycemic effect. Hepatoprotective activity	4-phenylcoumarins, 4-n-propylcoumarins, one 4-n-pentylcoumarin, 1,5-dihydroxyxanthone and 1-methoxy-5-hydroxyxanthone	[77-79]
Microdesmis puberula Hook.f. ex Planch.	Analgesic and anti-stress agent	keayanidines A, B, C and keayanine A. Saponins, cardiac glycosides, deoxysugars, alkaloids and terpenes	[80-82]
Milicia excelsa (Welw.) C. C. Berg	Wound healing and antibacterial effects. Used for the management of Type 2 diabetes	Tannins, alkaloids, flavonoids and saponins. Melicilamide A. 3,4-dimethoxybenzyl beta-D-xylopyranosyl -beta-D-glucopyranoside, lupeol acetate, ursolic acid, triacontyl (E)-ferulate, and 2-(3,5-dihydroxyphenyl) benzofuran-5,6-diol. Polyphenol, phenol, triterpenes and glycosides	[83-86]
Mimosa pudica L.	Antimicrobial activity. Hypolipidemic activity	C-glycosylflavones. Terpenoids, flavonoids, glycosides, alkaloids, quinines, phenols, tannins, saponins, and coumarins	[87-89]
Musa × *paradisiaca* L.	Antihyperglycemic activity. Anthelmintic activity. Antioxidant activity. Hypoglycemic activity	Tannins, eugenol, tyramine. Serotonin, levarterenol, norepinephrine and dopamine. Alkaloids, glycosides, steroids, saponins, flavanoids and terpenoids/steroids	[90-93]
Musanga cecropioides R.Br. ex Tedlie	Antihypertensive. Antioxidant activity	Cecropic acid methyl ester	[94-96]
Nauclea diderrichii (De Wild.) Merr.	Antitrypanosomal effects, Genotoxic activity	Alkaloids, flavonoids, terpenes and glycosides. Quinovic acid glycosides	[97,98]
Newbouldia laevis (P. Beauv.) Seem.	Antimicrobial activity. Hepatoprotective action. Antihyperglycemic activity	Chrysoeriol, newbouldiaquinone; 2-acetylfuro-1,4-naphthoquinone, 2-hydroxy-3-methoxy-9,10-dioxo-9,10-dihydroanthracene-1-carbaldehyde, lapachol, beta-sitosterol-3-O-beta-D-glucopyranoside, oleanolic acid, canthic acid, newbouldiamide and 2-(4-hydroxyphenyl)-ethyltrioctanoate	[99-101]
Pennisetum purpureum Schumach.	Antioxidant enzyme. Nutritional and antinutritional. Herbicidal activity	Ascorbic acid, rutin, epicatechin, anthocyanins, p-coumaric acid, quercetin, and catechin. Alkaloids, cyanogenic glycosides, flavonoids, oxalates, phytales, saponins, and tannins	[102-105]
Peperomia pellucida (L.) Kunth	Anticancer, antimicrobial, antioxidant properties. Antidiabetic, analgesic and anti-inflammatory activities	Phytol, 2-Naphthalenol, decahydro, hexadecanoic acid, methyl ester and 9,12-octadecadienoic acid (Z, Z)-, methyl ester. Alkaloids, tannins, resins, steroids, phenols and carbohydrate. Flavonoids, glycosides, saponins	[106-108]
Persea americana Mill.	Hypoglycemic and hypocholesterolemic activities	Tannins, saponins, steroids/triterpenoids and flavonoids. Estragole, α-farnesene, β-caryophyllene, germacrene D, α-cubebene, and eugenol	[109-111]
Petroselinum crispum (Mill.) Fuss	Antioxidant and antibacterial activities. Anti-vibrio activity. Antidiabetic effect	Phenolics compounds. 1,3,8-p-menthatriene, β-phellandrene, apiol, myristicin, and terpinolene	[112-114]
Phaseolus vulgaris L.	Antihyperglycemic activity. Antioxidant and antiproliferative effects	Alkaloids, flavonoids, proteins, tannins, terpenoids, saponins, quercetin, anthocyanin and catechin. Gallic acid, chlorogenic acid, epicatechin, myricetin, formononetin, caffeic acid, and kaempferol	[115,116]
Picralima nitida (Stapf) T. Durand and H. Durand	Hypoglycemic activity. Antioxidant and antidiabetic activities	Flavonoids, terpenes, sterols, saponins, alkaloids and polyphenols	[117,118]
Piptadeniastrum africanum (Hook.f.) Brenan	Antifungal activity. Gastroprotective and ulcer healing effects	Alkaloids, saponins, coumarins, flavonoids, carbohydrates, phenolic compounds, and tannins. Piptadenine and piptadenamide	[12,119,120]
Pseudospondias longifolia Engl.	Antioxidant and antimicrobial properties	Total phenols, gallic acid, flavonoids, quercetin, tannins, tannic acid and proanthocyanidins procyanidin	[121]
Psidium guajava L.	Hypoglycemic and hypotensive properties. Antioxidant activity	Tannins, pentacyclic triterpenoids, guiajaverin, quercetin. Gallic acid, catechin, chlorogenic acid, caffeic acid, epicatechin, rutin, isoquercitrin, quercetin, kaempferol and luteolin, glycosylated campeferol, tocopherol, β-carotene and lycopene	[122,123]
Quassia africana (Baill.) Baill.	Antiamoebic activity. Antiviral activity. Larvicidal property	Tannins, alkaloids, saponins, steroids/terpens. Quassin and simalikalactone D	[124-126]
Santiria trimera (Oliv.) Aubrév.	Antimicrobial activity	Triterpenes. Alpha-pinene, beta-pinene. Alpha-humulene and beta-caryophyllene	[127-129]
Tabernanthe iboga Baill.	Insulinotropic effect	Ibogaine, tabernanthine, and voacangine	[130,131]
Tithonia diversifolia (Hemsl.) A. Gray	Antiamoebic activity. Antidiabetic effect. Antimicrobial activity	Flavonoids, tannins, saponins, steroids and terpens. Tannins and saponins. Sugars, sesquiterpenes lactones and phenolics	[124,132-134]
Vernonia amygdalina Delile	Antioxidant activity. Hypoglycemic and hypolipidemic agent	Flavonoids, terpenoids, saponins, tannins and reducing sugars, alkaloids, cardiac glycosides. Carbohydrates, sterols and balsams. Sesquiterpene lactone vernolide and vernodalol	[135-137]

(Contd...)

Table 3: *(Continued)*

Botanicals names	Biological properties	Phytochemicals compounds	References
Voacanga africana Stapf ex Scott-Elliot	Antioxidant activity. Antimicrobial activity	Anthranoids, anthraquinone, cardiac glycosides, phenols, phlobatanins, starch and tannins. Ibogamine, voacamine, vobasine, voacangine, voacristine, 19-epi-voacristine and 19-epi-heyneanine	[124,138,139]
Xylopia aethiopica (Dunal) A. Rich.	Hypoglycemic effects. Antihyperglycemic and antioxidant potentials	Alkaloids and polyphenols	[140,141]
Zea mays L.	Preventive effect of the diabetic nephropathy. Antioxidant activity. Therapeutic and antioxidative agents	Anthocyanins and phenolics compounds. Flavonoids, saponins, tannins, phlobatannins, alkaloids, cardiac glycosides, and terpenoids	[142-144]

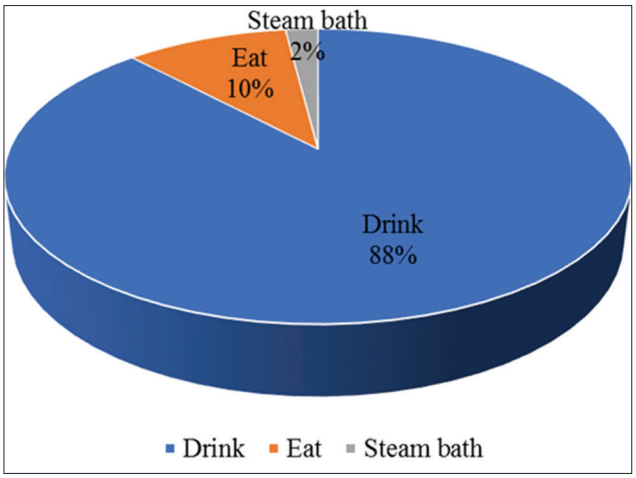

Figure 5: Mode of administration of recipes in the treatment of diabetes in some Gabonese regions

people from different regions for the management of diabetes. The literature also reports the use of some of these plants for diabetes treatment in others countries such as *A. boonei* has been studied in Nigeria [9]; *P. americana*, studied in Nigeria and Brazil [109,110]; *P. nitida* in Nigeria and Cameroon [117,118].

Moreover, the literature reports antidiabetic properties of many of these plants. 15 of them would have hypoglycemic, hypolipemia the case of *P. americana*, *P. guajava*, *C. citratus*, *C. pentandra*, *C. papaya*, *L. camara*, *A. muricata*, and *A. sativum* [22,109,110]. *C. pentandra* would have both antihyperglycemic and hypoglycemic effects [46]. *Guibourtia* would have antioxidant and hypoglycemic [69,70]. Since, the frequency of plant use citations by both traditional healers and literature is an indication of the pharmacological relevance of the plant and thus, of curative properties [156], one may argue the therapeutic properties of some of the investigated medicinal plants which were evidenced by their studied pharmacological properties.

CONCLUSION

The study highlights the drug discovery great potential of the Congo Basin Forest. Nowadays, the management of diabetes is not the only fact of modern medicine, many medicinal based plants recipes are proposed by healers worldwide and deserve to be valued and rationalize.

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Original Research

Bactericidal activity of herbal volatile oil extracts against multidrug-resistant *Acinetobacter baumannii*

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ABSTRACT

Aim: The aim of the study is to investigate the antibacterial activity of 10 volatile oils extracted from medicinal plants, including galangal (*Alpinia galanga* Linn.), ginger (*Zingiber officinale*), plai (*Zingiber cassumunar* Roxb.), lime (*Citrus aurantifolia*), kaffir lime (*Citrus hystrix* DC.), sweet basil (*Ocimum basilicum* Linn.), tree basil (*Ocimum gratissimum*), lemongrass (*Cymbopogon citratus* DC.), clove (*Syzygium aromaticum*), and cinnamon (*Cinnamomum verum*) against four standard strains of *Staphylococcus aureus, Escherichia coli*, *Pseudomonas aeruginosa, Acinetobacter baumannii,* and 30 clinical isolates of multidrug-resistant *A. baumannii* (MDR-*A. baumannii*). **Materials and Methods:** Agar diffusion, minimum inhibitory concentration, and minimum bactericidal concentration (MBC) were employed for the determination of bactericidal activity of water distilled medicinal plants. Tea tree oil (*Melaleuca alternifolia*) was used as positive control in this study. **Results:** The results indicated the volatile oil extracted from cinnamon exhibited potent antibacterial activity against the most common human pathogens, *S. aureus*, *E. coli*, *P. aeruginosa,* and *A. baumannii*. Most of volatile oil extracts were less effective against non-fermentative bacteria, *P. aeruginosa*. In addition, volatile oil extracted from cinnamon, clove, and tree basil possessed potent bactericidal activity against MDR-*A. baumannii* with MBC_{90} of 0.5, 1, and 2 mg/mL, respectively. **Conclusions:** The volatile oil extracts would be useful as alternative natural product for the treatment of the most common human pathogens and MDR-*A. baumannii* infections.

KEY WORDS: Medicinal plant, multidrug-resistant-*Acinetobacter baumannii*, volatile oil

INTRODUCTION

Acinetobacter baumannii is one of the most important drug-resistant pathogens worldwide. Recently, the World Health Organization indicated that drug-resistant A. *baumannii* is defined as the first priority pathogen, in which researches and developments for new antibiotics are urgently needed [1]. The bacteria has been revealed to persist on dry surfaces for a month and presented several drug-resistant mechanisms including drug efflux pumps, drug-inactivating enzymes, and drug target mutations [2]. Infected patients have many serious diseases including septicemia, pneumonia, and urinary tract infections [2,3]. The number of global drug-resistant A. *baumannii* was vary in estimation [4]; therefore, the high prevalence accounted to be approximately 54% and 77% of A. *baumannii* isolates have been revealed in Italy and India, respectively [5,6]. In Thailand, surveillance in the 2010 period indicated the rate of multidrug-resistant (MDR)-A. *baumannii* collected from clinical specimens was approximately 59% [7]. Regarding the limit of antibiotic treatment, many studies have focused on the alternative drugs and phytomedicine. Several studies revealed the effectiveness of extracted herbs on drug-resistant pathogens including methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, and MDR-A. *baumannii*, whereas the antimicrobial activity of volatile oils extracts was rarely reported [8,9]. Herein, 10 volatile oils extracted from various medicinal plants were determined for their inhibitory effect on the growth of the most common human pathogens and MDR-A. *baumannii*.

MATERIALS AND METHODS

Bacterial Strains

S. aureus ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and A. *baumannii* ATCC 19606 were purchased from the Department of Medical

Sciences, Ministry of Public Health, Thailand. 30 clinical isolates of MDR-*A. baumannii* were collected from the Diagnostic Laboratory, Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand, during February-April, 2012. Both biochemical tests followed by Constantiniu *et al*. [10] and molecular biology test using amplified ribosomal DNA-restriction enzyme analysis were performed for identification of *A. baumannii*. Primers used for 16S rDNA gene amplification were designed as followed by the previous report [11]. The antimicrobial susceptibility testing was performed using disk diffusion method following the Clinical and Laboratory Standards Institute (CLSI) guidelines [12,13]. The MDR was defined according to the unsusceptible of at least one in three agents of antimicrobial classes [14]. All 30 clinical isolates resisted to eight antibiotics in six antimicrobial classes consisting of amikacin, piperacillin/tazobactam, ciprofloxacin, cefoperazone/sulbactam, ceftazidime, trimethoprim/sulfamethoxazole, imipenem, and meropenem.

Volatile Oils Extraction

Volatile oils were extracted from 10 medicinal plants by water distillation. Galangal, ginger, plai, lime, kaffir lime, sweet basil, tree basil, lemongrass, clove, and cinnamon were selected in this study [Table 1]. The material was subjected to hydrodistillation using a Clevenger-type glass apparatus for 3-5 h [15]. Yields of the volatile oils obtained from the plants were calculated as the percent yield. All volatile oils were stored at 4°C until used.

Antibacterial Activity Testing

The antimicrobial activity testing was modified from Prabuseenivasan *et al*. [16]. Briefly, bacterial suspension was adjusted to McFarland standard No. 0.5 (1×10^8 CFU/mL) and spread over the Mueller-Hinton agar (MHA) plates using a sterile cotton swab. Each volatile oil was dissolved in 10% aqueous dimethyl sulfoxide (DMSO) with 0.5% v/v Tween 80 and sterilized by filtration. Sterilized disks (Whatman No. 5, 6 mm diameter) were impregnated with 20 μL of volatile oils and placed on the surface of MHA. The volatile dissolving buffer (10% aqueous DMSO, 0.5% v/v Tween 80) and tea tree oil were used as negative and positive control, respectively. After incubation at 37°C for 16-18 h, the inhibition zone was measured. All experiments were performed independently in triplicate and mean value was calculated.

Table 1: Medicinal plants used in this study

Common name	Botanical name	Families	Parts
Galangal	*Alpinia galanga* (Linn.) Swartz	Zingiberaceae	Rhizome
Ginger	*Zingiber officinale* Roscoe	Zingiberaceae	Rhizome
Plai	*Zingiber cassumunar* Roxb.	Zingiberaceae	Rhizome
Lime	*Citrus aurantifolia* Swingle	Rutaceae	Peel
Kaffir lime	*Citrus hystrix* DC.	Rutaceae	Peel
Sweet basil	*Ocimum basilicum* Linn.	Lamiaceae	Leaf/stem
Tree basil	*Ocimum gratissimum*	Lamiaceae	Leaf/stem
Lemongrass	*Cymbopogon citratus* DC. Stapf.	Poaceae	Leaf/stem
Clove	*Syzygium aromaticum* (L.) Merr. & Perry	Myrtaceae	Bud
Cinnamon	*Cinnamomum verum* J. Presl	Lauraceae	Bark

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Based on CLSI guidelines, MICs were determined by using broth microdilution method [17]. The preparation of water-insoluble volatile oils was slightly modified from the recommended CLSI guidelines. Each volatile oil was dissolved with 50% DMSO and serial 2-fold diluted in a 96-well microtiter plate ranging from 0.125 to 8 mg/mL. The bacterial suspension was diluted into approximately 1×10^6 CFU/mL, and 100 μL of bacterial suspension was applied to each well. The inoculum with 2.5% DMSO and media without inoculum were used as cell and media control, respectively. The microplates were incubated at 35°C for 20 h. Due to the turbidity of volatile oil suspensions, iodonitrotetrazolium chloride (INT) (BioChemica) was used as color indicator to visualize the bacterial growth [18]. The MIC was detected after added 50 μL of 0.2 mg/mL INT and further incubated at 35°C for 30 min. To determine the MBC, 10 μL of bacterial inoculums were taken aseptically from the wells with no color change and plated onto MHA plate and incubated at 35°C for 20-24 h. All experiments were separately performed in triplicate and calculated as mode, median, and 90th percentile. Median MIC value (MIC_{50}) represented the MIC value of one-half of the tested population. The 90th percentile (MIC_{90}) represented the MIC value of 90% of the tested population [19]. Likewise, MBC_{50} and MBC_{90} were the MBC values at which 50% or 90% of isolates in a tested population were killed, respectively.

Statistical Analysis

In this study, the inhibition zone of each volatile oil was compared with tea tree oil and statistically analyzed using independent Student's *t*-test (SPSS version 22). The MIC and MBC values in each of the tested volatile oils and tea tree oil were statistically analyzed by Mann–Whitney *U*-test (SPSS version 22).

RESULTS

The percent yields of the water-distilled volatile oils were calculated. The yields ranged from 0.1% to 4.3% w/w – ginger (0.1), lemongrass (0.2), tree basil (0.2), galangal (0.3), sweet basil (0.3), cinnamon (0.9), lime (1.0), plai (1.1), kaffir lime (2.1), and clove (4.3). A disk diffusion method was performed to preliminarily evaluate the antibacterial activity of the volatile oils against four reference bacterial strains (*S. aureus*, *E. coli*, *P. aeruginosa*, and *A. baumannii*). Except *P. aeruginosa*, the positive control tea tree oil represents antibacterial activity to the bacteria tested. No inhibition zone was observed in volatile dissolving buffer. The difference in the inhibition zones between tea tree oil and each volatile oil was analyzed using independent Student's *t*-test. The results indicated that cinnamon oil exhibited a high potency of antibacterial activity against all bacterial strains tested ($P < 0.01$). Sweet basil and lemon grass were highly active against *S. aureus* and *E. coli*; however, these volatile oils showed no significant activity when tested with both non-fermentative Gram-negative bacilli, *A. baumannii*, and *P. aeruginosa*. The volatile oils of clove, tree

basil, lime, and ginger were moderately active against some bacterial strains ($P < 0.05$). The antibacterial activity of plai and kaffir lime was rather inactive compared to tea tree oil. The inhibition zones of various volatile oils against *S. aureus*, *E. coli*, *P. aeruginosa*, and *A. baumannii* standard strains were shown in Figures 1-4, respectively. Many volatile oils showed an inhibitory effect against MDR-*A. baumannii* including tea tree oil [Figure 5]. However, the mean of the inhibition zones of the cinnamon and clove oils was significantly higher than tea tree oil ($P < 0.01$). Both standard strains of *A. baumannii* ATCC 19606 and MDR-*A. baumannii* isolates were determined for MIC and MBC by broth microdilution method. The MIC and MBC of the positive control tea tree oil against *A. baumannii* ATCC 19606 were 2 and 4 mg/mL, respectively. Cinnamon oil was highly active, with MIC and MBC values of 0.25 mg/mL. The MICs and MBCs of the volatile oils tested against *A. baumannii* ATCC 19606 were shown in Table 2. The MICs and MBCs of each volatile oil tested against MDR-*A. baumannii* isolates were statistically analyzed using Mann–Whitney *U*-test. The modes were equivalent to the medians. The tea tree oil exhibited anti-MDR-*A. baumannii* activity with MIC_{90} and MBC_{90} of 2 and 4 mg/mL, respectively. The mean MICs of four volatile oils, cinnamon, clove, tree basil, and kaffir lime were significantly lower than the positive control tea tree oil with the MIC_{90} of 0.25, 0.5, 1, and 1 mg/mL, respectively ($P < 0.05$). The MIC and MBC of the volatile oils against 30 clinical strains of MDR-*A. baumanni* were shown in Table 3.

