Isolation of gallic acid and flavonoids from antimicrobial extracts of *Terminalia brownii* leaves

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***Abstract*— The present work is concerned with the characteristics of active component in leaves of *Terminalia brownii*; as it is most likely to be responsible for some of the reported biological effects include antibacterial and antifungal activities.**

The extracts from dried *Terminalia brownii* leaves were investigated for antimicrobial effects against four types of bacteria and two fungi. The methanol extract possessed high activity against all tested bacterial organisms and *Candida albicans*. The minimum inhibition concentration for the active fractions were ranging between 4- 6 µg /ml. The most active constituents such as gallic acid, dihydroxy flavone, di-methoxy quercetin rhamnoglucoside and Kaempferol methoxy-sulphate were isolated and their chemical structures were identified by spectroscopic methods of analysis ^{1}HNMR and LC-MS.

***Index Terms*— *Terminalia brownii*, antimicrobial effects, chemical constituents, chemical structures.**

I. INTRODUCTION

Shaf or Subag is used in Sudan to designate the plant *Terminalia brownii* (Combretaceae), which has been used medicinally for treatment of many complaints.
Substantially, the water extract of the bark is used in Sudanese traditional medicine for treating diarrhea, cough and bronchitis [1, 2, 3].

Abdel Mageed [4] investigated the biological activity of *Terminalia brownii* stem extract, which exhibited pronounced antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Moreover, Phytochemical test showed that tannins, flavonoids, sterols, terpenes, coumarins and saponins were found to be present, as well as the isolation of Lupeol from the stems.

Miscellaneous studies of the phytochemical constituents and the biological effects of *Terminalia brownii* species including antibacterial and antifungal activities have been reported, which confirmed the significant activity against the tested organisms [5, 6]. The methanolic extracts of the stem bark and stem wood of *Terminalia brownii* were active against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella typhi* and *Bacillus anthracis*, *Candida albicans* and *Cryptococcus neoformans* [7].

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Kareru *et al.* [8] reported the high sensitivity of leaves and bark extracts of *Terminalia brownii* to *Escherichia coli* and *Staphylococcus aureus* .

Ahmed [9] reported the high antibacterial activity of Terminalia brownii leaves methanol extract against some standard organisms and in comparison with reference drugs; Ampicillin, Gentamicin and Cloxacillin, In addition to characterization of some active fractions.

The anti-microbial effects of phytochemicals found in the stem bark and root extracts of *Terminalia brownii* against the bacteria *Escherichia coli* and the fungi *Candida albicans* indicates their potential with wide spectrum of antibacterial and antifungal activities [10]. Oleanane type triterpenoid, ellagic acid derivatives, and 3-*O*-β-D-glucopyranosyl–β-sitosterol isolated from stem bark extract were found to be active against three species of fungi and one species of bacteria [11].

Terminalia. *brownii* roots gave the best antimycobacterial effects against *Mycobacterium smegmatis*; triacontanol, sitostenone and β-sitosterol were found in antimycobacterial hexane extracts of the stem bark, as well as *Terminalia brownii* could be a good source of new ellagic acid derivatives and tannins with antimycobacterial potential [12].

Most of the studies of *Terminalia brownii* stem bark, wood, roots and leaves extracts have indicated varied levels of antibacterial, antifungal and antiviral activity, but isolation and characterization of active compounds were limited. Therefore, the aim of the present work is to isolate and identify anti-microbial compounds from *Terminalia brownii leaves*.

II. MATERIAL AND METHODS

Chemicals

Methanol, ethyl acetate, chloroform, n-hexane, NaOH, HCl, sulphuric acid, glacial acetic acid and DMSO from Biosolve BV (Valkenswaard, The Netherlands), acetone, formic acid from JT Baker BV (Deventer, The Netherlands), anisaldehyde, silica gel 60, Diaion HP-20 from Acros Oragnic (Geel, Belgium), and MeOD NMR solvent from Eurisotop (Yvette, France). All solvents and reagents were analytical grade.

Plant materials

Terminalia brownii leaves were collected from Botanical garden/ Khartoum state and a voucher was deposited at

Herbarium of the Medicinal and Aromatic Plants Research Institute (MAPRI), Sudan.

Plant material Extraction and liquid fractionation

Powdered air-dried leaves of *Terminalia brownii* (500g) was extracted by methanol and sonicated at room temperature for 1 hr, and the solution was filtered. The procedure was repeated two times and the filtrate were centrifuged. The solvent was removed under reduced pressure. The dried extract was dissolved in 300 ml water and was partitioned with hexane (3x300 ml), chloroform (3x300 ml) and ethyl acetate (3x300 ml), respectively.

Phytochemil screening

Phytochemical tests were carried out on the methanolic extract of the leaves of *Terminalia brownii* using standard procedures of plant constituent's identification [13].