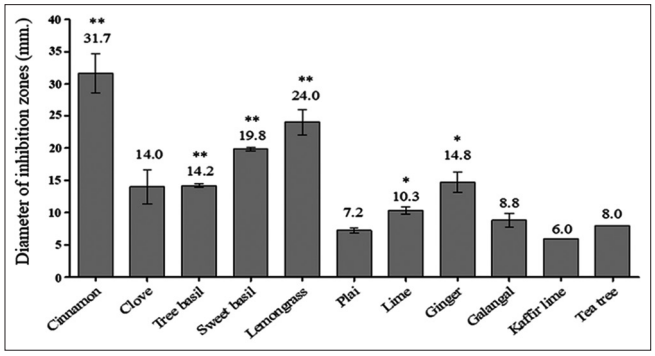

Figure 1: Inhibition zones of 11 volatile oils against *Staphylococcus aureus* ATCC 25923. *Indicated a significant difference at $P < 0.05$, and ** indicated a highly significant difference at $P < 0.01$

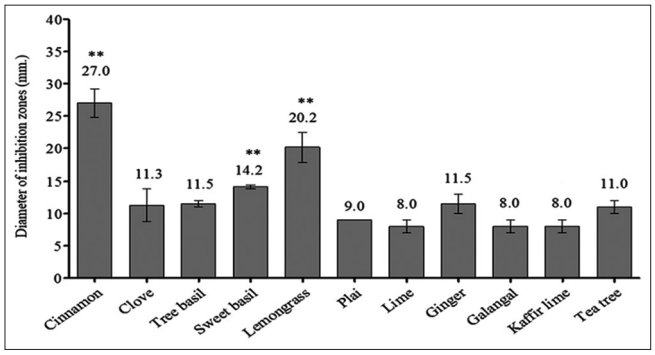

Figure 2: Inhibition zones of 11 volatile oils against *Escherichia coli* ATCC 25922. **Indicated a highly significant difference at $P < 0.01$

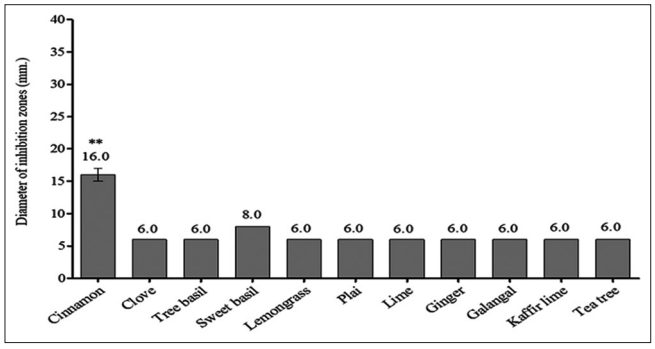

Figure 3: Inhibition zones of 11 volatile oils against *Pseudomonas aeruginosa* ATCC 27853. **Indicated a highly significant difference at $P < 0.01$

DISCUSSION

The most problematic of *A. baumannii* infections nowadays are the MDR and it becomes a serious issue, in which most antibiotics drug therapy are unable to cure the diseases. Finding new and effective antibacterial compounds against MDR-*A. baumannii* is urgent; volatile oils are one such compound worth screening. In this study, 10 volatile oils were determined for antibacterial activity against *S. aureus*, *E. coli*, *P. aeruginosa*, *A. baumannii*, and 30 isolates of MDR-*A. baumannii*. The antimicrobial activity of tea tree oil against aerobic bacteria has previously been

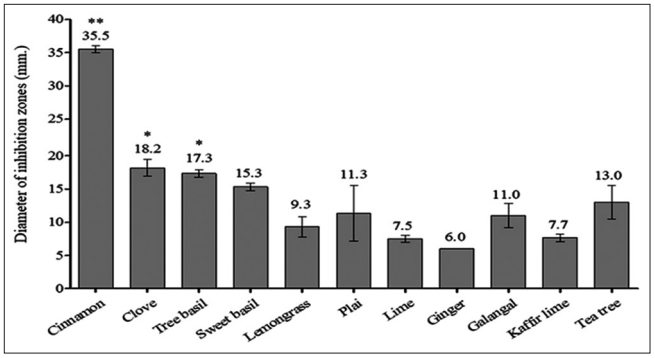

Figure 4: Inhibition zones of 11 volatile oils against *Acinetobacter baumannii* ATCC 19606. *Indicated a significant difference at $P < 0.05$, and **Indicated a highly significant difference at $P < 0.01$

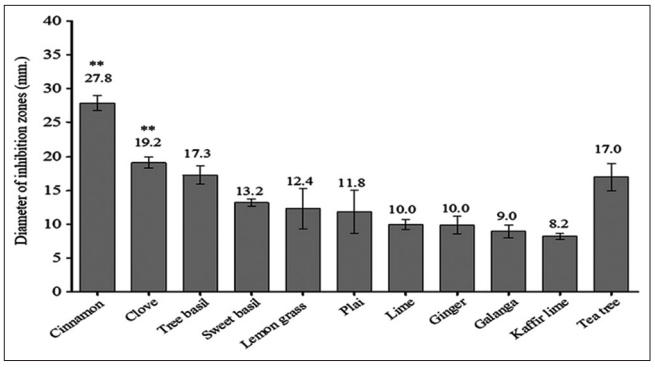

Figure 5: Average inhibition zone in diameter obtained from various volatile oils against 30 multidrug-resistant - *Acinetobacter baumannii* isolates. **Indicated a highly significant difference at $P < 0.01$

Table 2: MICs and MBCs of volatile oils against standard strain *A. baumannii* ATCC 19606

Volatile oils	MIC (mg/mL)	MBC (mg/mL)
Cinnamon	0.25	0.25
Clove	0.5	1
Tree basil	1	1
Sweet basil	1	8
Lemongrass	1	>8
Plai	2	>8
Lime	2	8
Ginger	2	4
Galangal	>8	>8
Kaffir lime	1	2
Tea tree	2	4

MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration, *A. baumannii: Acinetobacter baumannii*

Table 3: The MICs and MBCs of volatile oils against MDR-*A. baumannii*

Volatile oils	MIC (mg/mL)		MBC (mg/mL)	
	MIC_{50}	MIC_{90}	MBC_{50}	MBC_{90}
Cinnamon[a,b]	0.25	0.25	0.5	0.5
Clove[a,b]	0.5	0.5	1	1
Tree basil[a,b]	1	1	2	2
Sweet basil	2	2	4	8
Lemongrass[b]	2	2	2	4
Plai	2	4	4	>8
Lime	2	4	4	>8
Ginger	2	4	4	4
Galangal	>8	>8	>8	>8
Kaffir lime[a,b]	1	1	2	4
Tea tree	1	2	4	4

[a]Indicated the volatile oil that had the mean of MIC significantly lower than tea tree oil ($P<0.05$). [b]Indicated the volatile oil that had the mean of MBC significantly lower than tea tree oil ($P<0.05$). MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration, *A. baumannii: Acinetobacter baumannii,* MDR: Multidrug-resistant

published; the compounds involved in its antibacterial activity such as terpinen-4-ol, α-terpinene and γ-terpinene have been characterized [20]. Similarly to Carson and Riley's study, an inactive effect of tea tree oil against *P. aeruginosa* was observed in this study [20]. A. *baumannii* ATCC 19606 and MDR-A. *baumannii* isolates could be inhibited by tea tree oil with MIC_{90} and MBC_{90} concentrations of 2 and 4 mg/mL, respectively.

The extracted volatile oils were preliminarily screened for antibacterial activity by disc diffusion method. Among the medicinal plants tested, cinnamon oil exerted the highest activity to inhibit the growth of all bacteria while sweet basil and lemon grass strongly inhibited in some bacteria. Standard broth microdilution method was performed and revealed that the volatile oils of cinnamon, clove, tree basil, and kaffir lime showed strong antibacterial activity against MDR-A. *baumannii* isolates. The antimicrobial activity of cinnamon oil against *S. aureus*, *E. coli*, *Acinetobacter lwoffii*, and *P. aeruginosa* has previously been demonstrated [21]. Recently, Rath and Padhy indicated that the MIC and MBC of methanolic extract of both clove and cinnamon against MDR-A. *baumannii* were 1.51 and 3.41 mg/mL, respectively [22]. The inhibition zones of tree basil and tea tree oil were indifferent; the major constituents of tree basil volatile oil have previously been identified including thymol, γ-terpinene, eugenol, and ρ-cymene [23]. The mode of antibacterial action of thymol still unknown but it has been proposed to involve in outer and inner membrane disruption [24]. Cinnamon oil possessed the highest inhibition effect against all bacterial strains and MDR-A. *baumannii* isolates. Gas chromatography–mass spectrometry analysis was performed in this study to identify the active ingredients with antimicrobial activity. Thirteen peaks were observed and interpreted based on specific retention time compared to a reference database. The major ingredients in cinnamon oil were cinnamaldehyde (75.89%), *trans*-cinnamyl acetate (7.07%), hydrocinnamaldehyde (2.39%), and 1,8-cineole (2.17%) (data not shown). Cinnamaldehyde has previously been reported to inhibit in both Gram-positive and Gram-negative bacteria [25,26]. Noteworthy, aldehyde groups might be associated with the antimicrobial activity of cinnamon oil since these chemicals have an ability to covalently cross-link with the amine groups of DNA and proteins and interfere their functions in the cells. Although the mode of action of cinnamaldehyde is inconclusive [24], Gill and Holley demonstrated that cinnamaldehyde at a concentration of 30 mM could kill *L. monocytogenese* through its effect on the energy generation and membrane permeability of the bacteria [27,28]. In addition, the interaction of cinnamaldehyde with essential enzymes and bacterial cell wall damage at high concentration has been investigated [29]. Although cinnamaldehyde possesses potent antimicrobial activity against MDR pathogen, its cellular and *in vivo* cytotoxicity have been reported [30,31]. In addition, it has been reviewed to be a cause of allergic reaction in toothpaste [32]. Consequently, a dosage level at which no adverse effects is indispensable determined before use in the future application.

CONCLUSIONS

Our study indicated the antibacterial activity of volatile oils extracted from herbs against several bacteria, including MDR-A. *baumannii*. These plant extracts would be promising antimicrobial agents for further treating of human pathogens, including drug-resistant bacteria.

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Original Research

Traditional dentistry knowledge among Serbs in several Balkan countries

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ABSTRACT

Background/Aim: There are still unrevealed treasures of traditional dental medicine, that is, the reason to investigate and present various ways in treatment of oral and orofacial tissues, as well as magic and religious elements involved in representative areas among Serbs. **Materials and Methods:** Information was collected from the elderly non-professional folk dentists and herbalists with the additional help of local physicians and dentists that was done through questionnaire and personal interviews. **Results:** Classified and prepared material consists of total 1038 inquiry sheets. The 41 data were averagely obtained by inquiry form, i.e. 41,984 information for the whole research. The most voluminous was the group of 64 recipes, including 39 for gums diseases and 25 for toothache, while only seven ones were mentioned for magic way of treatment. Among them, 18 prescriptions were of nonherbal origin. The study revealed 84 herbal original prescriptions, including 67 plant species (29 families) including local name, synonyms, and preparation mode. Traditional healers used predominantly herbal recipes to treat painful tooth, gum disease, blisters - herpetic ulcers/lips and mouth/, stomatitis/painful mouth, ptyalismus/, maxillary sinusitis, bad breath, teeth cleaning and bleaching. Very few methods of treatment appeared as inadequate (magical practice), whereas majority were noted as beneficial ones (herbal medicine). Still many people in distant nonurban areas use various plant recipes, especially as the first aid in oral disease healing. **Conclusions:** The significance of plants obtained from unpolluted areas, whose active ingredients have not yet been used in dental pharmaceutics, should be further investigated in the future.

KEY WORDS: Dental ethnopharmacology, ethnomedicine, medicinal plants, phytotherapy, traditional dentistry

INTRODUCTION

Plants and other natural means have been used in traditional dental medicine for several millennia in the Balkan countries, as well as other ways of treatment. In the Balkan countries ethnodentistry (ETD), part of the ethnomedicine has been developed simultaneously by ethnopharmacy and ethnoveterinary medicine, presenting the branches of folk health culture [1-4]. ETD is to be considered interdisciplinary and composite field of science. Data obtained from investigation should be assumed much wider, i.e. systematically through the prism of archeology, history, general medicine, pharmacy, sociology, and tradition of the Balkan regions [5]. Based on the clinical and laboratory tests on the scope of phytotherapy, ETD investigations have been performed all over the world, mostly collecting the folk prescriptions in the large and middle-developed, but densely populated countries (China, Brazil, and India). Two of these (China and India) had the strong influence on the rest of the world in regard to traditional medicine, as well as dental medicine, having a long recorded history in the field of materia medica [6]. The reason for doing so was still high participation of traditional dental medicine in everyday life there, still cheap and more important, reachable and very applicable even in urban areas, where a high level of dentistry exists nowadays [6-19]. Investigations in those countries encouraged scientists to study the hidden recipes and active substances in medicinal herbs, useful to the modern man exposed to stresses and suffering from incurable diseases throughout its entire life. Contemporary studies conducted in the previous decade (India, Pakistan, Saudi Arabia, China, Japan, South America, etc.,) were directed to assess the

benefits of hundreds of traditional remedies, proofed by current medicine [6].

To study traditional medicine, as well as common customs of wild tribes and rarely investigated ethnical minorities of distant regions, comparative analysis can be applied around the globe. It was exposed as useful for the study in Eskimos, Papua Islands, East Africa, and Amazonian Brazil regarding ethnomedicine [11]. Although there were no comparative analyses among ETD studies done in the Balkan countries, unilateral studies provided encouraging results [20-22]. It was characteristic for all of them that people in rural areas would apply knowledge and experience of folk dentistry due to the lack of educated dentists [20-26], like in Serbia and Montenegro (SM), and areas where Serbs live [16,27,28]. Hence, compound interventions have been applied only on the establishment of independence of Serbia principality and wider where Serbs lived (middle of the 19th century), when advanced culturally population and many educated doctors of dentistry schooled abroad began to return home [29].

In the past centuries, folk dentistry played an important role concerning many wars, wounds, diseases, and injuries left behind. Thus, the book The Serbian Dictionary described around 130 medicinal plants from the 19th century [27]. However, the deeply studious and large pioneer investigation began only in 1972 through the Belgrade School of Dental Medicine (BSDM) [1].

Concerning methodology, a preliminary study was designed to find a good way for data collection. Students in their final years of BSDM were included into the research from 1976 onward, through the summer field work. Some of them collected the data used inquiry on return to their domicile regions. All activities were conducted and supervised by dentistry professors, including newly trained associate members (lecturer's staff), where beginners' mistakes were dismissed [30]. There was a need to record the ancestors' dental medicine tradition as credibly as possible, due to the strong and modern influence of the West, East, and Mediterranean regions, which threatened to erase that ample canon of folk dentistry. Here was author Tucakov to say: "...It would be a great irresponsibility on history's part not to collect such precious folk medicine treasure created through the sufferings, pains, and tears...[30]." Moreover, around developed countries, a great amount of medical and dentistry drugs were of herbal origin, as well as their extracts that exposed high financial benefit [3]. Concerning the aforementioned, the close aims of this study were:

- Collecting the folk tradition knowledge about mouth and tooth ailments and diseases (dental care, oral hygiene, and preventive deeds).
- Study of specific folk dental practice (professional traditional dentists, their curing doctrine and instruments, dentistry trade teaching, level of dental health culture, sorcerers and witch-doctors, patients' attitude toward folk dentists and contemporary dentistry).
- Study of influence of magic and religious elements to the folk dentistry.

MATERALS AND METHODS

Study Area

Geographical area where Serbs live, i.e., SM lies between 46°11″ to 41°52″ North latitudes and 18°26″ to 23°00″ East longitude, encompassing area of 102350 km^2, embracing Mediterranean and continental climate types.

Data Collection

This research was conducted during June-September period from 1981 to 1991 on the small groups of domicile population, to whom traditional dentistry was not official profession, several folk herbalists/healers/, folk dentists, as well as old persons. Informants were 30-80 years old. They were from small towns and distant villages and settlements, who dealt with folk dentistry long in the past and at the moment of the study. Subjects were asked to show and describe the plants used (their parts) on site, explain the period of collection. The investigators collected information on local names of plants, preparation way, and administration. The herbs were identified through botanical taxonomy. Collected data from all of them were recorded from memory, i.e., through the family background, unfortunately without written papers, which means that data arised from their own practice and lessons from teachers. Data from certain regions gave doctors of dentistry, general practioners and pharmacists, who secondarily gathered them from local traditional healers and old experienced persons-patients, folk-dentists, and herbalists. This collecting method was proposed to be authentic enough for all studied regions.

The preliminary study pointed out that the most appropriate way of data collecting was immediate terrain method, whether it was individual or team work. That method assumed direct data obtaining from all of the pooled subjects through a previously designed questionnaire. The research was conducted with the help of the trained pollsters: Doctors of dentistry and students of BSDM (final academic years), where pharmacists participated in the plant identification. Data gatherers were warned about language during the poll, where inquiry answers should be respected in the sense of exact noting in the local dialect. Doing so, the original idioms could be regularly and linguistically interpreted and explained.

The data were obtained from 39 representative places around SM [Figures 1 and 2]. The neighboring regions of Serbia were also investigated by the same methodology where Serbs are prevailing, by the help of BSDM students who originated from those places for further comparative analysis [Figure 3].

The research is still in progress in the areas of East Serbia and Banat to accomplish the whole country. The study was conducted by a questionnaire (Sheet 1) contained plain Serbian folk language considered five groups.

Sheet 1 - Five groups of questions that were requested from the subjects:

I. Recipes about oral, teeth and gum hygiene, teeth bleaching, tooth eruption and replacement and correspondent folk terminology
II. Recipes about mouth sore (thrush, cold sore, aphthae, and herpes) diseases of the lips, the tongue and the skin around the mouth, and related folk expressions
III. Recipes and ways of treatment of tooth, gum and jaw diseases, and related folk terms
IV. Data about profession of folk dentist, training and their instruments and practice
V. Magical (sorcery) rituals about tooth eruption care of sound and treatment of rotten teeth.

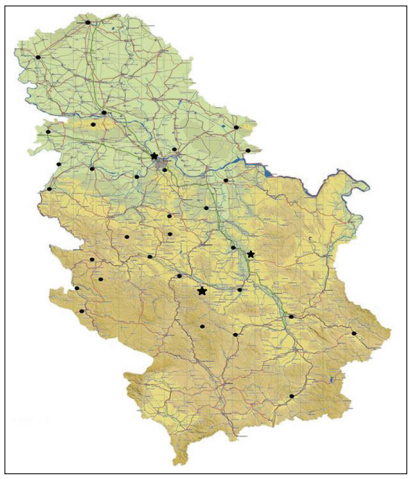

Figure 1: Map of studied places in Serbia (•) and headquarters (Belgrade, Krusevac, and Cuprija) (★)

The filled in lists of questionnaire were classified by places [Table 1] displayed by Figure 1 (Serbia) and Figure 2 (Montenegro).

The most inquiry lists were harvested in the region of Sumadija highlands, where the highest number of research points were situated on, especially during summer training course and voluntary masses. The rest Serbian regions gave just a few inquiries of mostly plain terrains (Vojvodina).

The results of the collected and partially worked out data were stored at the Department for History of Dentistry (FSB). The classified and prepared material consisted of total 1038 inquiry sheets - 1025 for Serbia and 13 for Montenegro, collected during the investigation period. 41 pieces of data were obtained on average per inquiry form, i.e., 41984 pieces of information for the whole research.

Upon deep and patient analysis of herbal names (synonyms) obtained from various regions, the list of corresponding botanical names was arranged, which were previously translated into English.

Figure 2: Inquired places (black crosses) in Montenegro (coastal and landlocked areas)

Table 1: Distribution of 1038 classified questionnaires by enquired places around Serbia and Montenegro (Montenegrin places are underlined)

Localities in the study area	No.
Fruska Gora, Pancevo, Sabac, Bukulja/Vencac, Loznica, Pozarevac, Bogatic, Smederevs, Palanka, Tara, Cacak, Nis, Vranje, Kursumlija, Kopaonik, Prijepolje	2
Divcibare, East Srem, Obrenovac, Pirot, Priboj, Rudnik, Sombor, Subotica, Vrsac, Zlatibor	3
Kraljevo	7
Bela Crkva	11
Novi Sad	13
Beograd	34
Cuprija	297
Krusevac	649
Podgorica	3
Kotor	3
Herceg Novi	7
Niksic	3
Zabljak	2
BijeloPolje	2
Pljevlja	2
Total	1038

Taxonomic identification was conducted by the authors, and plant nomenclature followed the Flora Europaea [31], the Angiosperm Phylogeny Group III system, and the plant list database (The Plant List [Version 1.1] 2013) [32].

RESULTS

Recipes of Mostly Nonherbal Origin

Analyzing seven categorized groups of oral ailments/diseases, eighteen prescriptions were noted mostly for toothache (five) as dominant mouth problem, that was expected [Table 2 and Figure 4].

Prescriptions Consist of Mostly Herbal Origin

The list of collected traditional recipes includes the used plant parts, modes of preparation and usage, and vernacular names (English and Serbian), displayed in Table 3.

The survey presented 84 herbal original prescriptions, including overall 61 plant taxa from 27 families, which are listed by alphabetical order, including local name and synonyms, as well as preparation mode and usage. Some of them were reported for use concerning two or more oral ailments/diseases (for an example toothache and gum bleeding). The most frequently used plants belong to family Compositae (10), *Lamiaceae* (9), *Rosaceae* (7), Leguminosae (4), *Amaryllidaceae* (3), and *Amaranthaceae* (3), like all others they were presented with two or one species. The first three families cover 42.6% from established plants. Half of the plants is cultivated (C), 39.6% from wild localities (W), and 10.3% may be collected whether as wild or cultivated (W/C). This study did not identify the folk names that directly show the use of plants in the care of teeth.

Figure 3: Investigated areas around Balkan countries (small blue stars: BiH – Republika Srpska, FYROM) and headquarters spots - Serbia

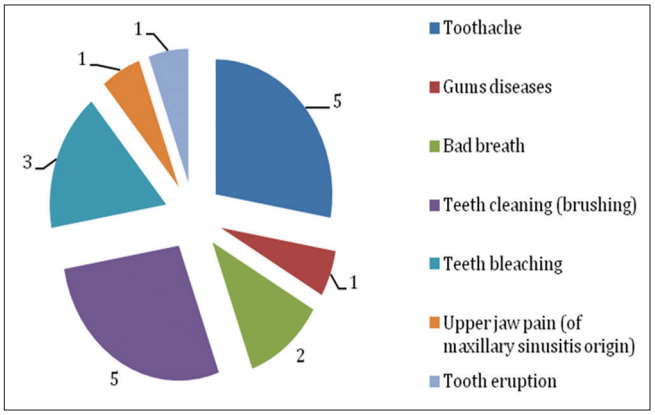

Figure 4: Prescriptions of dominant nonherbal origin``

Traditionally used medicinal plants were within reach from early spring to the end of autumn as fresh ones and in conservated form for the winter season (dry, cream, tincture, etc.). According to traditional beliefs, picking up the herbs was done only during the daylight period.

The most frequently used part of plants for medicinal preparations were their leaves, as well as the other parts (bark, fruit, seed, flower, stems, and roots).

The preparation mode for dental treatment in descending order teas was decoction, infuse, maceration, juices, dishes, pastes, as well as fresh plants. The noted mode of use was drinking/gargling, rubbing into the gums, inserting into the tooth cavity, inhalation, etc.

Some plants, such as *Achillea millefolium* and *Hypericum perforatum*, were noted as panacea for many diseases (not only oral).

A lot of recipes consisted of herbal parts mixed with other artificial materials, unvivid or inorganic substances (alcohol,

Table 2: Established recipes of mostly nonherbal origin

Component	Diseases/symptoms	Mode of usage
Ants	Alleviate pain in gums	Chewing
Bee wax	Alleviate pain in gums	Chewing
Bread	Teeth cleaning	Brushing by crumbled overbaked bread and rinsing by cold water
Bread from corn (proya dish)	Gums massage	Chewing
Cattle dung ash or dried dung	Teeth cleaning	Brushing by fingers or wollen peace
Coal powder	Teeth cleaning	+ Baking soda: Then crumble, permit to dry out and brush the teeth
	Teeth bleaching	
Clay powder	Teeth bleaching	Brushing
	Swollen gums	Gums massage
Flaxen textile	Tooth eruption	Rub the place where tooth is to erupt
Gasoline	Teeth cleaning	Brushing
	Teeth bleaching	
Honey	Toothache	+ Cinnamon (1:3): Dense cream rub of and around the painful tooth
Milk	Bad breath, after hard smoking	Gargling
Plant ash	Teeth cleaning	Brushing by fingers or woolen peace
Propolis	Upper jaw pain of maxillar sinusitis origin	Instilling drops of propolis
	Tooth eruption	Rub the place where tooth is to erupt
Rain water	Bad breath, after hard smoking	Gargling
Salt	Toothache without swelling	+ Perfume liquid: Place into the tooth decayed cavity+Alum
	Throbbing toothache	powder (1:1): Pack in the tooth cavity and around for 4-5 times a day

Table 3: List of 84 herbal prescriptions including 61 plant taxa from 27 families

Taxon, family and voucher specimen code	Recorded local name (s)	English name	Status	Plant part (s) used	Treated pathology (-ies) Recorded preparation Medicinal use (s)
Achillea millefolium L. s.l. Compositae	Stolisnik, hajducka trava	Common yarrow	W	Aerial parts	• Bad gums; oral ulcers (aphtae) • Brandy tincture; infuse • Rinse mouth; rinse mouth and drink
Allium cepa L. *Amaryllidaceae*	Crni luk	Onion	C	Bulb	• Toothache • Smashed bulb • Bandage where pulse is to be found (over the veins of the left hand) until the dental pain alleviates
Allium sativum L. *Amaryllidaceae*	Cesnjak, beli luk	Garlic	C	Bulb	• Toothache; mouth blisters, ulcers; painful gums, toothache • Place smashed garlic clove inside the external ear canal until the toothache passed away, add salt in the smashed clove or eau de Cologne and apply in the tooth cavity; soaking of clove garlic juice; fresh cloves; tea • To place smashed garlic clove inside the external ear canal until the toothache passed away; Add salt in the smashed clove or eau de Cologne and apply in the tooth cavity; both halves of garlic clove to place into each nostrils fixed by honey; mouth gargle of juice made of smashed garlic and sea salt
Allium ursinum L. *Amaryllidaceae*	Divlji luk, sremus	Wild garlic	W	Leaf, bulb	• Painful gums and mouth • Fresh • Massage and chewing
Antennaria dioica (L.) Gaertn. Compositae	Smilje, srcopuc, zecje nozice	Cat's foot	W	Leaf, corymb	• Gums bleeding; toothache • Decoct; cigarette • Mouth wash; cigarette smoking
Arctium lappa L. Compositae	Veliki cicak, repuh, lopuh	Common burdock	W	Root	• Oral abscess, gums bleeding • Extract, decoct • Mouth wash
Armoracia rusticana P. Gaertn., B. Mey and Scherb Brassicaceae	Ren, hren-pitomi	Horse radish	C	Root	• Bleeding gums, toothache • Finely chopped row root • Chewing
Artemisia vulgaris L. Compositae	Pelin divlji	Mugwort	W	Aerial parts	• Bad gums • Brandy tincture • Rinse mouth
Beta vulgaris L. Amaranthaceae	Blitva	Beet	C	Leaf, root	• Bleeding and swelling gums • Boiled • Consume
Beta vulgaris L. var. *rubra* *Amaranthaceae*	Cvekla, Cikla	Chard	C	root	• Bleeding and swelling gums • Chopped row • Chewing long

(Contd...)

Table 3: (***Continued...***)

Taxon, family and voucher specimen code	Recorded local name (s)	English name	Status	Plant part (s) used	Treated pathology (-ies) Recorded preparation Medicinal use (s)
Brassica oleracea L. Brassicaceae	Kupus	Cabbage	C	Leaf	• Toothache, bleeding gums; painful mouth at ptyalismus • Leaves brain; third strained water of boiled bran • Rinse mouth; gargling
Calendula officinalis L. Compositae	Neven, zutelj	Marigold	W, C	Corymb	• Painful gums, aphtae, ptyalismus • Decoct • Mouth wash
Cinnamomum verum J. Presl Lauraceae	Cimet	Cinnamon	C	Bark	• Toothache • Mix: Cinnamon powder, honey, dense cream • Rubbing and spread around the painful tooth
Citrus limon (L.) Osbeck Rutaceae	Limun	Lemon	C	Oil, fruit	• Swollen gums; mouth/lips ulcers, aphthae • White clay powder mixture of the olive oil and lemon juice; mixture from lemon and eucalyptus oil • Massage the gums; Soaking the painful and ulcerous places by cotton ball dipped in the mixture
Clinopodium nepeta subsp. *glandulosum* (Req.) Govaerts. (syn. *Calamintha officinalis* Moench) *Lamiaceae*	Bosiljak divlji	Calamint	W	Aerial parts	• Bleeding gums • Brandy tincture • Rinse mouth
Corylus avellana L. Betulaceae	Lesnik	Walnut	W, C	Leaf	• Ptyalismus, oral blisters • Cold decoct • Gargling
Equisetum arvense L. Equisetaceae	Rastavic, preslica	Field horsetail	W	Aerial parts	• Tooth absscess, gums bleeding • Decoct • Mouth wash
Eucalyptus globulus Labill. Myrtaceae	Eukaliptus	Eucalyptus	C	Leaf	• Upper jaw toothache, maxillary sinusitis; painful and ulcerous places; oral blisters • Infuse; mixture (eucalyptus oil and lemon) • Inhalation; soaking painful and ulcerous places by cotton ball dipped in the mixture
Fragaria×ananassa (Duchesne ex Weston, Duchesne ex Rozier) *Rosaceae*	Jagoda bastenska	Strawberry	C	Fruit	• Mouth blisters, ulcers; inflamed gums/oral mucosa, bad breath • Fresh, juice; leaves decoct • Enormous gargling and juice consummation; rinse mouth
Fraxinus excelsior L. Oleaceae	Jasen beli	European ash	W, C	Brunch	• Bad breath • Fresh • Interdental cleaning
Genista tinctoria L. Leguminosae	Zutilovka	Dyer's broom	W	Aerial part	• Painful gums • Herbal tea, monocomponent or+CuSO4 • Gargling
Geum urbanum L. *Rosaceae*	Blazenak, zecja stopa, srcenik	Wood avens	W	Root	• Painful oral diseases, bad breath, mouth ulcers • Decoct • Gargling
Glycine max (L.) Merr. Leguminosae	Soja	Soya	C	Seed	• Lip and mouth ulcers • Dishes with hot pepper • Consume
Glycyrrhiza glabra L. Leguminosae	Slatko drvce, sladic	Licorice	W	Leaf	• Painful gums • Herbal tea • Gargling and drink
Helianthus annuus L. Compositae	Suncokret	Sunflower	C	Oil, achene	• Jaw joint pain; bad breath • Oil; mixture of coal powder, rose petal and sunflower mixed with honey • Placed inside the external ear canal of nonpainful side of the head; chewing the mixture
Helichrysum arenarium (L.) Moench Compositae	Smilje	Immortelle	W	Leaf	• Bleeding gums • Decoct of white wine • Gargling
Hyoscyamus niger L. Solanaceae	Bunika, balam, svinjarac	Henbane	W	Leaf	• Toothache • Infuse-tea • Gargling
Hypericum perforatum L. Hypericaceae	Kantarion, borodicina trava, sentjanzevka, gospino zelje	St John's worth	W	Aerial parts	• Bleeding, swollen gums, ulcers, aphthae • Brandy tincture of mixture (plantain, yarrow, klamath weed, sweet basil or savory) • Rinse mouth

(Contd...)

Table 3: (***Continued…***)

Taxon, family and voucher specimen code	Recorded local name (s)	English name	Status	Plant part (s) used	Treated pathology (-ies) Recorded preparation Medicinal use (s)
Juniperus communis L. Cupressaceae	Kleka, smreka, borovac	Juniper	W	Galbula	• Bad breath • Raw • Chewing
Lavandula angustifolia subsp. *pyrenaica* (DC.) Guinea (syn. *Lavandula vera* DC.) *Lamiaceae*	Lavanda	Levander	C	Flower	• Bleeding gums • Decoct • Rinse mouth
Malus domestica Borkh. *Rosaceae*	Domaca jabuka	Apple	W, C	Fruit	• Mouth blisters, ulcers • Fresh, juice • Slowly chewing and garling
Matricaria chamomilla L. Compositae	Bolivac, milica , milanka titrica, milica-trava, milanka	German (wild) chamomile	W, C	Aerial parts	• Sinusitis • Infuse • Gargling, inhalation
Matricaria chamomilla L. (syn. *M. recutita)* Compositae	Kamilica, gorcak, gamilica, ramenak, kamil-tej, kokosnjak	Chamomile	W, C	Corymb	• Mild toothache, gums bleeding, swollen (sore) gums, oral linings, painful mouth/tongue diseases, teething, bad breath, lip ulcers • Infuse • Gargling and drinking
Melissa officinalis L. *Lamiaceae*	Maticnjak, limun trava, pcelinjak	Lemon balm	W	Leaf	• Bleeding gums, oral blisters; bad breath • Infuse; oil • Gargling; oil soaking
Mentha× *Piperita* L. *Lamiaceae*	Nana pitoma	Peppermint	C	Leaf	• Bleeding gums, bad breath; bad breath • Infuse; fresh leaves • Rinse mouth; chewing the leaves
Nicotiana tabacum L. Solanaceae	Duvan, duhan	Tobacco	C	Leaf	• Bad breath • Cigarette of tobacco leaves and wild rabbit excrement foiled by newspaper • Hard smoking by keeping the smoke inside the mouth
Nigella sativa L. Ranunculaceae	Curukot, curekot, Mackov brk, cupava kata	Black seed	C	Oil, seed	• Toothache • Oil of seed dissolve into the hot water • Mouth rinse, rubing with oil
Ocimum basilicum L. *Lamiaceae*	Bosiljak pitomi	Sweet basil	C	Aerial parts	• Bleeding gums • Brandy tincture • Rinse mouth
Olea europaea L. Oleaceae	Maslina	Olive	C	Oil	• Swollen gums • Mixture of the olive oil and lemon juice • Gums massage
Phaseolus vulgaris L. Leguminosae	Pasulj	Bean	C	Seed	• Oral blisters • Boiled • Chewing for a long and eat
Pinus sylvestris L. Pinaceae	Beli bor	Scots pine	W, C	Resin	• Bad breath; very painful tooth with swelling • Pine resin; decoct of the stumps; pine incense • Chewing around 10-15 min; teeth brushing; hot piece of pine incense placed into the tooth decayed cavity provokes swollening and rupture of tooth structure relieving exudate and pain
Piper nigrum L. Piperaceae	Papar crni, biber	Pepper	C	Fruit	• Bleeding gums • Alcohol tincture (mint and pepper); dried fruits • Gums massage; chewing
Plantago lanceolata L. Plantaginaceae	Uskolisna (muska) bokvica	Plantain	W	Leaf	• Toothache • Brandy tincture of the plantain, yarrow, klamath weed, sweet basil or savory • Gargling
Prunus domestica L. *Rosaceae*	Modra sljiva	Common plum	C	Fruit	• Swallowed painfull tooth • Several freshly extracted plum pits • Press by tongue in oral cavity thus abscessed tooth relieves pus then cold compress over the skin
Pyrus communis L. *Rosaceae*	Kruska domaca	Common pear	C	Fruit	• Mouth blisters; mouth blisters, ulcers • Fresh fruits; • Chewing for long; enormous garling and consummation of juice

(Contd…)

Table 3: (***Continued...***)

Taxon, family and voucher specimen code	Recorded local name (s)	English name	Status	Plant part (s) used	Treated pathology (-ies) Recorded preparation Medicinal use (s)
Quercus robur L. Fagaceae	Hrast luznjak	Oak	W	Leaf	• Painful mouth • Decoct, leaves: water: vinegar 1:1 • Gargling
Rosa spp. *Rosaceae*	Ruza	Rose	C	Flower	• Painful gums; mouth blisters; bad breath • Infuse of red rose petal; rose oil; mixture of coal powder, rose petal and sunflower mixed with honey • Gargling; soaking; chewing the mixture
Rubus caesius L. *Rosaceae*	Kupina	Blackberry	W	Leaf, fruit, root	• Loose teeth; bad gums/oral mucosa • wine decoct root or fruit; decoct of leaves • Long rinse
Salvia officinalis L. *Lamiaceae*	Zalfija, kadulja, pitomi pelin	Sage	W, C	Leaf	• Bleeding gums, toothache • Decoct • Gargling
Satureja hortensis L. *Lamiaceae*	Cubar vrtni	Savory	C	Aerial part	• Bleeding gums • Brandy tincture • Gargling
Spinacia oleracea L. *Amaranthaceae*	Spanac, Spinat	Spinach	C	Leaf	• Bad gums, mouth ulcers • Coocked, spinach dish • Chew long
Symphytum officinale L. Boraginaceae	Gavez, volovski jezik		W	Root	• Swollen gums, painful mouth • Tea • Gargling
Syzygium aromaticum L. Merr&L.M.Perry Myrtaceae	Karanfilic, klincic	Clove	C	Flower bud	• Bad breath; toothache • Dried; extracted oil • Chewing; put into tooth cavity
Taraxacum sp. Compositae	Maslacak, vetrokaz mlecac, mislovka popino guvno	Dandelion	W	Leaf, root	• Toothache; bleeding and swelling gums • Latex from root; boiled leaves • Soaking by gauze and place in painful tooth cavity; chewing leaves
Thymus serpyllum L. *Lamiaceae*	Majcina dusica, babja dusica, tamjanika, vrisak, divlji bosiljak, materinka, papric, bukovica	Wild thyme	W	Aerial part	• Oral mucosa ulcers (aphtae) • Infuse • Rinse mouth
Thymus vulgaris L. *Lamiaceae*	Timijan vrtni	Garden thyme	C	Aerial part	• Oral ulcers (aphtae) • Infuse • Rinse mouth, gargling, drink
Tilia tomentosa Moench Malvaceae	Lipa	Lime tree	W, C	Brunch	• Bad breath • Fresh • Interdental cleaning by small branch
Urtica dioica L. Urticaceae	Kopriva, zara	Nettle	W	Leaf	• Bleeding/swelling gums • Boiled leaves • Chewing and eat
Vaccinium vitis-idaea L. Ericaceae	Brusnica	Cowberry	W	Fruit	• Gums diseases • Raw • Long chewing
Vitis vinifera L. Vitaceae	Crno/Belo grozdje	Common grape	C	Fruit	• Toothache, bleeding gums; mouth blisters, ulcers • Immortelle wine decoct; Press grains • Gargling; long and mouth juice gargling
Zea mays L. Poaceae	Kukuruz	Maize	C	Grain	• Toothache; painful gums/teeth; stomatitis, ptyalismus • Boiled water of the white unriped maize grains; Boiled white unriped grains with CuSO4; third strained water of the boiled maize bran • Gargling

C: Cultivated plants, W: Wild plants, W/C: Wild or cultivated plants

clay, wood derivatives, salt, spiritus, milk, $CuSO_4$, excrements, honey, propolis, baking soda, etc.), enabling adjuvant and corrective function.

The storage of materia medica was done by woolen textile, paper, leather or linen bags, and clay, wooden or glass vessels.

The common mouth and teeth problems for treatment were dental pain, swelling around tooth, gums bleeding/swelling, and painful tooth eruption.

The most inquiry lists were harvested in the region of Sumadija (Serbia), where the highest number of research points were

situated, especially during summer training course and voluntary masses - 1000 (96.3%). Precisely, the most plentiful Sumadija spots, i.e., headquarters were Krusevac (649) and Cuprija (297) that involved mostly woodland areas [Figure 3]. The plain Vojvodina region gave just a few inquiries - 2.7%.

Montenegrin research locations were just a few, presenting 13 inquired subjects - 1.3% out of all inquiries [Table 1 and Figure 2].