Determination of antimicrobial activity of the extract

The antimicrobial activity of crude extract and fractions were tested against *Bacillus subtilis* (*B.s*) NCTC19659; *Staphylococcus aureus* (*S.a*) ATCC 6538, *Escherichia coli* (*E.coli*) ATCC10936; *Pseudomonas aeruginosa* (*Ps.a*) ATCC 9027; and antifungal properties for *Candida albicans* (*C.alb*) ATCC 10231; *Aspergillus niger* (*As.n*) ATCC 9642 using the agar diffusion method [14, 15].

Determination of minimum inhibitory concentration

Various concentrations of active components from *Terminalia brownii* ranging between 4.0 and 6.0 µg/ml were introduced into different test tubes inoculated with an overnight culture of micro organisms diluted to give a final concentration of 10^6 cells per ml. The tubes were incubated at 37ºC for 24 h. The least concentration of component that did not permit any visible growth of the inoculated test organism in broth culture was regarded as the minimum inhibitory concentration (MIC) in each case [15].

Column and thin layer chromatography

Column chromatography was performed with silica gel 60 (230-400) mesh in a 5 x 58 cm column and Diaion HP-20 in a 2.5 x 40 cm column. Preparative and analytical TLC were performed using aluminum TLC plates of 20 x 20 cm silica gel 60 F 254.

Isolation of compounds

The chloroform extract of *Terminalia brownii* leaves was chromatographed on a silica gel column and eluted stepwise with a gradient ($CHCl_3$-MeOH) to give fractions, which were further tested for their antimicrobial activities. Aqueous extract of *Terminalia brownii* was chromatographed on Diaion HP-20 column and eluted subsequently with 500 ml of H_2O, 25% MeOH, 50% MeOH, 75% MeOH, 100% MeOH and CH_3COCH_3 to give one active fraction 100% MeOH. Fractionation and isolation were monitored by TLC with visualization under UV (λ_{max}254 and 365 nm), using the chloroform/ ethyl acetate (7:3) solvent system.

NMR measurement

Each pure compound was dissolved in Deuterated methanol (MeOD) for ^{1}HNMR, in which the spectra was recorded on 500 MHz Bruker DMX 500 Spectrometer. Chemical shifts (δ) are given in ppm.

APCI Mass Spectrometry

A spectrum was recorded on Agilent LC-MS Spectrometer using probe positive-ion and phenomenex RP 18 (4.6 x 150 mm, 5 micron) column. The mass scan range was 100 – 800 m/z. The solvent system was MeOH: H_2O: Formic Acid (90:10:1) with flow rate of 1 ml/minute and injection volume of 10 µL/minute. The mass Spectroscopy data was subjected to the library search using metabolomics query bank.

III. Results and Discussion

Phytochemical screening of *Terminalia brownii* leaves revealed the presence of saponins, coumarins, sterols, tannins and flavonoids. The methanol extract of the leaves of *Terminalia brownii* possessed high activity against all bacterial organisms and antifungal activity against *C. alb*, but inactive against *As.n*. The inhibition zones ranging between (21 - 27 mm) at 50% concentration with bacteria and 17 mm with *C. alb*. The extract was slightly more active against Gram negative bacteria than the Gram positive one. The result confirmed the antibacterial activity of alcoholic extract of *Terminalia brownii* all parts, which was similar to that previously reported [4, 5, 6, 7, 9].

Out of the solvents used for partitioned fractionation, the water and chloroform extracts showed high activity since they gave inhibition zone ranging between (17 -30 mm) for the bacteria and (19-29 mm) for *C. alb* (Table 1). One active fraction (T) isolated from water extract by Diaion HP-20 column was identified. The minimum inhibition concentration (MIC) for the fraction T was 4.5 µg /ml against *E. coli* and 4.0 µg /ml against *Ca.alb*, which exhibited its powerful antifungal activity. Furthermore, three fractions were collected from silica gel column chromatography using chloroform, methanol as the eluent for chloroform extract.

T1 was the most active one with MIC 5.0 µg /ml against *B. s.*, 5.5 µg /ml against *S.a.*, 4.0 µg /ml against *E. coli*, 4.5 µg /ml against *Ps. a.*, and 6 µg /ml against *Ca. alb*. It was subjected to column chromatography which leads to the isolation of three sub fractions **1**, **2** and **3**.

All the fractions were identified by comparing their spectroscopic data with the literature values.

Structures elucidation

APCI-MS spectra of fraction T (Fig 1), a yellowish crystals melts at 212-214˚C, shows molecular ions peak $[M+H]^+$ at m/z 381 approximately resemble the significant antifungal agent Kaempferol 7-O-methoxy-3-sulphate (scheme1)

molecular weight 380.63 and in agreement with it molecular formula $C_{16}H_{12}O_9S$ [16, 17].