Data for the Five Groups

The most answers (27 from Group I) were more or less similar by their ingredients. These recipes and those from Group II and III could be summarized as tea (decoction, infusion, and macerate), extract, herbal mixture with or without combination of edible fruits, tea combined of mineral ingredients and/or woody parts of plants, herbal mixtures with addition of pure chemicals (alcohol and methylated spirit) or Mediterranean or subtropics fruits (lemon, lavender, rosemary, clove, coffee grains, and cinnamon) and some animal products (milk, honey, and cattle excrement).

The ample sources of stomatitis treatment exhibited Group II - 26 recipes. Those were healing recipes of herbal mixtures and dietetic dishes. They were required to be the longer, the better in contact to oral mucosa to expose salutary remedy effect. Many idioms were recorded for variants of mouth sore conditions.

Based on the gathered data, the most voluminous was Group III (64 recipes). That goes without saying if consider the great significance of dental/gingival pain for common peasant, who was away even from provincial city and occupied by everyday farmer chores, having no time for a qualified dentist visit. Although the most of those remedies just numb the pain and do not point to the cause, they used referred recipes to help them even for a while. This group consisted of the recipes mostly for gum diseases (39) and toothache (25). Those pathologies were noted as dominant for traditional healers in the study around Burkina Faso [13].

Considering Group IV, nine folk dental therapists (men) were enquired about their way of treatment. They were classified into four character types: (a) Dental trade that was a well-kept secret learned from their ancestors, handing on to succeeding generation within the family (4 subjects). They are believed that trade secret discovering out of the family would bring the loss of curing power; (b) dental trade learned from the "teacher," handing down to the others (one answer); (c) type was like type b, but it kept healing secret only for itself (three enquired persons); (d) self-taught dental skillfulness, where "secret of treatment" confided only to its close relatives (one inquired). If someone else had discovered the secret of dental trade, the power was believed to vanish.

Some of them learned dental treatment interventions during war or military service, especially extractions in position of doctor's assistant (apprentice). In peacetime, at homeland, they used their own instruments (forceps) made of iron or combined with wooden handle, or equipment brought from military ambulance. There were "operators" who charged for the services by reasonable prices, some used compensation payment, while others treated free of charge.

Group V contained only seven different "recipes of magic power" (5.5% out of all). Some of those included: Buying a white present (upon first milky tooth eruption), or placing the first milky extracted tooth under the home bearing beam, or white pillow, giving it to a dog to eat it, etc., All those habits were to stress the whiteness as sound tooth color and home beam and dogs as symbols for power. The majority of those answers pointed out to the high rate of failure by that way of treatment, among the people who was more and more enlightened, even in isolated spots. They gave up more and more magic remedies, accepting new therapy methods, but still preserved the custom of folk traditional substantial prescriptions (Group I, II, and III recipes) empirically approved as beneficial ones.

DISCUSSION

The authors of this study put the great effort into the interpretation of medicinal herbs names and their synonyms, as well as for classification on the similar, slightly varied prescriptions by composition and preparation mode that might be useful for botanical classification/systematization of the species possessing phytotherapeutical role.

Considering the study as a whole, the aim of this research was to explain the good and bad ways of traditional treatment and remedies used throughout the centuries and to distinguish the medically correct doctrine from what was unscientific, wrong and harmful to one's health inefficient, even toxic.

Sorcery and magic rituals could be noted as bad ways of treatments. These options have been used only if the substantial (mostly herbal) or operative mode failed. Precisely, dysfunction of feeding (chewing and swallowing), as well as accompanied pain of well innervated orofacial region was often unbearable for the patient, who then decided to ask for suggestive/hypnotic episodes by the help of sorcerers. Having been alleviated of oral troubles by these ways just for a while, the end solution would be extraction or incision of pus collection.

The copious resulting material might be of confidential origin, due to voluminous results and the direct method of data collecting, immediately obtained from the subjects such as "folk therapists" - healers and common people, often illiterate ones, even from isolated highlanders, and plainsmen.

The contemporary dental pharmacology and pharmacognosy should benefit from recorded recipes, using herbs from still unpolluted areas of investigated regions. The similar situation has already occurred in India on completion of ETD research done in 2004, where 16 brand new herbal species were discovered for the treatment of dental pain, aphthae, alveolar pyorrhea, dental plaque, and dental caries prevention. Those plants, up

to that time, had not been recognized and classified in Indian ethnobotanical and phytotaxonomical literature [14,33].

Usefulness of comparative analysis showed the ethnomedicinal studies in the regions of Eskimos, Papua Island's tribes, East Africans, Amazonian Brazilians, etc., [6,12]. Hence, the similarity in dental recipes was found in literature: Italian ethnologists conducted the same work recorded several herbal recipes in Albanian immigrant population in South Italy, unknown up to then. The added animal constituents, what they used [9], were similar to the Serbian mixture prescriptions which probably came from the Albanian nation from Kosovo into central Serbian regions. The second example was the case when plain population, mostly villager and farmers, were compelled to use cheap and "within reach" medicaments, what was amazingly similar between Serbian [34] and Dinka population (African mostly Christian Ethiopian tribe). (To remember that Orthodox Christianity in Ethiopia was established in the 4th century). Both populations used ash mixture of burned cow dung for teeth cleaning by fingers. The third case was copper sulfate mixed by herbs powder as a common recipe for toothache and painful gums relief in Serbia [34], as well as in India [14,33].

Some of the recorded plants in our ETD study had the similar beneficial effect like sage. What a pity they are still not in use, concerning the presence of sage extracts in many dental products for oral care, exposing even six useful effects, such as antioxidant, antimicrobial, antifungal, astringent, anti-inflammatory, and odorant drugs. Those plants might be categorized into several pharmacological groups, namely, sialogogues, antisecretolytics, tonics, adstringents, styptics, antiseptics, sedatives, antineuralgics, anesthetics, vulneraries, antibiotics, and corrigenses. They exposed useful effect in dental medicine through the following active principles: Alkaloids, heterosides, saponins, essential oils of aromatic herbs, tannins, flavonoids, mucinous matters, phytocides, vitamins, etc., most of them demonstrated antioxidant, immunostimulate, and anticancer effects.

According to our best knowledge, beside mineral substances, the following plant species were noted in this ETD study: Broom, rosemary, coltsfoot flower, yellow iris, licorice, marigold, black grapes, cranberry, spinach, henbane, celandine, walnut leaf, onion, petal and oil of red rose, immortelle, garlic, root/leaf of blackberry, dandelion juice, yarrow, nettle, plantain, basil, chips of pine log, madder, common mallow, radish, grape hyacinth, rue, bilberry, blueweed, barberry, elder, calamus, hedge bindweed, pine marten resin, grain brandy, and incense. These species have not been yet applied nor their derivatives in the recent dental phytotherapy. The benefit of the study might be attributed to botany, botanic terminology, pharmacognosy, and ethnopharmacy.

As far as the wrong mode of traditional dental treatment, the most of sorcery and magic curing was noted in rural and isolated places. Nowadays, in those regions more and more experienced advantageous herbal prescriptions are in use even near contemporary dental offices. However, traditional dental recipes are often adjuvant and even competitive to modern ones, because they are cheap and previously experienced as very effective ones.

Unfortunately, there are still no ways to collect the ETD treasure from Kosovo and Metohija region. That region would be of great interest due to the existence of numerous isolated settlements with preserved customs in time interval too distant from contemporary life, concerning ethnical and religious aspect of mixed population situated there.

It is appropriate to quote the urgent and pressing words of Serbian pioneers in ETD, ethnomedicine and ethnopharmacy directed to generations to come. They pointed out even in 1976 of necessity to speed up the data analysis of ETD research in much detail because "…there is a need to record the grandfather's medicine as soon as possible and authentically because accelerating urban changes threaten all of that heritage bring out of oblivion up to the end of the 20th century…." The confirmation of aforementioned note could be found in Prof. K. Todorovic foreword of book "700 years of medicine in Serbs." Here he wrote "...Serbs were going through the history through the different phases of military glory and power, advanced social structure and cultural ascent, then fall and internal trouble, discord, and mutual rivalry and clashes, military ups and downs as well as long slavery, migrations and suffering where even bare existence of the nation was sometimes endangered...[30]."

Besides mainly biological aspects of ETD in this study, sociocultural focus might be of great importance for social science (ethnolinguistics, history, etc.) analyzed by professional persons in those fields. The recorded data in our investigation frequently showed the union of religious elements and herbal healing treatment. Overall mentioned points to the significance of performed ETD research and data operations, what altogether calls for further extension of our study, encompass the multidisciplinary approach and comparative analysis thoroughly.

The above-mentioned findings should be supported for industrial utilization of some herbs, which can be useful for dental pharmaceuticals due to the opulent biodiversity of Balkan regions. They might present a significant source of export earnings. Having in mind that around 80% of the world's population use herbal-based recipes, this cheap way of treatment, especially in the Third World countries, should be stressed.

The majority of collected interesting folk terminology data ("folk dental idioms") have still not worked out and should be classified by specific methodology (further research is under way).

Furthermore, there were many folk jargons recorded about anatomical dental terms in all questionnaire groups, as well as for diseases and medicinal plants [34] that are interesting material for further ethnolinguistical research.

The obtained data from the neighboring countries (Republika Srpska, FYROM – Macedonia), i.e., spots where Serbs are prevailed, have not yet been operated in the scope of comparative analysis. That would be useful for the study of

immigrant change of life conditions and adaptation to new environments, similarity and differences of folk dental medicine knowledge in the studied areas and abroad where Serbs live, among neighboring nations, ethnic, and religious groups.

CONCLUSION

We should emphasize the significance of plants obtained from unpolluted areas, whose active ingredients have not yet been used in dental pharmaceutics, which can be a promising field for further researches.

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Hereby, we pay a tribute to Prof. Dr. Vera Gavrilovic – Doctor of dentistry (In memoriam) who began this study as early as 1972 as pioneer in ethnostomatological research around Balkan peninsula and ran a great deal of this investigation in the past decades.

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Original Research

Toxicity status and antiulcerative potential of *Sansevieria trifasciata* leaf extract in Wistar rats

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ABSTRACT

Aims: The lethal dose 50% (LD_{50}) and antiulcerative potentials of *Sansevieria trifasciata* (ST) leaf extract were investigated. **Materials and Methods:** LD_{50} was determined through two routes of administration (intraperitoneal [i.p] and oral [p.o]) using the method of Lorke. The antiulcerative activity was evaluated in indomethacin-induced ulcer model (40 mg/kg body weight [BW], i.p, single dose) against a reference drug, cimetidine (100 mg/kg BW, p.o). ST was assessed at two different doses (200 and 400 mg/kg BW, p.o). Treatments were done twice daily at 8 h interval for 7 days before indomethacin administration. **Results:** The i.p LD_{50} was determined as 774.60 mg/kg BW and oral administration of the extract at 18,000 mg/kg BW dosage did not cause any negative behavioral changes in the animals, and no mortality was recorded after 24 h of the experiment. ST-pre-treated animals showed some improvement against indomethacin-induced ulceration. The extract curtailed indomethacin-induced reduction in gastric volume (36.1%), free acidity (55.3%), total acidity (35.6%) while minimizing the increase in pH by 13.3%. Moreover, the extract showed 17.92% and 14.96% ulcer protective ability at 200 and 400 mg/kg BW, respectively. The phytochemical analysis of ST extract revealed the presence of phytoconstituents such as glycosides, saponins, flavonoids, terpenoids, alkaloids, tannins, anthraquinone, and glycosides. **Conclusions:** ST apparently has a promising antiulcerative potential, and is safe for use in folk medicine. This valuable medicinal property is probably due to the array of important phytochemicals contained in the plant as observed in this study. However, a further study involving bioassay-guided identification of the main antiulcerative compound in ST is required to establish the use of the plant as a viable antiulcerative agent.

KEY WORDS: Gastric ulcer, indomethacin, *Sansevieria trifasciata*, toxicity status

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INTRODUCTION

Gastric ulcer remains a health burden on almost 10% of the world populace, and the need for antiulcerative agents from natural sources, such as plants is becoming popular and acceptable. This is probably because formulated drugs are relatively expensive and often associated with adverse side effects. Physiological imbalance between aggressive factors (pepsin and hydrochloric acid) and protective factors (bicarbonate and mucus) in the stomach is adjudged the primary cause of gastric ulcer [1,2]. Bad dietary habits, stress, *Helicobacter pylori* infection, and excessive use of nonsteroidal anti-inflammatory drugs (NSAIDs) are other factors which may result in gastric ulcer [3,4]. Patients with gastric ulcer have over the years relied on orthodox drugs such as cimetidine, ranitidine, antacids, and omeprazole for the management of the disease. However, in recent times, for different reasons, including those aforementioned there is an increased public interest in plant therapies [5].

Sansevieria trifasciata (ST) is one of the 70 species of *Sansevieria* genus. It is a species of flowering plant in the family Asparagaceae, native to tropical West Africa. The plant is often referred to as viper's bowstring hemp, snake plant, mother-in-law's tongue, or Saint George's sword (in Brazil). It has significant therapeutic utilization in folklore medicine [6]. In Africa, the plant is used as a protective charm against evil or bewitchment [7-9]. The use of ST in folk medicine for the treatment of different ailments such as ear-ache, ulcer, jaundice, pharyngitis, skin itches, urinary diseases, analgesic and antipyretic is well known [10]. This study, therefore, sought to evaluate the antiulcer potential of the ethanol leaf extract of the plant.

MATERIALS AND METHODS

Collection of ST Leaves

Fresh ST leaves were harvested in the month of December 2015 from Ibadan south west Local Government Area of

Ibadan, Nigeria. The harvested leaves were authenticated at the Department of Botany, University of Ibadan. Where a specimen was deposited and assigned a voucher number, UIH 22435.

Preparation of ST Leaf Extracts

The plant material was freed of extraneous materials; air dried at room temperature and milled to a fine powder, using a Warring blender. 300 g of the powdered sample was macerated in 2.5 L of the extracting solvents (ethanol) at room temperature (27 ± 2°C). The mixture was allowed to stand for 72 h and stirred intermittently with a glass rod to facilitate extraction. Sieving of the mixture was achieved with a muslin cloth (maximum pore size 2 mm). The resulting filtrate on sieving was further filtered through Whatman filter paper (No. 42) and subsequently reduced in volume with a rotary evaporator at 40°C. Final elimination of solvent and drying was done using a regulated water bath at 40°C.

Collection and Management of Animals

Male rats of the Wistar strain (102-151 g) were used for the study. The rats were purchased from the Animal Breeding Unit, Department of Anatomy, University of Ibadan. All procedures for maintenance and sacrifice (care and use) of animals were carried out according to the criteria outlined by the National Academy of Science published by the National Institute of Health [11]. The animals were handled humanely, kept in plastic suspended cages, placed in a well-ventilated and hygienic rat house under suitable conditions of room temperature (27 ± 2°C) and humidity. They were provided rat pellets with water *ad libitum* and subjected to a natural photoperiod of 12 h light and 12 h dark cycle. The animals were allowed 2 weeks of acclimatization before the commencement of all animal model experiments in this study.

Lethal Dose Determination of Ethanol Leaf Extract of ST

Lethality studies to determine the lethal dose 50% (LD_{50}) of the extract were performed according to the combined procedures described by Lorke [12] and OECD guidelines-425 [13]. It was assessed through two routes of administration; intraperitoneal (i.p), and oral (p.o). For i.p determinations, 40 male rats were randomly assigned to 10 groups, with each group having 4 animals. They were, respectively, treated with 200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800, and 2000 mg of the extract per kg body weight (BW) of the animals. The animals were then returned to their respective cages, allowed free access to pellets and drinking water 3 h later. They were thereafter monitored for clinical signs, symptoms, behavioral change, feeding pattern and mortality within 24 h of the experiment. Animals were observed individually once during the first 30 min after dosing, periodically during the first 24 h (with more attention during the first 4 h), and daily for a period of 14 days.

For the oral LD_{50} determination, three different sets of animals were used. The first set of animals was randomized into five groups, each containing four rats. They were treated with 1000, 2000, 3000, 4000, and 5000 mg/kg BW with no mortality recorded after 24 h. In the second phase, doses of 6000, 8000, 10,000, 12,000, and 14,000 mg/kg BW were administered to another set of animals. When no mortality was recorded, a third set of animals equally assigned to five groups were, respectively, treated with doses of 15,000, 20,000, 30,000, 40,000 and 45,000 mg/kg BW of the extract. They were closely observed for negative behavioral changes and mortality within 24 h of the experiment.

The lethal dose of the extract through the different routes was calculated using the formula by Lorke [12]:

$$LD_{50} = \sqrt{D_0 \times D_{100}}.$$

Where D_0 = Maximum dose that produces 0% mortality, D_{100} = Minimum dose that produces 100% mortality.

Indomethacin Induced Gastric Ulcer Model

A total of 30 adult male Wistar rats of weight between 130.30 and 163.00 g were randomly assigned to five groups as shown in Table 1. Groups I and II were administered saline, while Groups III, IV, and V were pretreated with cimetidine (100 mg/kg BW), ST (200 mg/kg BW), and ST (400 mg/kg BW) for 7 days, respectively. On the 8th day, gastric ulcer was induced by the method described by Bhattacharya *et al*. [14] with slight modification. Indomethacin (40 mg/kg BW) was administered to rats in Groups II, III, IV, and V after animals were fasted for 24 h. The animals were then sacrificed 4 h after the administration of indomethacin. The stomach of each animal was removed, incised along the greater curvature and the gastric content was emptied into appropriately labeled sample bottle for determination of gastric volume, pH, pepsin activity, free acidity, and total acidity. The stomach was then washed with normal saline, pinned on a flat surface to observe for lesions/ulcer in the glandular portion.

Quantification of Ulceration

The ulcerative index and percentage ulcer protection of the different treatments were estimated using the method described by Szabo and Hollander [15]. Based on the intensity of ulceration as observed with a hand lens, the score were given as: 0 = Normal

Table 1: Experimental design and treatment

Experimental group	Treatment/dose/route	Code
Group I	Rats were normal control	NC
Group II	Indomethacin rats were left untreated	UC
Group III	Ulcerated rats were treated with cimetidine (100 mg/kg BW/p.o)	PC
Group IV	Ulcerated rats were treated with plant extract (200 mg/kg BW/p.o)	ST 200
Group V	Ulcerated rats were treated with plant extract (400 mg/kg BW/p.o)	ST 400

NC: Normal control, UC: Untreated negative control, PC: Treated positive control, ST 200: 200 mg/kg BW/p.o ST extract, ST 400: 400 mg/kg BW/p.o ST extract, ST: *Sansevieria trifasciata*

mucosa; 1 = Vascular congestion; 2 = One or two lesions; 3 = Severe lesions, 4 = Very severe lesions, 5 = Mucosa full of lesions. The ulcer index was determined using the formula:

Ulcer index = (Ulcerated area/total stomach area) × 10

Percentage protection = (Uc−Ut) × 100/Uc

Where Uc = Ulcer index of control group and Ut = Ulcer index of treated group.

Estimation of Free Acidity

The gastric contents were centrifuged at 1000 rpm for 10 min. One mL of the supernatant liquid was pipette out and diluted to 10 mL with distilled water. The solution was titrated against 0.01 N NaOH using Topfer's reagent (dimethyl-amino-azo-benzene) as an indicator, to the end point when the solution turned to orange color. The volume of 0.01 N NaOH needed was taken as corresponding to the free acidity.

Estimation of Total Acidity

Titration was further continued by adding two drops of 1% solution of phenolphthalein till the solution gained the pink color. The volume of 0.01 N NaOH required was noted and was taken as corresponding to the total acidity.

Acidity was expressed as:

$$\text{Acidity} = \frac{\text{Volume of 0.01 N NaOH} \times \text{normality} \times 100 \text{ mEq/L/100 g}}{0.1}$$

Estimation of Pepsin Activity

The assay mixture contained 0.1 mL gastric juice supernatant (centrifuged at 5000 ×*g* for 10 min) and 1 mL of bovine albumin (0.5% w/v in 0.01 N HCl, pH 2) which was incubated for 20 min at 37°C. 2 mL of 10% trichloroacetic acid was added to stop the hydrolysis. All tubes were heated in boiling water for 5 min to denature the proteins and cooled. The precipitate was removed by centrifugation (9000 ×*g* for 10 min), and 1 mL of the supernatant was mixed with 0.4 mL of 2.5 N NaOH and 0.1 mL of the Folin–Ciocalteu reagent and the volume was make up to 10 mL with distilled water. A control set up in which 1 mL albumin was replaced with 1 mL of distilled water was run simultaneously. The absorbance was measured at 700 nm. The peptic activity was calculated in terms of micrograms of tyrosine liberated per milliliter of gastric juice according to the method described by Prino *et al.* [16].

Qualitative Phytochemical Evaluation of ST Extracts

Standard procedures as described by Sofowara [17]. Edeoga *et al.* [18], Trease and Evans [19] Harbone [20] were used with some modifications to detect the phytochemicals present in the extract of the plant.

Statistical Analysis of Data

The data obtained were statistically analyzed using GraphPad Prism statistical software, version 6.4. Hypothesis testing was by one-way analysis of variance followed by least significant difference test. $P < 0.05$ was considered statistically significant. Results are presented as mean ± standard deviation ($n = 5$).

RESULTS

LD_{50} Determination

i.p, the maximum dose of the extract that produced 0% mortality was 600 mg/kg BW and the minimum dose that produced 100% mortality was 1000 mg/kg BW.

The i.p LD_{50} of ST was estimated as 774.60 mg/kg, while the oral LD_{50} was estimated to be >18,000 mg/kg BW.

Antiulcerative Effects of ST

Compared to the control animals, single i.p administration of indomethacin at a dose of 40 mg/kg BW elicited a significant reduction in gastric juice volume, free acidity, total acidity, and pepsin activity as well as a concomitant increase in pH in experimental rats. However, oral pre-treatment of the animals with ST leaf extract at 200 and 400 mg/kg BW for a period of 7 days showed some significant effects in reducing the negative alterations caused by indomethacin in the animals [Table 2]. The extract was able to minimize the indomethacin-induced reduction in gastric volume (by 36.1%), free acidity (by 55.3%), and total acidity (by 35.6%) while minimizing the increase in pH (by 13.3%). Moreover, the extract showed 17.92% and 14.96 % ulcer protective ability at 200 and 400 mg/kg BW, respectively [Table 3]. This mild protection is substantiated by the photomicrographs shown in Figure 1 (a,b,c,d and e).

Qualitative Phytochemical Constituents of ST Leaf Extract

The phytochemical contained in ST ethanol leaf extract is shown in Table 4.

Table 2: Effect of ST extract on pH, free and total acid, and pepsin activity of the gastric juice in indomethacin-induced gastric ulcer in Wistar rats

Group	Gastric juice (ml)	pH	Free acidity (mEq/L)	Total acidity (mEq/L)	Pepsin activity (mg/mL)
NC	7.5±0.6	2.6±0.2	35.2±3.4	49.0±6.9	189.0±2.1
UC	3.6±0.4	5.6±0.4	9.6±0.6	20.8±2.5	131.0±33.6
PC	5.8±0.3*	3.5±0.5*	26.5±2.2*	37.3±3.0*	144.5±6.2*
ST 200	4.6±0.7	4.5±0.9	21.5±4.1*	30.7±3.2*	122.7±10.0
ST 400	4.9±0.5	3.9±0.3*	21.2±4.4*	32.3±4.1*	127.1±6.1

Values are expressed as mean±SD, n=5. *Significantly different (P<0.05) compared to indomethacin untreated group. SD: Standard deviation, NC: Normal control, UC: Untreated negative control, PC: Treated positive control, ST 200: 200 mg/kg BW/p.o ST extract, ST 400: 400 mg/kg BW/p.o ST extract, ST: *Sansevieria trifasciata*

DISCUSSION

Phytochemicals are chemical compounds formed during the plants' normal metabolic processes and have been associated with the biological or pharmacological effects elicited by plants. The medicinal functionality of several phytochemicals including alkaloids, flavonoids, coumarins, glycosides, phenols, tannins, terpenes, and terpenoids is well documented [21,22].

According to Martins *et al*. [23], the antitumor, anti-inflammatory, and antimicrobial properties of plant extracts are due to the presence of alkaloids. Flavonoids are known for their antioxidant characteristics [24]. Phenolics such as flavonoids and tannins have been linked with anti-helmenthic properties [25]. Saponins, in addition to their industrial uses such as foaming agents and detergents, have a wide range of medicinal applications [26].

In this study, the phytochemical screening of the ethanol leaf extract of ST shows that the plant constitutes most of these aforementioned biologically active phytochemicals, thus, signifying its use in folk medicine for treatment of different ailments including gastric ulcer. Moreover, the presence of these phytochemicals in ST suggests that the plant leaves possess valuable medicinal potential yet to be explored.

Despite the medicinal relevance of plants, studies have suggested that some plants or vegetable species are potentially toxic to humans and animals [27]. The chemical compounds responsible for the toxic effects of plants are probably produced as part of the plant's defense mechanism against pest and herbivores or to gain an advantage over competing plants. The toxicity status of a compound is often measured by its LD_{50}. LD or lethal concentration 50% is generally referred to as the dose or concentration of a test material (plant, chemical, drug, etc.) that causes mortality in 50% of the animals (rats, mice, etc.) in a dose group. LD_{50} values are useful in comparing the relative acute hazards of substances, especially when no other toxicology data are available for the substances. More importantly, the LD_{50} value of a material communicates its safety dose range through different routes.

Table 3: Inhibitory activity of ST leaf extract on indomethacin-induced ulcer in Wistar rats

Group (treatment)	Ulcer index (mean±SD)	Ulcer inhibition (%)
NC	0±0.0	-
UC	7.42±0.28[a]	-
PC	4.36±0.28[c]	41.24
ST 200	6.09±0.28[b]	17.92
ST 400	6.31±0.28[b]	14.96

Values with different superscripts are statistically significant ($P \leq 0.05$) to each other. SD: Standard deviation, NC: Normal control, UC: Untreated negative control, PC: Treated positive control, ST 200: 200 mg/kg BW/p.o ST extract, ST 400: 400 mg/kg BW/p.o ST extract, ST: *Sansevieria trifasciata*

Table 4: Phytochemical screening of ST

Phytochemicals	Result
Saponin	+
Flavonoid	+
Terpenoid	+
Cardiac glycoside	+
Alkaloid	+
Tannin	+
Anthraquinone	+
Glycoside	+

+: Present, ST: *Sansevieria trifasciata*

The LD_{50} values of the investigated leaf extract of ST through i.p route was 774.60 mg/kg BW. This value connotes a substantial degree of safety for the use of ST leaf extract in terms of toxicity level assessment through this route [28]. More importantly, oral administration of the extract at a dose of 18,000 mg/kg did not cause any negative behavioral changes in the animals, and no mortality was after 24 h of the experiment.

The strikingly high oral safe dose of ST could possibly be due to biotransformation of the active component(s) of the extract into nontoxic metabolites in the gastrointestinal tract of the animals by the action of certain modifying or detoxifying enzymes.

This study also evaluates the antiulcer activity of ethanol leaf extract of ST in indomethacin-induced ulcer model. Indomethacin is a NSAIDs which reduces pain, fever, and inflammation by inhibiting cyclooxygenase-1 (COX-1) and COX-2 (enzymes that produce prostaglandins, which promote pain, inflammation, and fever) [29]. COX-1 produces an additional type of prostaglandin that protects the stomach lining from stomach acid. Thus, inhibiting the enzyme makes the mucosal cells vulnerable to pepsin-acid damage, and consequently increases the risk of ulcers and gastrointestinal bleeding [30]. This is evident in the results of this study in which i.p administration of indomethacin at a dose of 40 mg/kg significantly altered valuable parameters associated with gastric secretion, and caused severe damage to the lining of the stomach [Figure 1a and 1b]. Nonetheless, oral pre-treatment of the animals with ST leaf extract at doses of 200 and 400 mg/kg BW for a period of 7 days significantly minimized the negative alterations caused by indomethacin in the experimental animals and showed some level of ulcer inhibition by the plant (17.92% and 14.96%, respectively) [Figure 1d and 1e]. The observed antiulcer activity of ST was however not comparable to that of cimetidine, a standard antiulcer drug, in that it was as much as 50% less effective than the drug [Figure 1c]. Nevertheless, the ulcer-inhibitory activity of ST in this study was estimated to be statistically significant relative to the ulcerated animals left untreated. Besides, the antiulcerative effect of cimetidine (41%) obtained in the study was below expectation, suggesting that the dosage of indomethacin used in this study may have caused severe gastric ulcer condition which appeared to have slightly subdued the potency of cimetidine. Anyways, considering the fact that the dosage employed (100 mg/kg BW) has been used in the previous studies [31,32] in which cimetidine was reported to offer better ulcer protection, it is, therefore, possible that the pharmacological response of the species of rats used in this study may have also played a significant role in the performance of the drug. In the same vein, any of these factors may have also

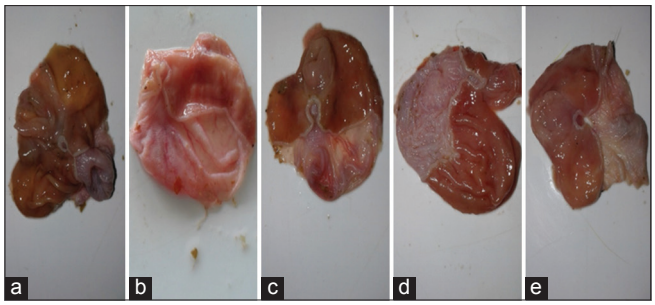

Figure 1: Photomicrographs of the stomach linings of indomethacin-exposed rats treated with cimetidine and *Sansevieria trifasciata* (ST) extract, (a) NC: Normal control, (b) UC: Untreated negative control, (c) PC: Treated positive control, (d) ST 200: 200 mg/kg BW/p.o ST extract, (e) ST 400: 400 mg/kg BW/p.o ST extract

affected the therapeutic performance of the plant (ST) extract. These postulations call for further investigations involving the use of a lesser dose of indomethacin/increased dose of ST on the antiulcerative potential of ST extract. Moreover, isolation and identification of the major antiulcerative compound in ST and subsequent evaluation of its antiulcerative potential will be necessary to arrive at a definite and meaningful conclusion on the effective use of ST as a remedy for gastric ulcer. Regardless, it is important to forecast the scientific basis for the antiulcerative potential of ST demonstrated in this study. The presence of antioxidant phytochemicals such as flavonoids and tannins in ST leaf extract may be partly accountable for its gastroprotective ability noted in this study. These compounds probably play a role in the metabolic activation of the COX enzymes that produce prostaglandins [29], particularly the COX-1 which produces an additional type of prostaglandin that protects the stomach lining from stomach acid and making the mucosal cells less vulnerable to hydrochloric acid and pepsin damage [30].

Although the indomethacin-induced increase in gastric pH noted in this study is at variance with the observation made in some previous studies [33,34], it may, however, be due to adaptive response of the experimental animals to the induced ulceration. In fact, it has been reported that the administration of indomethacin stimulates gastric bicarbonate secretion [35,36] which in turn can results in an increase in gastric pH.

CONCLUSION

The outcome of this study suggests that ST has a promising antiulcerative potential, along with herbal safety which is a major concern in the therapeutic utilization of plants. Further studies involving bioassay-guided identification of the main antiulcerative compound in ST is necessary to affirm and maximize the possible use of the plant as a therapeutic remedy for gastric ulcer.

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Review Article

Antimicrobial activity of Nigerian medicinal plants

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ABSTRACT

Antimicrobial resistance (AMR) is currently one of the major threats facing mankind. The emergence and rapid spread of multi- and pan-drug-resistant organisms (such as vancomycin-, methicillin-, extended-spectrum β-lactam-, carbapenem- and colistin-resistant organisms) has put the world in a dilemma. The health and economic burden associated with AMR on a global scale are dreadful. Available antimicrobials have been misused and are almost ineffective with some of these drugs associated with dangerous side effects in some individuals. Development of new, effective, and safe antimicrobials is one of the ways by which AMR burden can be reduced. The rate at which microorganisms develop AMR mechanisms outpaces the rate at which new antimicrobials are being developed. Medicinal plants are potential sources of new antimicrobial molecules. There is renewed interest in antimicrobial activities of phytochemicals. Nigeria boasts of a huge heritage of medicinal plants and there is avalanche of researches that have been undertaken to screen antimicrobial activities of these plants. Scientific compilation of these studies could provide useful information on the antimicrobial properties of the plants. This information can be useful in the development of new antimicrobial drugs. This paper reviews antimicrobial researches that have been undertaken on Nigerian medicinal plants.

KEY WORDS: Antimicrobial activity, antimicrobial resistance, Nigerian medicinal plants

THE ANTIMICROBIAL RESISTANCE BURDEN

AMR is the ability of microorganisms such as bacteria and fungi to grow despite exposure to antimicrobial (antibacterial or antifungal) agent designed to inhibit their growth [1]. In general, microorganisms exhibit AMR by innate (e.g., absence of drug target site) and/or acquired (e.g., enzymatic degradation of drug) mechanisms conferred by AMR genes (ARGs) acquired via horizontal gene transfer (HGT) (transformation, transduction, and conjugation) from other microorganisms [1,2]. AMR includes two levels of resistance, the cellular level resistance (CELR), and the community level resistance (COLR) [3,4]. CELR develops via endogenous gene mutation or via HGT of resistance determinants from other microorganisms [4] while COLR occurs when a group of organisms become tolerant to environmental stress [5]. COLR is often observed among persisters (organisms that change their physiological state to become tolerant to lethal effect of drugs) in bacterial biofilms [4,5]. It was earlier thought that AMR evolved after the development of penicillin in 1940s, but historical reports, as well as studies on bacterial organisms from permafrost, had revealed that microorganisms exhibited innate AMR long before the development of any antimicrobial agent [6-8]. However, it is inappropriate use (abuse, misuse or overuse) of antimicrobials in human, animal, and plant settings that triggered the emergence of acquired AMR in microorganisms [8].

Currently, AMR is one of the major threats to global health and factors such as global climatic change, globalization (increased international travel and food importation/exportation), and change in demographics are worsening the crisis [4,9,10]. The emergence and rapid spread (due to mobile genetic elements) of multi-drug- and pan-drug-resistant organisms (such as vancomycin-, methicillin-, extended-spectrum β-lactam-, carbapenem-, quinolone- and colistin-resistant bacteria) which exhibit resistance to virtually all antimicrobial agents currently known to man, has put the whole world in a dilemma [11]. These organisms (superbugs) jeopardize antimicrobial therapy resulting in untreatable and fatal infections [7]. There is no need reiterating their involvement in hospital-, nosocomial-, and community-linked infections worldwide [7,8]. The economic and health impact of AMR on a global scale is enormous and dreadful [4,7,10]. A recent review on "global crisis of AMR" chaired by Jim O'Neill, underestimated that about 700,000 lives are lost worldwide annually due to antimicrobial-resistant infections [11]. The report also estimated that by 2050, the societal and financial cost of not tackling the AMR crisis will be US$100 trillion [11,12]. Other recent studies estimated population reduction of between 11 million and 444 million people and a reduction in the size of the global economy by 0.1-3.1% by 2050, if effective antimicrobial agents are not developed [13,14]. The cost of developing a new antimicrobial has been estimated to be US$1 billion [7] and an estimation of US$30 billion is needed to tackle AMR crisis now before it

becomes uncontainable [11,12]. The impact of AMR is worse in developing nations, including Nigeria, where the cost of treatment of resistant infections and associated deaths are unaccounted for [15].

SEARCH FOR NEW PHYTOCHEMICALS WITH ANTIMICROBIAL ACTIVITY

It is largely recognized that most of the currently available antimicrobials which are mainly synthetic are almost inefficient and most of these agents elicit terrible effects to recipients [16-18]. For example, Stevens-Johnson syndrome and toxic epidermal necrolysis, and hypersensitivity reactions are associated with the administration of antimicrobials such as sulfonamide and fluoroquinolones, and penicillin, respectively [19,20]. All the experts that proposed strategies/solutions to tackle the AMR crisis recognized that development of new and safe antimicrobials is more critical than any other proffered solutions/strategies [7,11,12,21]. Many initiatives and programs have been set up by many countries/organizations with the aim of developing new, effective, and safe antimicrobials [21]. For instance, the 10x'20 initiative proposed in 2010 is aimed at developing 10 new, safe, and effective antibiotics by 2020 [22]. Thus, researchers/scientists are now looking at every ecological niche including soil, plant, animal, and marine for potentially new and safe antimicrobial agent [23,24]. Unfortunately, the rate at which microorganisms develop AMR outpaces the rate of discovery/development of new drugs [7,11].

The African traditional medicine is the oldest medicinal system and often culturally referred to as the Cradle of Mankind [16,25]. Traditional herbal medicines have been used to treat infectious diseases for thousands of years in various parts of the world [26,27]. There has been a renewed interest in indigenous medicine worldwide because orthodox medicine is not widespread [17,27]. In poor countries, the health care has been sustained by other practices based on cultural alternatives [27]. In many developing countries, including Nigeria, 80% of patients use indigenous herbal remedies to treat infectious diseases [17,24,28]. Despite the availability of modern medicine in some communities, herbal medicines (medicinal plants) have continued to maintain popularity for historical and cultural reasons, in addition to their efficacy and cheaper cost [17,24,27]. They also represent sources of potentially important new pharmaceutical substances since all parts of a plant, from roots to seed heads and flowers, are employed in traditional remedies and can, therefore, act as sources of lead compounds [17]. Moreover, molecules from natural products have represented about 80% of drugs that have been put into the market [17,29]. The use of plant remedies has steadily increased worldwide in recent years as well as the search for new phytochemicals that potentially could be developed as useful drugs for the treatment of infectious diseases [16,24,28].

Nigeria is located in West Africa on the Gulf of Guinea. It is bordered in the East by Cameroon (1,690 km), Northeast by Chad (87 km), North by Niger (1,497 km), and West by Benin (773 km) and by the Atlantic Ocean in the South [Figure 1] [17]. The country is divided administratively into the Federal Capital Territory (Abuja) and 36 states [17], these states are grouped into 6 geographical regions. Covering an area of 923,768 km^2, Nigeria is a country rich in biodiversity, possessing an array of fauna and flora including about 20,000 species of insects, almost 1,000 species of birds, 247 species of mammals, 123 species of reptiles, about 1,000 species of fish [17,30]. Nigeria boasts a unique and diverse botanical heritage with over 7,895 plant species of which *ca*. 3000 species are used therapeutically [30-32]; although, many of its plant species are at risk of extinction due to inadaptability problems attributed to natural factors such as climate change (e.g., desertification) as well as anthropogenic factors (e.g., deforestation due to timber logging, bush clearing and burning, oil spillage, over-grazing and urbanization), among others [30,33]. The humid tropical climate of Nigeria supports the growth and development of many plant species that have been used in Nigerian traditional medicine even before the advent of Western medicine [17]. Not only is the Nigerian flora rich in diversity but it is also mostly endemic [16,34]. In addition to this unique botanical heritage, Nigeria has a cultural diversity with traditional healing being integral to each ethnic group [16,17].

Despite the well-documented ethnobotanical literature, very little scientific information (e.g., efficacy, phytochemistry) has become available on indigenous medicinally used plants in Nigeria [17,27,35]. From available literature, the earliest documents on antimicrobial activity of Nigerian plants seem to be those of Dalziel [36,37] in 1937 and 1957, respectively. Two decades later, few other publications on chemistry and antimicrobial activities of Nigerian plants appeared in the literature [38,39]. In the 1980s, few studies on antimicrobial activity of Nigerian plants became available in the literature [40-42]. However, from 1990 to date, there has been an avalanche of publications in the literature on the chemistry and antimicrobial properties of Nigerian medicinal plants. This recent emergence in the scientific validation of antimicrobial activities of Nigerian medicinal plants may be a result of increased public awareness, method advancements and a number of citations in local books confirming the need for such studies [16,36,37,43]. Further reasons for advancement of work on Nigerian medicinal plant include searching for new lead compounds to be developed as drugs or as templates for analog synthesis and the evaluation of traditional medicine and herbal medicinal products [17,24,44]. Medicinal effects of Nigerian plants are attributed to interaction of phytochemicals (such as alkaloids, tannins, phenols, saponins, flavonoids, and essential oils) and bioactive compounds contained in their tissues [16,45]. Scientific compilation of studies on antimicrobial activity of Nigerian plants would enhance understanding of the extent of research that has been undertaken to elucidate the antimicrobial potential of these plants. Such study could arouse interest on Nigerian plants with potential antimicrobial activity from which new antimicrobial molecules could possibly be isolated. This review highlights the findings of studies that have been undertaken on antimicrobial activity of Nigerian medicinal plants 1971-2016.