^{1}HNMR spectra of T fraction showed two doublets signals at δ 6.17 ppm and 6.35 ppm assigned to protons at A ring, and two doublets signal at δ 7.87 ppm and 6.75 ppm were due to protons of the B ring typical Kaempferol type and confirm that this compound is flavonoid (Table 2) [18, 19]. Moreover, Lassaigne's confirmatory test revealed the presence of sulphur in the compound [20]. This compound was not previously identified from *Terminalia brownii*.

The APCI-MS spectra of the active fraction T1 (Fig 2) isolated from chloroform extract afforded five molecular ions peaks. Therefore, T1 was a mixture of five compounds possibly three of them were isolated individually by TLC.

The molecular ion peak **1** $[M+H]^+$ at m/z 171 matches with the molecular weight of gallic acid 170.1 and in agreement with its molecular formula $C_7H_6O_5$ [21]. Moreover, ^{1}H NMR spectroscopic data to fraction 1 showed doublet signals at δ 7.02 ppm denoted to the proton of aromatic ring. Thus, compound **1** was identified as gallic acid. The molecular ions peaks **2** $[M+H]^+$ at m/z 255 accord with 7,4`-dihydroxy isoflavone (Scheme 2) molecular weight 254 and matches its molecular formula $C_{15}H_{10}O_4$ [22, 23]. The ^{1}HNMR spectra of the compound showed a similar assignment of flavone type, since it gave signals between δ 6.0-7.0 ppm attributed to the protons adjacent to the hydroxyl group in benzene ring A and B, or the proton signal of H-3 in ring C; and the signals above δ 7.0 ppm which are attributed to the protons with meta position to hydroxyl group in benzene ring B. Furthermore the existence of the singlet at δ 6.68 (H-2) (Table 3) confirms that compound **2** is identified as hydroxy flavones [24], and it was not previously reported from *Terminalia brownii*.

The molecular ions peaks **3** $[M+H]^+$ at m/z 636 corresponding to 7, 3'-di-methoxy quercetin rhamnoglucoside (Scheme 3) molecular weight 635 and its molecular formula $C_{29}H_{31}O_{16}$. The ^{1}HNMR spectra of this compound revealed the characteristics of flavones ^{1}H NMR spectra. Therefore, it showed signals between δ 6.0-7.0 ppm attributed to the protons adjacent to the hydroxy or methoxy group in benzene ring A and B, and the signals above δ 7.0 ppm which are attributed to the protons with meta position to hydroxy and/ or methoxy group in benzene ring B typical to quercetin skeleton. Furthermore, two sugar anomeric doublets at δ 4.86 ppm and 4.21 ppm were identified, which undergo method for quantitative determination of reducing sugars by heating with alkaline copper tartrate reduce the copper from the cupric to cuprous state and thus cuprous oxide is formed [25]. The singlet δ at 3.82 ppm attributed to methoxy group (Table 4). This compound exhibited molecular weight and NMR spectral data consistent with literature values of a same compound isolated from *Hyphaene thebacica* leaves extract [19], and it was characterized from *Terminalia brownii* leaves for the first time.

Table 1 Antimicrobial activity of *Terminalia brownii* leaves extracts and reference drugs against standard organisms

Sample	Standard organisms used*/MDIZ (mm)					
	B.s	***S.a***	***E. coli***	***Ps.a***	***As.n***	***C.alb***
Chloroform	20	17	23	22	-	19
Methanol	24	21	27	25	-	17
Water	22	19	30	24	-	29
Fraction T	16	15	23	18	-	30
Fraction T1	22	20	27	24	-	18

***** MDIZ = Mean diameter of inhibition zone (mm). Conc. used 100 mg/ml at 0.1 ml/ cup. MDIZ: >15 Sensitive, 14 – 15 Intermediate, <14 Resistant, - No inhibition zone

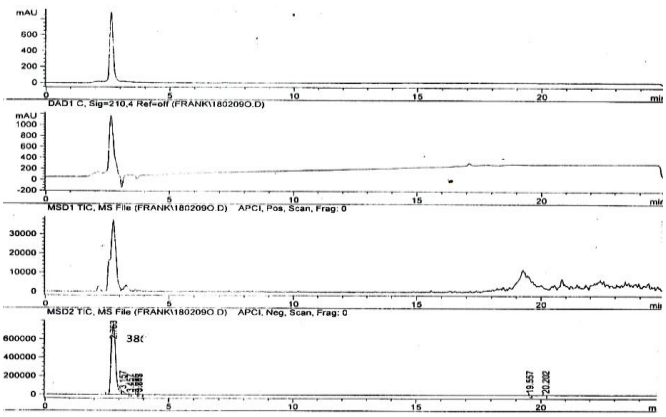
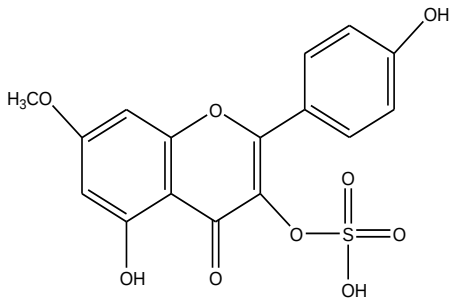