Figure 1: Map showing Nigeria and her neighbors [17,46]

EXPERIMENTAL APPROACH USED IN ANTIMICROBIAL INVESTIGATION OF NIGERIAN PLANTS

Several steps are taken in evaluation of plants for antimicrobial activity. Selection of plant for antimicrobial screening is necessary to avoid unnecessary waste of time and resources [16]. Four standard approaches used for selecting plants include: (1) Random selection followed by chemical screening, (2) random selection followed by antimicrobial assays, (3) follow-up of antimicrobial activity reports, and (4) follow-up of ethnomedical or traditional uses of plants against infectious diseases [27,45-47]. Of these four approaches, the random selection followed by antimicrobial assay of plants against infectious diseases was the most common approach used by studies cited in this review [Table 1]. Selection of plant for antimicrobial investigation based on ethnomedical use is the best approach to avoid waste of resources and time [16,48]. It is important that selected plant is identified by an expert/plant taxonomist and deposited in a reliable herbarium for future identification and reproducibility of study [27]. While most of the studies on antimicrobial activity of Nigerian medicinal plants reported expert identification of selected plant(s), only few papers [49-60] reported deposition of plant in herbarium with the accompanying voucher number. Selection of the plant part to be evaluated may be based on ethnomedical use, randomly or follow-up of antimicrobial activity [61]. Different parts of a plant may contain varying types and amount of phytochemicals [45], thus the extent of extraction of these bioactive substances depends on the type of solvent used for extraction and the degree of binding with other substances in the plant material [24,61]. The majority of the antimicrobial screening studies on Nigerian plants assayed the leaf, while few analyzed the root, stem bark, fruit, and/or seed of selected plant(s) [Table 1].

The process of extraction in antimicrobial studies is critical as it determines to a large extent the result of the study [24,61]. In cases where the study is based on ethnomedical approach, an important factor to consider is the preparation of extract as described by the traditional healers to mimic as much as possible the way the herbal remedy is indigenously used [16,32]. In this way, the use of the plant in the traditional medicine can be correctly validated or invalidated. In cases where the antimicrobial activity of the plant is not based on ethnomedical approach, selection of solvent system largely depends on the specific nature of the bioactive compound being targeted [24]. In general, however, a good solvent used in plant extraction for antimicrobial bioassay should (i) have low toxicity, (ii) have relatively low boiling point so as to be easily removed from the compound after extraction, (iii) promote rapid physiological absorption of the desired compound in the extract in specific body compartments, (iv) have preservative action and inability to cause the quenching or dissociation of active principles, and (v) not interfere with the bioassay as the end product in extraction will contain traces of residual solvent [24,61]. Although aqueous (water) extraction is commonly used by the traditional healers, it has been shown that plant extracts obtained using organic solvents give more potent and consistent antimicrobial activity result than aqueous extract [16,24,62,63]. Studies indicated acetone as the most favorable solvent for plant extraction in antimicrobial studies [16,24,64]. Some of the antimicrobial screening papers in this review instead of using water or ethanol that is used in traditional medicine used organic solvent including ethyl acetate, methanol, butanol, petroleum ether and hexane for extraction [Table 1]. These organic solvents are not acceptable in indigenous preparation of plant extracts, thus the result could have been affected in a way [32].

Consideration should also be given to time and temperature of extraction as these as well as the solvent determines the extraction yield [24]. Some screening papers in this review

Table 1: Antimicrobial screening assays performed on extracts from Nigerian plants

Screening approach	Number of plants studied	Extract tested	Plant part analyzed	Assay	Highest activity	Reference
Antimicrobial activity	6	Ethanol	Leaf	AWD, MIC	*V. amygdalina,* 25 mg/ml against *S. typhi*	[86]
Pharmacological study	5	Methanol	Leaf	AWD	*A. djalonensis,* against *Proteus* spp., *E. coli* and *Shigella* spp.	[163]
Antimicrobial activity	3	Petroleum ether, methanol	Leaf	DD, MIC	*T. roka, T. procumbens* and *M. angolensis* (methanol extracts), 6.25 mg/ml, broad-spectrum	[249]
Antibacterial activity	3	Methanol	Leaf	AWD, MIC	*G. latifolium,* 75 mg/ml against *P. monteilli*	[206]
Pharmacological study	50	Ethanol	Various	MIC	*A. senegalensis,* 0.0625 mg/ml against *Bacillus* (*B.*) *subtilis*	[137]
Pharmacological study	13	Methanol	Various	DD	*A. cissampelli* and *G. arboretum* (root extracts), against *B. subtilis* and *E. coli*	[246]
Antibacterial activity	8	Ethanol	Leaf and bark	AWD	*A. schimperi* (leaf extract), against *P. aeruginosa*	[150]
Spices	5	Aqueous, ethanol, hexane	Various	AWD	*X. aethiopica* (ethanol seed extract), against *B. cereus* and *S. dysenteriae*	[81]
Curcubitaceae	3	Aqueous, ethanol	Leaf	AWD, MIC	*M. charantia, L. cylindrical and T. cucumerina* (ethanol extract), 2-9 mg/ml, broad-spectrum	[250]
Orodental hygiene	18	Aqueous	Various	AWD, MIC	*C. ferruginea* (fruit extract), *B. ferruginea* (stem/twigs extract), *A. leiocarpus* and *T. glaucescens* (root extracts), <2 mg/ml, broad-spectrum	[50]
Pharmacological study	6	Aqueous, ethyl acetate, ethanol, methanol, butanol	Stem bark or root bark	AWD	*M. senegalensis* (ethanol root bark extract), against *S. aureus*	[248]
Antimicrobial activity)	5	Aqueous (hot and cold), ehanol	Leaf	AWD	*A.* africana (ethanol extract), against *K. pneumonia*	[184]
Pharmacological study	11	Methanol, chloroform, hexane, ethanol, aqueous	Various	AWD, MIC	*M. tomentosa* (leaf), broad-spectrum; *T. heudelotti* (leaf), broad-spectrum	[48]
Orodental hygiene	4	Aqueous, ethanol	Root and stem	AWD, MIC	*A. schimperi* (ethanol root and stem extract), 1.56 mg/ml against *S. aureus*	[251]
Orodental hygiene	9	Phosphate buffered saline	Root and stem	MIC	*S. werneckei*, 0.25 μg/ml against *B. gingivalis*	[42]
Pharmacological study	3	Aqueous, ethyl acetate, methanol	Leaf	AD, MIC	*H. opposita* (methanol extract), against *Klebsiella* spp.	[252]
Antimicrobial activity	5	Aqueous, ethanol, methanol, ethanol: Aqueous	Various	AWD	*B. paradoxum* (ethanol extract), against *Klebsiella* spp.	[253]
Antibacterial activity	4	Aqueous (hot and cold), ethanol	Various	AWD	*A. indica* (ethanol leaf extract), against *E. coli*	[180]
Wound healing	9	Aqueous, ethanol	Leaf or stem	MIC	*A. wilkesiana* (ethanol extract) and *P. globosa* (ethanol and aqueous extract), 0.31 mg/ml against *S. aureus;* *A. conyzoides* (ethanol extract), *L. inermis* (ethanol and aqueous extract) and *P. globosa* (aqueous extract), 0.31 mg/ml against *B. subtilis*	[141]
Antimicrobial activity	4	Aqueous, ethanol	Leaf	DD	*C. papaya* (aqueous extract), against *A. butzleri*	[94]
Orodental hygiene	10	Aqueous, methanol	Stem	AWD	*G. kola, A. leiocarpus, T. glaucescens, S. warneckei* and *V. doniana* (aqueous extracts), against methicillin-resistant *S. aureus*, vancomycin-resistant *Enterococcus*, multidrug resistant *B. cepacia* and *P. aeruginosa*	[187]
Pharmacological	2	Methanol	Root or stem bark	DD, MIC	*T. avicennioides* and *A. leiocarpus,* 0.3 mg/ml against *S. pyogenes* and *B. subtilis*	[166]
Pharmacological study	4	Aqueous (hot and cold), ethanol, methanol	Leaf	AWD	*P. macrophyla* (methanol extract), against *S. aureus*	[254]
Pharmacological study	5	Ethanol	Leaf	AWD	*B. nitida, C. alata* and *G. arboretum* against *T. rubrum, E. floccosum* and *B. haptosporus*	[255]
Antimicrobial activity	4	Aqueous (hot), methanol	Leaf	AWD	*E. hirta* (methanol extract), against *Pseudomonas* spp.	[161]
Antimicrobial activity	2	Aqueous: methanol	Leaf	MIC	*E. camaldulensis*, <0.0625 μg/ml against *C. albicans*	[112]

(Contd...)

Table 1: *(Continued)*

Screening approach	Number of plants studied	Extract tested	Plant part analyzed	Assay	Highest activity	Reference
Pharmacological study	2	Aqueous, ethanol, acetone, methanol	Leaf	AWD, MIC	*V. doniana* (acetone extract), 0.78 mg/ml against *E. coli*	[256]
Pharmacological study	2	Aqueous, ethanol	Leaf	AWD	*B. alba* (ethanol extract) against *M. luteus*	[158]
Orodental hygeine	2	Methanol	Stem	AWD, MIC	*A. leiocarpus*, 3.125 mg/ml against *C. krusei*	[111]
Spices	4	Aqueous, ethanol	Seed	AWD, MIC	*P. guineense* (aqueous extract), 30 mg/ml against *S. aureus*	[153]
Pharmacological study	4	Ethanol	Leaf	AWD, MIC	*S. mombin*, 4 mg/ml against *S. aureus*	[257]
Anti-infective activity	2	Methanol	Stem and root bark	DD	*A. leiocarpus* against *Lactobacillus* spp.	[92]
Antimicrobial activity	2	Ethanol	Stem	AWD	*O. gratissimum* against *Salmonella* spp.	[168]
Pharmacological study	4	Aqueous	Leaf	DD, MIC	*A. hybridus, C. esculenta,* and *C. bicolor,* 6.33 mg/ml against *C. albicans, E. coli*, and *S. aureus,* respectively	[106]
Orodental hygiene	5	Aqueous, ethanol, ethyl acetate	Root	AWD	*V. doniana* against *S. aureus*	[258]
Pharmacological study	3	Ethanol	Leaf	AWD, MIC	*P. osun*, 0.25 mg/ml against *K. pneumoniae*	[142]
Antifungal activity	8	Methanol	Various	AWD	*C. occidentalis* (root extract) against *A. fumigatus*	[174]
Pharmacological study	4	Aqueous, ethanol	Root and bark, or bark	AWD, MIC	*T. glaucescens* and *A. leiocarpus* (ethanol root extract), 0.625 mg/ml against *E. coli, S. aureus,* and *S. dysenteriae*	[143]
Skin infections	2	Aqueous, ethanol	Leaf and bark	DD	*M. oppositifolius* (aqueous extract), against *Penicillium* spp.	[79]
Pharmacological study	3	Aqueous (hot), ethanol	Seed or leaf	DD	*V. amygdalina* (aqueous and ethanol extracts), against *S. aureus* and *C. albicans*	[181]
Pharmacological	2	Ethanol	Leaf	AW	*S. mahogoni*, against *S. aureus*	[155]
Antimycobacterial	10	Ethanol, hexane, methanol	Various	MIC	*A. leiocarpus* and *T. avicennioides* (hexane extract), 312 μg/ml against *M. tuberculosis* and BCG antigen	[259]
Pharmacological study	3	Ethanol, acetone	Leaf	DD, MIC	*O. basilicum* (ethanol extract), 0.5 mg/ml against *E. coli, V. amygdalina* and *G. latifolium* (ethanol and acetone extract), 0.5-1 mg/ml against *K. pneumoniae, P. aeruginosa,* and *E. coli*	[105]
Orodental hygiene	8	Aqueous	Various	AWD	*T. glaucescens* against *P. gingivalis, P. intermedia, F. nucleatum, E. corrodens* and *C. rectus*	[169]
Antimycobacterial	11	Aqueous: methanol	Various	MIC	*P. corymbosa* (leaf and twist extract), 800 μg/ml against *M. tuberculosis*	[55]
Pharmacological study	4	Aqueous, ethyl acetate, butanol, hexane	Leaf	AWD	*C. acontifolius* and *A. digitata* (butanol extract), against *B. subtilis*	[201]
Antimicrobial activity	3	Aqueous	Leaf and stem bark	AWD	*A. schimperi, C. occidentalis* and *B. thonningi* (leaf extract), against *S. aureus* and *S. typhi*	[176]
Antibacterial activity	3	Aqueous, ethanol	Leaf	AWD	*M. lucida*, against *Flavobacterium* spp.	[262]
Antiviral activity	5	Methanol	Various	Cell culture /RNA probe	*A. muricata* (stem bark methanol extract), 5.8 μg/ml (EC_{50}) against Hepatitis C virus	[261]
Antibacterial	5	Aqueous, ethanol	Leaf or stem bark	MIC	*T. avicennioides* (ethanol stem bark extract), 18.2 μg/ml against methicillin-resistant *S. aureus*	[49]
Orodental hygiene	3	Aqueous, ethanol	Stem	AWD	*A. leiocarpus* against *C. krusei*	[188]
Antimicrobial activity	7	Methanol	Various	MIC	*S. mombin* (stem bark extract), 61.1 μg/ml (91% inhibition) against *M. tuberculosis*	[122]
Orodental hygiene	9	Aqueous	Various	MIC	*S. werneckei* (bark and pulp extract), ≤1% concentration against *B. melaninogenicus, B. oralis, B. ginivalis* and *B. asaccharolyticus*	[133]
Bryophyta	2	Ethanol, methanol, acetone	Whole	MIC	*C. erosum* and *B. coronatum* extracts, <0.0625-5 mg/ml, broad-spectrum	[145]
Antimycobacterial activity	12	Aqueous, ethanol	Various	Proportion	*A. acalonicum* (aqueous and ethanol leaf extract), *T. glaucescens* (aqueous and ethanol stem extract and *A. cepa* (aqueous and ethanol bulb extract), and *S. longepedunculata* (ethanol stem extract), 0.05 g/ml against *M. tuberculosis*	[235]

(Contd...)

Table 1: *(Continued)*

Screening approach	Number of plants studied	Extract tested	Plant part analyzed	Assay	Highest activity	Reference
Pharmacological study	10	Aqueous, ethanol	Stem or root	DD	*D. benthmianus* (ethanol extract), against *T. mentagrophyte*	[80]
Antimicrobial activity	4	Ethanol	Leaf	AWD	*J. nigra* against *C. albicans*	[164]
Antibacterial activity	10	Aqueous, ethanol	Twigs	AWD	*Azadirachta indica* (ethanol extract), against *S. mutans*	[240]
Antibacterial activity	8	Aqueous, methanol	Various	AWD, MIC	*P. africana* (methanol extract), *G. senegalensis* and *D. microcarpum* (aqueous leaf extract), 0.156-0.625 mg/ml against *S. typhi*, *P. aeruginosa*, *E. coli*, *K. pneumoniae*, *P. vulgaris* and *Citrobacter* spp.	[148]
Antidiarrheic	2	Aqueous, ethanol	Leaf	AWD, MIC	*A. occidentale* (ethanol extract), 0.05-0.10 mg/ml, broad-spectrum	[135]
Pharmacological study	2	Aqueous, ethanol	Leaf	AWD	*S. alata* (aqueous extract), against *A. niger*	[239]
Orodental hygiene	3	Aqueous, ethanol	Stem	AWD	*F. zanthoxyloides* against *S. aureus*	[190]
Antimicrobial activity	6	Ethanol	Various	MIC	*A. indica* and *J. mimosaides* against *S. typhi* and *S. dysenteriae*	[260]
Pharmacological study	3	Ethanol	Various	AWD, MIC	*N. latifolia* (root extract), *C. penduliflorus* and *A. precatorius* (seed extracts), 3.025 mg/ml against *K. pneumoniae*	[247]

AWD: Agar well diffusion; DD: Disc diffusion; MIC: Minimum inhibitory concentration, *E. coli: Escherichia coli, P. aeruginosa: Pseudomonas aeruginosa, C. ulcerans: Candida ulcerans, S. pyogenes: Streptococcus pyogenes, K. pneumoniae: Klebsiella pneumoniae, N. gonorrhoeae: Neisseria gonorrhoeae, M. bovis: Mycobacterium bovis, S. aureus: Staphylococcus aureus, A. niger: Aspergillus niger, C. albicans: Candida albicans, M. tuberculosis: Mycobacterium tuberculosis, S. typhi: Salmonella typhi, P. mirabilis: Proteus mirabilis, S. faecalis: Streptococcus faecalis, A. precatorius: Abrus precatorius, N. latifolia: Nauclea latifolia, C. penduliflorus: Croton penduliflorus, A. indica: Azadirachta indica, S. dysenteriae: Shigella dysenteriae, F. zanthoxyloides: Fagara zanthoxyloides, J. mimosaides: Jacaranda mimosaides, A. occidentale: Anacardium occidentale, S. alata: Senna alata, P. vulgaris: Proteus vulgaris, D. microcarpum: Deterium microcarpum, G. senegalensis: Gueira senegalensis, J. nigra: Juglan nigra, D. benthmianus: Distemonath benthmianus, T. mentagrophyte: Trichophyton mentagrophyte, S. longepedunculata: Securidaca longepedunculata, T. glaucescens: Terminalia glaucescens, A. cepa: Allium cepa, C. erosum: Calymperes erosum, B. coronatum: Bryum coronatum, B. asaccharolyticus: Bacteroides asaccharolyticus, B. ginivalis: Bacteroides ginivalis, B. oralis: Bacteroides oralis, B. melaninogenicus: Bacteriodes melaninogenicus, S. werneckei: Serindeia werneckei, S. mombin: Spondias mombin, A. leiocarpus: Anogeissus leiocarpus, A. digitata: Adansonia digitata, C. acontifolius: Cnidoscolus acontifolius, P. corymbosa: Pavetta corymbosa, C. rectus: Campylobacter rectus, E. corrodens: Eikenella corrodens, F. nucleatum: Fusobacterium nucleatum, P. intermedia: Prevotella intermedia, P. gingivalis: Porphyromonas gingivalis, V. amygdalina: Vernonia amygdalina, O. basilicum: Ocimum basilicum, S. mahogoni: Swietenia mahogoni, M. oppositifolius: Mallotus oppositifolius, P. osun: Pterocarpus osun, V. doniana: Vitex doniana, E. camaldulensis: Eucalyptus camaldulensis, E. hirta: Euphorbia hirta, B. haptosporus: Basidiobolus haptosporus, E. floccosum: Epidermophyton floccosum, C. alata: Cassia alata, T. rubrum: Trichophyton rubrum, G. arboretum: Gossypium arboretum, B. nitida: Baphia nitida, P. macrophyla: Pentaclethra macrophyla, B. cepacia: Bulkhoderia cepacia, S. warneckei: Sorindeia warneckei, G. kola: Garcinia kola, A. butzleri: Arcobacter butzleri, C. papaya: Carica papaya, P. globosa: Parkia globosa, L. inermis: Lawsonia inermis, A. conyzoides: Ageratum conyzoides, A. wilkesiana: Acalypha wilkesiana, B. paradoxum: Butyrospermum paradoxum, B. gingivalis: Bacteroides gingivalis, T. heudelotti: Trichilia heudelotti, M. tomentosa: Markhamia tomentosa, A. africana: Aspilia africana, M. senegalensis: Maytenus senegalensis, B. ferruginea: Bridellia ferruginea, C. ferruginea: Cnestis ferruginea, T. cucumerina: Trichosanthes cucumerina, L. cylindrical: Luffa cylindrical, M. charantia: Momordica charantia, X. aethiopica: Xylopia aethiopica, A. cissampelli: Adenia cissampelli, A. senegalensis: Annona senegalensis, P. monteilli: Pseudomonas monteilli, G. latifolium: Gongronema latifolium, A. fumigatus: Aspergillus fumigatus, C. bicolor: Caladium bicolor, C. esculenta: Colocasia esculenta, A. hybridus: Amaranthus hybridus, O. gratissimum: Ocimum gratissimum, P. guineense: Piper guineense, M. luteus: Micrococcus luteus, B. alba: Basella alba, H. opposita: Hoslundia opposita, M. angolensis: Maerua angolensis, T. procumbens: Tridax procumbens, T. roka: Trichilia roka, A. djalonensis: Anthocleista djalonensis*

reported the use of hot aqueous solvents for extraction may be to increase yield or to mimic the extraction procedure used by the traditional practitioners [Table 1]. Nasir *et al.* [24] suggested that in studies where the aim is initial screening of plants for potential antimicrobial activities, the process may begin by using the crude extracts prepared from different organic and aqueous solvents and then followed by the utilization of various organic solvents for fractionation. Some publications on Nigerian medicinal plants reported fractionation of extracts with various organic solvents [65-72]. The storage conditions of plant material (whole/extract) have been shown to affect the result of antimicrobial studies by impacting microbial efficacy [16,64,73]. Some papers on Nigerian plants reported storage of extract in refrigerator at 4°C [18,59,60,74]. This storage condition is good because the activity/growth of possible extract-contaminating organisms would be inhibited and this in a way enhances the reliability of the result [16,75].

In vitro or *in vivo* method employed in assay of antimicrobial activity of plant extracts is critical. This relates to the fact that microbiological methods incorporate viable test microorganisms; therefore, predictability of the outcome is not always clear and subject to many environmental influences that may impact on a response [16,61]. Hence, there is need for standardization of methods which is often encountered with many problems [16,61]. The Clinical and Laboratory Standards Institute and the European Committee on Antimicrobial

Susceptibility Testing have standardized some of the methods used in antimicrobial assay [76] but it should be noted that these methods are standardized for standard drug preparations and not really for plant extracts [61]. Various *in vitro* methods are employed in assay of plant extracts for antimicrobial activity; these methods have been extensively reviewed [24,61,76,77]. The most critical step in *in vitro* assay of plant extract for antimicrobial activity is the inoculum size quantification of selected organism [24,76]. While bacterial/yeast inoculum size quantification is commonly done using the McFarland's turbidity standard method [76], quantification of mold inoculum size is more difficult and requires biosafety equipment because they produce spores. Hemocytometric method is considered the best for quantifying fungal spores [78]. Few papers on antimicrobial activity of Nigerian plants against mold reported the use of hemocytometeric method in quantifying spores of selected organism [79-81].

Review of over 400 papers on antimicrobial investigation of Nigerian medicinal plants revealed the use of various antimicrobial assay methods. Figure 2 represents proportion of studies and the methods used to assess antimicrobial activity of Nigerian plants from 1971–2016. Minimum inhibitory concentration (MIC) and Agar well diffusion (AWD) assays are the two most common methods used to investigate the antimicrobial activity of Nigerian medicinal plants. The MIC assay is a quantitative method of measuring antimicrobial activity based on the principle of contact of a test organism to a series of dilutions of test substance [16,24,76]. MIC is the lowest concentration of the antimicrobial agent that prevents visible growth of a microorganism under known conditions [24,76,82]. Assays involving MIC methodology (such as macro [test tubes] and micro [microtitre plates] broth dilution and agar dilution) are widely used and an accepted criterion for measuring the susceptibility of organisms to inhibitors [16,83]. This is supported by majority of publications on Nigerian plants representing 58.1% for extract and 1.9% for essential oil. The AWD is a widely used method of assay possibly because it indicates the concentration of the plant extract that exhibit the highest microbiostatic effect on the test organisms [24,76]. The method is qualitative and based on the principle of contact of a test organism to an equal volume of different concentrations of test substance inoculated into wells of equal depth and diffusing into cultured agar [24,76]. Although AWD resembles disc diffusion, it is preferred to disc diffusion because it gives a more consistent result [24,76] and this is supported by high proportion (57.6% for extract and 1% for essential oil) of publications on Nigerian medicinal plants which utilized this method. Some papers presented result obtained using both AWD and MIC assay [84-89]. However, variation in data obtained using MIC assay may be influenced by factors such as the inoculum size, the type of growth medium, the incubation time, and the inoculum preparation method [76,90,91].

The fact that a lower proportion of publications on antimicrobial activity of Nigerian plants (15.7% for extract and 1% for essential oil) used disc diffusion is a further indication that the investigators adopted more quantifiable methods of assaying antimicrobial activity of plants [16]. The use of disc diffusion in these studies may possibly be due to its simplicity and capacity to analyze a large number of test samples [16,24,76]. Some studies on Nigerian plant extract presented only disc diffusion data [80,92-99] while some papers (7.1%) reported results using both disc diffusion and MIC [48,69,100-106]. Although disc diffusion methodology is a quick simple means of screening for antimicrobial activity, it is associated with problems which may arise when investigating oil samples [16]. The associated problems that could yield inconsistent result with disc diffusion assay include variation in diffusion rates due to differences in chemical nature of the particular sample, lipophilic substances like essential oil or water-insoluble samples do not easily diffuse through the agar even with a pre-diffusion time allocation of 1 h [16,76,107]. Thus, false negatives may still be encountered and the possibility of activity could be underestimated [16]. Volatility of oily samples is another prominent factor to be considered [16]. Excessive incubation

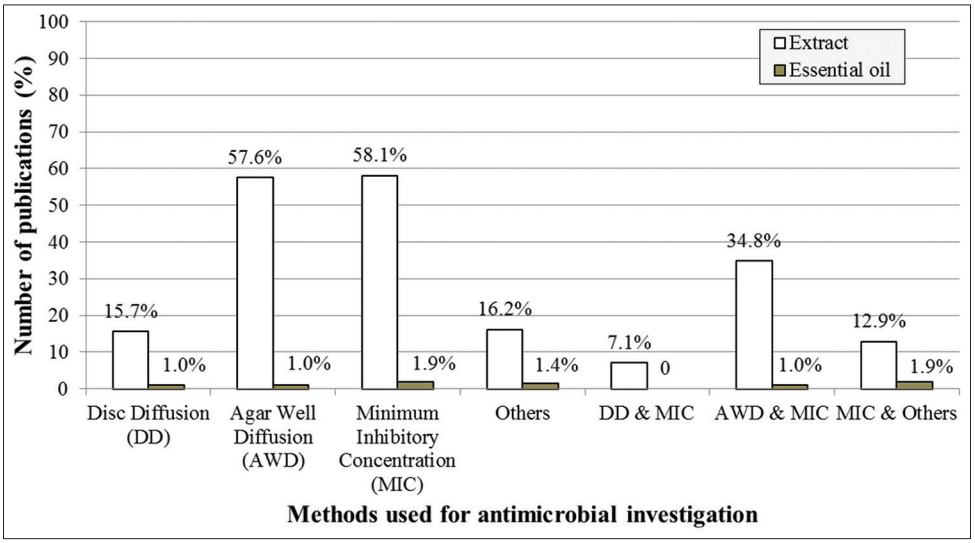

Figure 2: Methods used to assess antimicrobial activity of Nigerian plant extracts

time and temperature (such as during long incubation period 2-7 days of fungal incubation) may result in loss of a proportion of the oil due to evaporation, this too may impact on the false negatives [16,108]. Antimicrobial activity against a proportion of plant sample (especially essential oils) may be assessed following loss of hydrocarbon which is postulated to be very prone to evaporation [16,109].

Other antimicrobial investigation methods used to assess antimicrobial activity of extracts and essential oils of Nigerian plants include killing kinetics and bioautographic assays [16,76]. Although killing kinetics is labour-intensive and requires a number of steps where variables may be introduced, it provides descriptive information on the relationship between bacteriostatic and/or bactericidal activity in relation to the concentration of test substance over time (i.e. the method gives valuable information of the cidal action over time) [16,110]. Some publications on Nigerian plant extract reported data using killing kinetics [42,53,57,111]. Bioautographic assay (involving chromatography and biological systems) is mainly used to evaluate antimicrobial activity of isolated bioactive compounds [16,24,76], but it is also useful for assessing antimicrobial activity of crude plant extracts [24]. Antimicrobial activity of Nigerian plant extracts [65,68,70,71,112-127] and essential oils [32,128] have been assayed using bioautography [Figure 2]. However, no study on Nigerian plants has utilized biointeractive methods [16,129].

ANTIMICROBIAL INVESTIGATION ON NIGERIAN PLANT EXTRACTS

The majority of the papers on Nigerian medicinal plants were dedicated to antimicrobial activity of extracts. Identification of plants with potential antimicrobial activity from screening publications for further investigation is usually the first choice in antimicrobial studies, thus papers on antimicrobial screening are usually sought after [16]. Some screening studies in which several Nigerian medicinal plants were studied are presented in Table 1. It is widely accepted that extracts having activities where MIC values are below 8 mg/ml are considered to possess some antimicrobial activity [16,130] and natural products with MIC values below 1 mg/ml are considered noteworthy [16,131,132]. Some publications reported antimicrobial activities of Nigerian medicinal plant extracts with MIC less than or equal to 1 mg/ml against selected organisms [42,49,51,53,54,58,71,103,104,114,121,133,134-148] [Table 1]. Inhibitory zone diameter (IZD) is measured in millimeter (mm) when DD or AWD assays is used [76,149,150]. Kudi *et al*. [150] reported that plant extracts exhibiting IZD of 6mm and above against a selected pathogen are considered to posses some antimicrobial activity while Udgire and Pathade [151] suggested IZD of 10 mm and above. Because many organisms are now exhibiting high resistance to most antimicrobials, this study proposes that plant extract exhibiting IZD greater than or equal to 10 mm against selected organisms should be considered to possess antimicrobial activity whereas those showing IZD ≥20 mm against selected organisms are considered noteworthy. Some papers [32,101,121,145,152-159] on Nigerian plant extract reported IZD ≥20 mm.

Some publications were dedicated on study of specific genus of Nigerian plant such as *Pterocarpus* spp. [142], *Eucalyptus* spp. [69], and *Amaranthus* spp. [106]. Some Nigerian plant genus whose antimicrobial activity has been investigated by several authors include *Stachytarpheta* spp. [66,69,85,133], *Euphorbia* spp. [96,160-162], *Ocimum* spp. [97,142,105,163-168], *Terminalia* spp. [49,92,111,112,143,165,169-173], *Cassia* spp. [103,114,151,174-177], and *Allium* spp. [57,102,104,178-180]. Some specific species have been investigated by several researchers such as *Vernonia amygdalina* [86,94,97,105,141,179,181,182], *Psidium guajava* [87,121,163,183], *Jatropha curcas* [88,138,184], *Crinum jagus* [59,118,127], *Moringa oleifera* [147,185,186], *Anogeissus leiocarpus* [50,111,171,138,153,167,186-189], *Gongronema latifolium* [128,136], and *Fagara* (*Zanthoxylum*) *zanthoxyloides* [39,190] while some papers were dedicated to the study of specific species such as *Terminalia avicennioides* [58,71,117], *Stachytarpheta angustifolia* [69,70], and *Struchium sparganophora* [191,192].

Geographical and seasonal variations have been reported to affect the phytochemical constituents of plant and this in turn affects the result of antimicrobial activity [16,61]. No study has been conducted to assess the effect of seasonal change on Nigerian medicinal plants. South African studies reported little antimicrobial variability of plant extracts within 2 months interval [16,193]. Some papers on Nigerian plants focused on the antimicrobial screening together with other pharmacological investigations including toxicity [18,42,54,60,85,95,120,191,194,195]. Although medicinal plants are considered generally safe, they are known to contain potentially toxic, mutagenic, and/or carcinogenic substances [16]; therefore, it has been recommended that pharmacological studies should always be accompanied by toxicology screening [16,196,197]. Several papers included additional pharmacological assays to complement the antimicrobial investigations, such as the study on Nigerian *Stachytarpheta* spp. which includes antispasmodic properties [66,85]. Some authors investigated wound healing properties of plants together with antimicrobial property [18,59,60,141,155,198] while some studies reported antioxidant and antimicrobial properties together [48,59,60,99,199-203]. Ajali and Okoye [194] reported the anti-inflammatory and antimicrobial properties of *Olax viridis* and MIC assay showed considerable broad-spectrum activity (MIC 2000-4000 μg/ml) against selected pathogens. Williams *et al*. [204] reported antidiarrheal and antibacterial properties of *Guiera senegalensis* and MIC assay showed broad-spectrum activity (MIC 3.13 mg/ml) against selected pathogens. Kasim *et al*. [191] reported antitumor (using cell lines) and antimicrobial properties of *Struchium sparganophora* and MIC assay showed considerable activity (MIC 6.25 mg/ml) against *P. aeruginosa* and *C. albicans*. Some studies were dedicated to investigation of antimicrobial activity of Nigerian plants against multidrug resistant organisms such as methicillin-resistant *S. aureus* (MRSA) [49,124,147,187,205] and extended-spectrum β-lactamase (ESBLs)-producing organisms [206]. To ensure that resistant isolates were used, some investigators went

further to confirm resistance in isolates using disc diffusion assay [65,119,162,181,182,207].

It was observed that not much have been done on subterranean Nigerian plants. Few studies investigated the antimicrobial activity of subterranean plants such as *Allium* species [57,102,104,178-180] [Table 1] and *Dioscorea bulbifera* (Dioscoreaceae) which neither methanol nor ethanol tuber extracts showed promising antimicrobial activity (MIC 25 mg/ml against *S. aureus* and *E. coli*) [89]. This study recommends that further anti-infective studies be undertaken on medicinal bulbous plants of Nigeria. It was also observed that the majority of researchers on Nigerian plants investigated antimicrobial activity of leaf of selected plants whereas plant roots were barely studied [Tables 1 and 2]. The reason for avoidance of studies using root may be due to the destructive harvesting nature [16]. Elsewhere, it was shown that root and shoot of plants may exhibit similar antimicrobial activities [16,208]. Higher concentrations of secondary metabolites (alkaloids, tannis, flavonoids, saponins, etc.,) that are responsible for the antimicrobial activity of plants occur in bark, heartwood, roots, branch bases, and wound tissues [45,61]. The concentration varies from one plant species to another and from season to season and environment [61,209]. The mechanisms of antimicrobial activities of these metabolites have been extensively reviewed [24,45,210].

Antimicrobial Synergistic Activity of Nigerian Medicinal Plants

Because of the slow pace in development of new antimicrobials, there has been renewed interest in plants that have potential of increasing the effect of available standard (allopathic) antimicrobials. Time-kill kinetics showed that combining plant products together with allopathic antimicrobial drugs is effective in treating multidrug resistant infections [211]. In addition to antimicrobial activity of Nigerian plant extracts, some studies also investigated antimicrobial synergistic effect of these extracts with standard drugs [72,161,212,213] or antimicrobial synergistic effect of extracts from different plants [102]. Some of the studies used the agar diffusion checkerboard (ADCB) method in which the fractional inhibitory concentration (FIC) of the substances is determined by dividing the MIC of each of the substance in combination by the MIC of the substance alone, summation of the FICs gives the FIC index which is then used to classify the effect of the combination ratio as additive, synergistic, antagonistic or indifferent [72,75,76,214]. The overlay inoculum susceptibility disc (OISD) method involves measuring the IZD when antibiotic agar base containing sub-inhibitory concentration of the extract is overlayed with inoculated agar on which standard antibiotic disc is placed, percentage difference of IZD of the test in comparison with the control is then used to classify the effect of the extract as additive, synergistic, indifferent or antagonistic [72,214].

By ADCB method, methanol leaf extract of *Euphorbia hirta* exhibited synergistic effect in combination with nystatin (ratio, 6-9:4-1) against *C. albicans* [160]. Methanol leaf extracts of *Phyllanthus muellerianus* exhibited synergistic effect with ciprofloxacin against *Pseudomonas aeruginosa* and *Proteus mirabilis* using ADCB and OISD methods, respectively [72]. Using OISD method, Nweze and Onyishi [212] demonstrated that ethanol and methanol fruit extracts of *Xylopia aethiopica* exhibited synergistic effect in combination with different antimicrobials (ciprofloxacin, ofloxacin, gentamicin, fluconazole, and ketoconazole) against various organisms including *P. aeruginosa*, *E. coli*, *B. subtilis*, *S. aureus*, *C. albicans*, and *Aspergillus flavus*. The major concern surrounding the combined use of plant extracts and standard drugs is the toxicity effect that could result from interaction of phytochemicals with the active principle(s) in the drug [61,212]. Therefore, there is need for *in vivo* interaction and toxicity studies on Nigerian plants that showed potential antimicrobial synergism with standard drugs.

Antimicrobial Investigation on Essential Oils of Nigerian Medicinal Plants

Essential oils are volatile oils from aromatic plants responsible for the characteristic scent, odor or smell they exude [45]. Essential oil is found in the volatile steam distillation fraction of plants [45]. Several endemic Nigerian plants belonging to the family Lamiaceae, Myrtaceae, Compositae, Asteraceae, Liliaceae, and others are rich in essential oils [16,45]. Phytochemical screening of plants in these families in many publications [Tables 1 and 2], in this review, revealed the presence of terpenoid essential oils both mono and sesquiterpenes. Some studies investigated antimicrobial activity of essential oils of Nigerian plants in different families [32,52,69,128,179,192,215-221]. It was observed that studies on geographical and seasonal variation have not been undertaken on essential oil of selected aromatic Nigerian plants. There is need to conduct these studies because they are important for potential commercialization [16]. Reporting the chemical composition of essential oil (both quantitative and qualitative) together with the antimicrobial activity could give some indication as to how the climatic or geographical factors may influence the phytochemicals and their resultant biological activity [16]. It has been reported that with antimicrobial studies, the chemical composition should ideally be used to correlate any structure activity relationships [16]. Studies on antimicrobial activity of Nigerian plant oils have indicated that the correlation between chemical structure and biological activity are integrated and the essential oil chemistry has provided insight into the antimicrobial activity [16,128,192]. Isolation of the bioactive compound β-caryophyllene from essential oil obtained from *Gongronema latifolium* and *Struchium sparganophora* [128,192] are attributes that enable the plant oils to elicit antimicrobial activity. Nonetheless, it should be noted that bioactive compounds from essential oil cannot independently and in combination be responsible for the overall activity of the plant [16,222].

There has been lack of set criteria in the literature by which essential oil is classified as having good, moderate or poor activity, and many researchers base the assessment on their own

Table 2: Antimicrobial compounds isolated from Nigerian traditional medicinal plants

Plant	Plant part used	Solvent	Compound	Antimicrobial activity			Reference
				Bioactivity	Highest activity	Range	
P. crassipes K. Schum (Rubiaceae)	Leaf	Aqueous (hot)	Quercetin-3-O-rutinoside	Antibacterial	*E. coli P. aeruginosa* and *C. ulcerans* *S. pyogenes, K. pneumoniae* and *N. gonorrhoeae*	6.25 mg/ml 6.25-12.5 mg/ml	[224]
T. avicennioides Guill. & Perr. (Combretaceae)	Root bark	Petroleum ether, ethyl acetate, chloroform, methanol	Friedelin	Antimycobacterial	Bacille Calmette Guerin (*M. bovis*) antigen	4.9 μg/ml	[117]
		Hexane, ethyl acetate, chloroform, methanol	Arjunolic acid			156 μg/ml	[71]
G. latifolium (Benth.) (Asclepiadaceae)	Leaf	Hydroditillation (essential oil)	β-caryophyllene	Antibacterial	*E. coli*	39 μg/ml	[128]
C. alata Linn. (Leguminosae)	Seed	Ethanol	4-butylamine 10-methyl-6-hydroxy cannabinoid dronabinol	Antibacterial and antifungal (yeast and mold)	*P. aeruginosa* *S. aureus, E. coli, K. pneumoniae, A. niger* and *C. albicans*	6.25 mg/ml 12.5-50 mg/ml	[177]
S. mombin Linn. (Anacardaciae)	Stem bark	Methanol	Mombitanes I and II	Antimycobacterial	*M. tuberculosis*	40 μg/ml	[122]
F. zanthoxyloides (Lam.) Zipern and Timler. (Rutaceae)	Root	Petroleum ether, chloroform, ethanol, aqueous	Canthine-6-one, chelerythrine and berberine	Antibacterial			[39]
S. sparganophora Linn. Ktze (Asteraceae)	Aerial part (essential oil from stem and leaf)	Hydrodistillation	β-caryophyllene, germacrene D, a-humulene, caryophyllene oxide and 1,8-cineole	Antibacterial	*S. typhi* *P. aeruginosa, Proteus mirabilis, B. cereus* and *B. subtilis*	0.1 mg/ml 0.1-1 mg/ml	[192]
	Stem and root	Hexane, chloroform, methanol	Vernodalin Luteolin 3 methyl 2,6, hexacosedienol	Antibacterial Antibacterial and antifungal (yeast and mold)	*K. pneumonia* *K. aerogenes* *K. aerogenes, E. coli, C. albicans,* and *A. niger*	25 μg/ml 6.25 μg/ml 50 μg/ml	[191]
R. communis Linn. (Euphorbiaceae)	Seed	Hexane	Cineole Limonene	Antibacterial	*S. aureus* Broad spectrum, mold and yeast	6.25 mg/ml 12.5-25 mg/ml	[207]
C. nigricans Vahl. (Leguminosae)	Leaf	Petroleum ether, methanol	Hydroxyestranic acid ethy ester	Antibacterial	S. pyogenes *P. aeruginosa, C. albicans, N. gonorrhoeae* and *S. typhi*	2000 μg/ml 3000 μg/ml	[114]
P. guineense Schumach. and Thonn. (Piperaceae)	Fruit	Hydrodistillation	Myristicin	Antibacterial	*P. aeruginosa*	5 mg/ml	[215]
J. gossypifolia Linn. (Euphorbiaceae)	Seed	Methanol	9-acetoxynerolidol (from chloroform partition)	Antifungal	*C. albicans*	5 mg/ml	[68]
I. secundiflora Poir. (Fabaceae)	Aerial	Methanol, acetone	Quercetin 3, 3′, 4′-trimethyl ether	Antibacterial	*S. aureus, Bacillus subtilis, E. coli* and *P. aeruginosa*	200 μg/ml	[116]
L. pterodonta (DC.) Sch. Bip. (Asteraceae)	Aerial part (stem and leaf)	Hexane, ethyl acetate	Triacontyl methyl ether	Antibacterial	*K. pneumoniae, K. ozonae* and *B. cereus*	50 μg/ml	[125]
			Di-eicosanyl glycol or ethane-1,2-dieicosanoate		*K. pneumoniae, B. cereus, B. subtilis, S. aureus* and *K. ozonae*	50 μg/ml	

(Contd...)