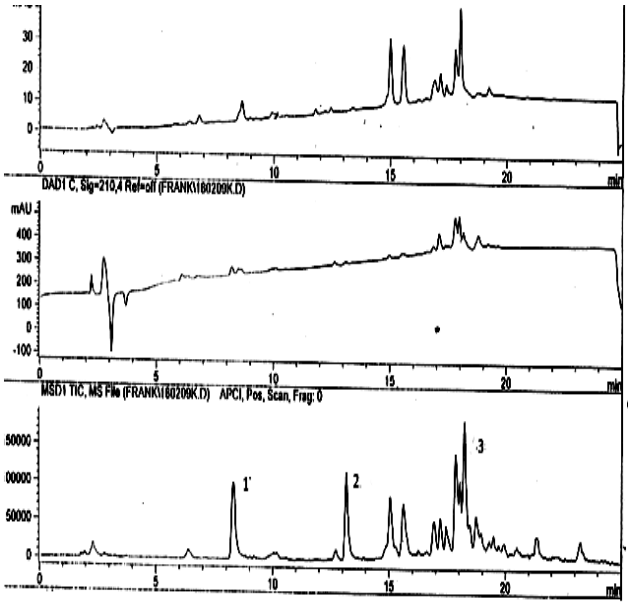
Fig (1) APCI-MS molecular ion peak of T

Table 2 ^{1}H (500 MHz) Chemical shift of Kaempferol methoxy-sulphate in MeOD (ppm)

Position	Chemical shift
6	6.17(1H,d)
8	6.35(1H,d)
2', 6'	7.87(1H,d)
3', 5'	6.75(1H,d)
7-CH_3O	3.80(3H,s)



Scheme 1: Kaempferol 7-methoxy-3-sulphate



Fig(2) APCI-MS molecular ion peak of T1

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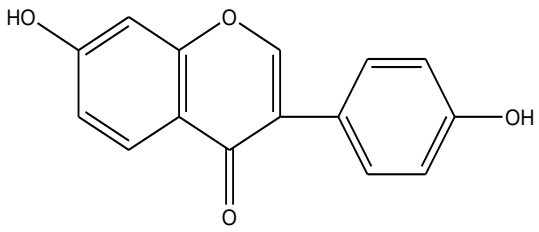
Table 3 ^{1}H (500 MHz) Chemical shift of dihydroxy isoflavonein MeOD(ppm)

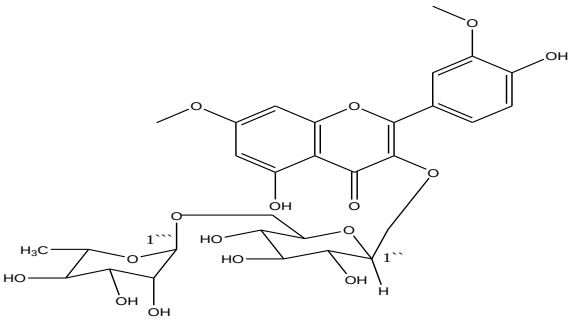
Position	Chemical shift
2	6.68(1H,s)
5	6.49(1H,d)
6	6.15(1H,dd)
8	6.78(1H,d)
2', 6'	7.45(1H,d)
3', 5'	7.86(1H,d)



Scheme 2: 7, 4'-dihydroxyisoflavone

Table 4 ^{1}H (500 MHz) Chemical shift of di-methoxy quercetin rhamnoglucoside in MeOD (ppm)

Position	Chemical shift
6	6.78(1H,d)
8	6.90(1H,d)
2'	7.73(1H,d)
5'	8.56(1H,d)
6'	7.64(1H,dd)
7-CH_3O	3.47(3H,s)
3'- CH_3O	3.82(3H,s)
1"	4.86(1H,d)
1"'	4.21(1H,d)
Other sugar protons	3.15, m



Scheme 3: 7, 3'-dimethoxy quercetin rhamnoglucoside

IV. Conclusion

The methanol extracts of *Terminalia brownii* leaves showed high antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and antifungal activity against *Candida albicans*. Gallic acid, dihydroxy flavone, di-methoxy quercetin rhamnoglucoside were isolated from chloroform extract of *Terminalia brownii* leaves and the powerful antifungal Kaempferol 7-O-methyl-sulphate from its aqueous one.

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