Table 2: *(Continued)*

Plant	Plant part used	Solvent	Compound	Antimicrobial activity			Reference
				Bioactivity	Highest activity	Range	
			Eicosanoic acid		*K. pneumoniae, K. ozaenae, B. subtilis, B. cereus, S. aureus, E. coli, S. faecalis* and *S. dysentariae*	50-100 μg/ml	
			Ethane-1,2-di-eicosenoate		*K. pneumoniae, S. aureus, B. cereus, K. pnuemoniae* and *K. ozaenae*	50 μg/ml	
			2-triacontoxyethyleicosanoate		*S. aureus, B. subtilis, B. cereus, K. pneumoniae* and *K. ozaenae*	50-100 μg/ml	
		Hexane, ethyl acetate	Taraxasteryl aceteate Ethane-1,2-dieicosanoate	Antimycobacterial	*M. tuberculosis*	691.48 μm 269.23 μm	[126]
		Hexane, ethyl acetate, methanol	Pterodiondol (5βH, 7βH, 10β-epi-cryptomeridiol) Stigmasterol Stigmasteryl-3β-O-D-glucopyranoside	Antibacterial	*S. aureus, K. pneumoniae, K. ozaenae, B. cereus* and *E. coli*	50 μg/ml 50-100 μg/ml 50-100 μg/ml	[123]
B. pinnatum (Lam) Oken. (Crussalaceae)	Aerial part	Aqueous: Ethanol	Gallic acid Luteolin and epigallocatechin 3-O-syringate	Antibacterial	*S. aureus* *S. aureus*	25 μg/ml >400 μg/ml	[263]
C. prostrata (Linn.) Blume (Amaranthaceae)	Aerial part	Ethyl acetate	Ethyl hexadeacanoate and 7, 9-Di-tert-butyl-1-oxaspiro (4, 5) deca-6, 9-diene-2, 8-dione	Antimicrobial	*B. subtilis, S. aureus, E. coli, A. niger, P. aeruginosa, C. albicans*	1-15 mg/ml	[264]

E. coli: Escherichia coli, P. aeruginosa: Pseudomonas aeruginosa, C. ulcerans: Candida ulcerans, S. pyogenes: Streptococcus pyogenes, K. pneumoniae: Klebsiella pneumoniae, N. gonorrhoeae: Neisseria gonorrhoeae, M. bovis: Mycobacterium bovis, S. aureus: Staphylococcus aureus, A. niger: Aspergillus niger, C. albicans: Candida albicans, M. tuberculosis: Mycobacterium tuberculosis, S. typhi: Salmonella typhi, P. mirabilis: Proteus mirabilis, N. gonorrhoeae: Neisseria gonorrhoeae, S. faecalis: Streptococcus faecalis, C. alata: Cassia alata, S. mombin: Spondias mombin, F. zanthoxyloides: Fagara zanthoxyloides, S. sparganophora: Struchium sparganophora, R. communis: Ricinus communis, C. nigricans: Cassia nigricans, J. gossypifolia: Jatropha gossypifolia, I. secundiflora: Indigofera secundiflora, L. pterodonta: Laggera pterodonta, B. pinnatum: Bryophyllum pinnatum, C. prostrata: Cyathula prostrata

particular data attained [16]. Considering that essential oils have lower antimicrobial activities than extracts, they need to be classified differently [16]. It has been proposed that essential oils with MIC value less than or equal to 2mg/ml could be considered noteworthy [16]. Publications on Nigerian medicinal aromatic plant essential oils which reported antimicrobial activities MIC of less than or equal to 2mg/ml against selected pathogens include studies on *Ocimum gratissimum* (Lamiaceae) [223], *Gongronema latifolium* (Asclepiadaceae) [128], *Allium sativum* (Liliaceae), and *Citrus reticulata* (Rutaceae) [179]. There is need for studies on time-kill kinetics of essential oils of Nigerian medicinal plants to be undertaken to elucidate their microbicidal effect.

Bioactive Compounds from Nigerian Medicinal Plants

Different bioactive compounds with antimicrobial properties have been isolated from various Nigerian medicinal plants [Table 2]. This is made possible due to engagement of multidisciplinary approach (by collaboration between chemists, botanists, and microbiologists) which is integral to achieve high-quality research [16]. It has been proposed that isolated compounds with antimicrobial activities of 64–100 μg/ml are accepted as having clinical relevance [16,131]. Some authors specify that compounds with activities less than or equal to 10 μg/ml are noteworthy [16,132]. Reports on isolated bioactive compounds from Nigerian plants exhibiting MIC values less than or equal to 10 μg/ml against selected organisms include Bello *et al*. [224], Mann *et al*. [117], and Kasim *et al*. [191] [Table 2]. Despite the availability of a number of publications on the isolation and identification of bioactive compounds from Nigerian plants, it should be noted that the complexity of plants and a single compound may not be responsible for the observed activity but rather a combination of compounds (either major or minor) interacting in an additive or synergistic manner [16,225].

IN VIVO AND FORMULATION STUDIES ON NIGERIAN MEDICINAL PLANTS

Considering that traditional medicines are esteemed not only for their therapeutic value but also from a holistic administrative approach in which the plant is given to treat the patient on various levels, one must not forget that there may be other physiological effects on the body that act beyond the symptomatic treatment of the disease when studying traditional medicine [16]. Although many studies on Nigerian medicinal plants have identified specific plant species as having antimicrobial activity in an *in vitro* model, it is necessary to subject these plants to animal models and human subjects to determine their efficacy in metabolic environments [16]. Two studies [59,226] on antimicrobial activity of Nigerian plants focused on *in vivo* models. Mice injected intraperitoneally with *S. aureus* and then dosed orally with 25-200 mg/kg body weight of *Alchornea cordifolia* aqueous:ethanol leaf extract improved significantly when compared to the control [226]. Topical treatment of excision wound on rats contaminated with *S. aureus*, *B. subtilis*, *C. albicans* and *P. aeruginosa* with 5% and 10% *Crinum jagus* methanol bulb extract ointment, resulted in significant reduction in isolation rate of these organisms except *P. aeruginosa* [59]. However, these studies used solvents/solvent mixture that are not acceptable in traditional medicine, therefore, there is need to include aqueous extract in *in vivo* screening assays to mimic traditional use of plant material [16]. This will enable adequate assessment of the efficacy of plants as used in traditional medicine. Nevertheless, it has been reported that solvent-derived extracts exhibit more antimicrobial activities than the aqueous extracts [16,227], thus raising concern in terms of antimicrobial efficacy when the traditional method is applied [16]. Therefore, there is great need to translate the applied knowledge gained from intricate assays and make it meaningful to the ethnic people who rely on traditional medicine [16].

Establishing suitable formulations that retain the efficacy demonstrated in the *in vitro* screening procedures has been reported to be the next logical step in the investigation of the antimicrobial efficacy of plant extracts and essential oils [16]. Formulations could be in form of tinctures, concoctions, teas, ointments, capsulation or tablets. Some studies on Nigerian plants focused on ointment formulations of plant extract which is applied topically. Suara *et al*. [228] examined the potential application of methanol extract of *Plukenetia conophora* into cream formulation, the ointment showed highest activity MIC 1mg/ml against *Proteus mirabilis*. Azubuike *et al*. [229] also examined ointment formulation of *Azadirachta indica* and *Aloe barbadensis* which gave highest activity MIC 2.5 mg/ml against *B. subtilis*, *S. aureus* and *C. albicans*, and 2 mg/ml against *C. albicans*, respectively. Muinat *et al*. [230] examined ointment formulation of *Argemone mexicana*, AWD assay showed inhibitory effect of the formulation on *Trichophyton mentagrophyte*. Udegbunam *et al*. [18] examined *Pupalia lappacea* methanol leaf extract ointment formulation on surgical wounds in rats, wound healing parameters assessed as well as microbiological assay (culture) showed inhibitory effect of the ointment on common wound-contaminating pathogens. Soap and ointment formulated using extract from 4 Nigerian medicinal plants proved effective in management of skin infections in selected individuals [165]. Studies are needed on assessment of potential antimicrobial activity of Nigerian plants' essential oil formulations and plant extract formulations in other forms apart from cream ointment. Inclusion of studies assessing the feasibility and bioavailability/stability of the active ingredients of the formulations especially is important [16].

ACTIVITIES ATTRIBUTED TO THE SPECIFIC ETHNOBOTANY OF THE PLANT

It was observed in this review that the majority of plant directed antimicrobial studies focused on screening against a battery of pathogens, while very few ethnobotanical studies have been carried out on pathogen-specific infections where the selection of test organisms relate directly to the traditional use of the plant [16]. For adequate ethnobotanical study, it has been suggested that studies on plants should be done using organisms related to the diseases managed

with the plant in traditional medicine [16,27,61,142]. For instance, plants used for managing diarrhea should be tested against *E. coli*, *Salmonella typhi*, *Shigella dysenteriae*, *Vibrio cholerae*, *Campylobacter* spp., *Entamoeba histolytica*, etc., which are known to be associated with enteritis [231]. Plants utilized for skin infection should be tested against bacteria such as *Pseudomonas* spp. and *S. aureus* and fungal agents associated with skin diseases including *Epidermophyton* spp., *Trichophyton* spp., and *Microsporum* spp. [16,231]. Plants used for oral complaints should be tested against *Streptococcus mutans*, *Streptococcus sobrinus*, *Porphyromonas gingivalis*, and *Aggregatibacter actinomycetemcomitans* [16,232]. Although, studies relating to ethnobotanical use of Nigerian plants have been largely neglected (in terms of using pathogens targeted in ethnomedical setting), few studies focused on ethnobotanical use of studied plant on wound healing [18,60,142] and oral infection [42,133,169,170,233] taking into account the possible impact of microbial infection.

It was observed that few publications [135,168,204] have been dedicated to the antidiarrheal properties of Nigerian plants, and given the severity and mortality rates of diarrheal-related diseases especially in rural areas, not enough has been done on one of the most prevalent diseases affecting rural Nigerians [16]. Omojasola and Awe [135] studied 2 plants (*Anacardium occidentale* and *Gossypium hirsutum*) used traditionally to treat stomach ailments in Southwest Nigeria and reported MIC 0.05-0.10 mg/ml of their ethanol leaf extract against *S. aureus*, *E. coli*, *S. dysenteriae*, *Salmonella* spp., and *P. aeruginosa*.

Candida albicans is an organism (yeast) responsible for infections which are often prolific, requiring long-term antifungal treatment [16,233]. About 120 Nigerian medicinal plants were screened for anticandidal activity [Table 1]. Reference, clinical and nonclinical *C. albicans* strains were used in the studies. The MIC of plant extract against the organism varied depending on the plant, solvent used and the strain of *C. albicans* used [16]. Nigerian plant extracts with the most promising anticandidal activity include *Sesame radiatum* (methanol leaf extract, MIC 28.2 μg/ml) [138], *Amaranthus hybridus* (aqueous leaf extract, MIC 6.33 mg/ml) [106], *Pterocarpus santalinoides* (ethanol fresh leaf extract, MIC 0.75 mg/ml) [142], *Balanites aegyptiaca* (aqueous leaf and root extract, MIC 3.125 mg/ml) [234], *Eucalyptus camaldulensis* (aqueous:methanol leaf extract, MIC 0.0625 μg/ml) [112], *Commiphora africana* (ethanol root extract, MIC 2,000 μg/ml) [65], *Cymbopogon citratus* (chloroform leaf extract, MIC 32 μg/ml) [119], and *Cyathula prostrata* (ethanol leaf and stem extract, MIC 400 μg/ml) [154]. Kubmarawa *et al*. [137] reported anticandidal activity of ethanol extract from various parts of *Acacia tortilis*, *Anogeissus leiocarpus*, *Jatropha curcas*, *Nauclea latifolia*, and *Vitex doniana* with MIC ranging from 0.5 to 2 mg/ml. There is need for further clinical assessment of these Nigerian plant extracts with considerable anticandidal activity.

Some publications have been dedicated to investigation of antimycobacterial activity of Nigerian plant extract [54,55,57,118,122,125,189,235,236] [Tables 1 and 2]. Screening of antimycobacterial activity of 12 medicinal plants used in treating tuberculosis in Southwest Nigerian revealed that both methanol and ethanol extract of *Allium ascalonicum*, *Terminalia glaucescens*, *Allium cepa*, and ethanol extract of *Securidaca longepedunculata* inhibited *M. tuberculosis* at concentration of 0.05 g/ml [235]. On comparison with Bacille Calmette Guerin (BCG) (attenuated *Mycobacterium bovis*) antigen, 10 Nigerian medicinal plants used in herbal antitubercular recipes by traditional healers, showed potential antimycobacterial activity following preliminary MIC assay [189]. Hexane extract of various parts of *T. avicennioides* and *Anogeissus leiocarpus* exhibited activity (MIC 312 μg/ml) against *M. tuberculosis* and BCG antigen [236]. Chloroform root bark extract of *Uvaria afzelii* (Annonaceae) and hexane root bark extract of *Tetracera alnifolia* (Dilleniaceae) exhibited activity MIC 87.5 μg/ml and 93.31 μg/ml against *M. tuberculosis*, respectively [54]. Screening of aqueous:ethanol extract from various parts of 11 plants used for treating tuberculosis in the Northcentral region of Nigeria, showed that leaf/twist extract of *Pavetta corymbosa* exhibited the highest activity (MIC 800 μg/ml) against *M. tuberculosis* [55]. Methanol stem bark extract of *Spondias mombin* exhibited activity (MIC 61.1 μg/ml) against *M. tuberculosis* [122]. Bioactive compounds with potential antimycobacterial activity have been isolated from Nigerian medicinal plants [117,126] [Table 2]. The use of BCG antigen in conducting antimycobacterial test by some investigators may be possibly due to the difficulty (long incubation time, requirement for specialized media, and biosafety cabinet environment to avoid exposure) associated with culturing *M. tuberculosis*. Considering the zoonotic importance and the difficulty (long-term therapy and resistance - multidrug resistant tuberculosis [MDR TB] and extremely-drug-resistant TB [XDR TB]) in treating mycobacterial infections, there is need for more investigations on Nigerian plants for detection of potential antimycobacterial agent. Currently, there is awakened interest in search of new, safe, and effective antitubercular drugs globally [237].

Although Nigerian medicinal plants used in treatment of sexually-transmitted infections (STIs) showed antimicrobial activity [66,69,70,72,79,95,174,238,239], none of the studies used organisms such as *Neisseria gonorrhoeae*, *Treponema pallidum*, *Haemophilus ducreyi*, *Trichomonas vaginalis*, *Ureaplasma urealyticum*, and *Oligella urealytica* which are known to cause STIs [16]. Understandably, these organisms are fastidious and require more intensive culturing techniques, but their use is a necessary progression for further antimicrobial pharmacognosy studies since it can no longer be assumed that broad-spectrum activity is adequate for plants used for specific diseases [16].

STUDIES ON NIGERIAN MEDICINAL PLANTS BASED ON LOCALITY

A few antimicrobial-related papers have focused on the ethnobotany of specific geographical regions within Nigeria [92,117]. These studies investigated the antimicrobial activity of *Terminalia aviccenoides* commonly used in the Nupe

traditional medicine for treating microbial infections, with the root bark extract of *T. avicennioides* yielding Friedelin with strong antimycobacterail activity (MIC 4.9 ug/ml) [Table 2]. Some studies investigated antimicrobial activity of plants used as chewing stick for maintaining oral hygiene within Southwest Nigeria [39,42,124,133,169,170,187,188,190,218,233,240].

ANTIMICROBIAL STUDIES ON NONFLOWERING AND PARASITIC NIGERIAN PLANTS

Two studies reported the antimicrobial activity of the fungus *Ganoderma* (Ganodermataceae) [241,242]. Ofordile *et al*. [241] screened four *Ganoderma* species in which *G. colossum* showed potential antimicrobial activity, further studies on this species resulted in isolation of Colossolactone E and 23-hydroxycolossolactone E which inhibited the growth of *B. subtilis* and *Pseudomonas syringae* [241]. Extracts of bryophytes *Calymperes erosum* (Calymperaceae) and *Bryum coronatum* (Bryaceae) exhibited broad-spectrum activity against selected Gram-positive and Gram-negative organisms with both plants having MIC 0.625 - 5 mg/ml [145] [Table 1]. Screening of the parasitic plant *Viscum album* (Loranthaceae) by diffusion method showed no promising antimicrobial activity [98,243].

FUTURE ANTIMICROBIAL RESEARCH ON NIGERIAN MEDICINAL PLANTS

There are some considerations that investigators of Nigerian plants need to adopt for adequate validation of antimicrobial activity of these plants. Van Vuuren [16] highlighted these considerations: Methods should be standardized and only noteworthy activities considered for publication, disc diffusion assays should be avoided especially when considering essential oil studies, isolation of bioactive compounds should be directed to plants having antimicrobial activity as identified in the screening procedures, attention needs to be directed toward the ethnobotanical use of the plant and the rational antimicrobial screening that follows, the use of nonpathogenic organism such as *Bacillus subtilis* for anti-infectivity studies should be avoided, studies on the efficacy of plants against resident beneficial bacteria such as *Lactobacillus acidophilus* and *Bifidobacterium bifidum* could yield information that may make plant extracts or oils more appropriate than the presently administered allopathic antimicrobials, which inevitably destroy the beneficial commensal organisms together with the invading pathogens. Moreover, the majority of investigators on antimicrobial activity of Nigerian plants used isolates that were only identified to generic level. This may be due to limited facilities (such as molecular laboratories) for adequate identification of organisms to species level. However, for reproducibility of studies, there is need for the use of adequately characterized organisms in antimicrobial screening studies.

Because the identification of a single active chemical entity responsible for the antimicrobial activity of a plant is less likely, research should be focusing on the investigation of a combination of compounds to achieve a greater efficacy [16]. Incorporation of interactive phytochemical studies with existing practices is crucial in the search for novel chemotherapeutic agents [16]. Regular or consistent administration of antimicrobials to determine if enhanced efficacy would be achieved with regular subtherapeutic administrations in comparison with single acute administrations should be considered in future research designs [16,244,245]. Extended time-kill experiments monitoring the viability of plant extracts overtime with regular dosages is crucial in determining the cidal effect of an antimicrobial [16].

CONCLUSION

The reviewed reports showed that there is a progressive trend in studies on antimicrobial activity of Nigerian medicinal plants. With advancement in laboratory techniques coupled with renewed interest in the field and the scientific validation of the traditional uses of medicinal plants, traditional medicine is increasingly been recognized as an accepted alternate regimen to orthodox health-care systems [16]. It is clear that many endemic Nigerian plants used in treating microbial infections and various ailments in traditional medicine, potentially possess antimicrobial activity. Bioactive compounds with antimicrobial activity have been isolated from Nigerian plants in different families. With further researches, new chemotherapeutic agents could possibly be developed from these plants.

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