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Full Length Research Paper

Antimicrobial activity of extracts and a germacranolide-type sesquiterpene lactone from *Tithonia diversifolia* leaf extract

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The *in vitro* antimicrobial activity of the hexane, ethyl acetate and methanol extracts of the leaf of *Tithonia diversifolia*, and one sesquiterpene lactone 1 isolated from the ethyl acetate extract was studied. Of the fourteen strains of bacteria used, the ethyl acetate extract was the most active, showing inhibitory activity against five Gram +ve and two Gram –ve organisms. This was followed by the hexane extract and then methanol. The ethyl acetate fractions (TDE 2 – TDE 5, TDE 7, TDE 8 and TDE 10) showed varying degrees of inhibitory activity. The sesquiterpene lactone 1 showed activity against all the tested microorganisms, except *Staphylococcus aureus* and *Bacillus cereus* (MICs = 15.6 – 62.5 µg/ml for most stains). All the extracts, fractions and compound 1 showed activity against the fungus *Candida albicans*. The results of the present study indicate that the non-polar leaf extract of *T. diversifolia* could be useful in the treatment of some disease conditions and the sesquiterpene lactone 1 could be a good candidate as a phytotherapeutic agent against some bacterial infections.

Key words: *Tithonia diversifolia*, antimicrobial activity, sequiterpene lactone.

INTRODUCTION

Tithonia diversifolia (Hemsely) A. Gray (Asteraceae), commonly called Mexican sunflower, is a common shrub (weed) native to Central America but has become naturalized in many tropical countries, including Nigeria. It is found in Nigeria on road sides, crop fields and waste areas. The tradition of plant collection and plant-based medications has been handed down from generation to generation, usually by word of mouth among many cultures. A wide variety of natural products are used in the treatment of common infections in traditional medicine in most developing countries. *T. diversifolia* had been used in traditional medicine for the treatment of various ailments.

The hot water extract of the aerial parts of the plant is used for the treatment of malaria in Guatamala, Taiwan, Mexico (Calzada and Ciccio, 1978) and Nigeria. An oral decoction of the leaves and stem is used to cure hepatitis in Taiwan and gastrointestinal disorders in Kenya and Thailand (Johns et al., 1995). Also, the infusion of the leaves is used for the treatment of measles in Cameroon (Kamdem et al., 1986), while the dried leaves are applied externally on wounds in Costa Rica (Kuo and Chen, 1997). A decoction of the flowers is used for the treatment of skin eczema (Gurib-Fakun et al., 1996). Extracts of the various parts of the plant have been reported to exhibit antimalarial (Madureira et al., 2002), antiinflammatory (Rungeler et al., 1998), anti-proliferation (Gu et al., 2002), insecticidal (Hongsbhanich et al., 1979) analgesic and anti-inflammatory (Owoyele et al., 2004) and antibacterial (Bork et al., 1996) activities.

Several compounds, mostly sesquiterpenes, diterpenes, monoterpenes and alicyclic compounds have been isolated from the leaves, stem and flowers of *T. diversifolia* (Chon et al., 2000; Kuo and Chen, 1998; Agusta and Jamal, 1999; Lamaty et al., 1991). In this paper, we report the evaluation of the antimicrobial activity of the methanol, ethyl acetate and hexane

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extracts of Nigerian *T. diversifolia* leaves and a sesquiterpene lactone isolated from the ethyl acetate extract.

MATERIALS AND METHODS

General procedures

^{1}H and ^{13}C NMR spectra were run on a Jeol EX400 spectrometer (in $CDCl_3$). IR spectra were recorded on a BUCK Scientific spectrometer. Mass spectra (EI) were run on a Varian GCQ machine (Italy) fitted with a direct inlet probe.

Plant material

The plant materials used in this study consisted of the leaves of *T. diversifolia* collected in April, 2004, in Ile-Ife, Osun State, Nigeria. The plant was identified by Dr. H. C. Illoh of the Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria. A voucher specimen was prepared and deposited in the Herbarium of the Botany Department, Obafemi Awolowo University, Ile-Ife, Nigeria for reference.

Extraction and isolation procedures

Finely powdered, air-dried leaves of *T. diversifolia* (1 kg) were extracted successively at room temperature for 72 h with n-hexane (Hx), ethyl acetate (EA) and methanol (M). After filtering, the different extracts obtained were evaporated to dryness with a rotary evaporator to give crude solid residues (6.6 g, 0.7%, for Hx; 28.5 g, 2.8%, for EA and 36.2 g, 3.6%, for M). The crude solvent extracts were screened for antimicrobial activity as described below, in section “Antimicrobial screening”. The ethyl acetate extract was found to be the most active (in terms of broad spectrum). The EA extract of *T. diversifolia* was chromatographed over silica gel (60 – 100 mesh) column and eluted (taking 20 ml fractions in test tubes) starting with hexane, followed by toluene and then toluene-chloroform (1:1). Fractions with components having the same retention factor values on thin layer chromatography (TLC) were combined and coded.

Elution with hexane gave fractions TDE 1 (orange oil, 26 mg), TDE 2 (orange oil, 570 mg). Elution with toluene gave fractions TDE 3 (orange oil, 1.4 g), TDE 4 (brown oil, 760 mg), TDE 5 (brown oil, 350 mg), TDE 6 (green oil, 660 mg), TDE 7 (colourless oil, 2.5 g); while elution with toluene-chloroform (1.1) gave fractions TDE 8 (dark green semi-solid, 1.6 g), TDE 9 (green solid 6 mg), TDE 10 (semi-solid, 1.7 g) and TDE 11 (semi-solid, 1.1 g). TDE 11 was heated in toluene to give a clear solution and then left to stand to give colorless crystals of **1**, m.p. 142 – 144°C. Compound **1** was identified as a germacranolide type sesquiterpene lactone by its ^{1}H and ^{13}C NMR spectra.

Microorganisms

The cultures of microorganisms used were of the National Collection for Industrial Bacteria (NCIB) and some locally isolated organisms (LIO). The organisms included *Bacillus anthracis* (LIO), *Bacillus cereus* (NCIB 6349), *Bacillus polymyxa* (LIO), *Bacillus stearothermophilus* (NCIB 8222), *Bacillus subtilis* (NCIB 3610), *Clostridium sporogenes* (NCIB 532), Corynebacterium pyogenes (LIO), *Staphylococcus aureus* (NCIB 8588), *Streptococcus faecalis* (NCIB 755); *Escherichia coli* (NCIB 86), *Klebsiella pneumoniae* (NCIB 418), *Pseudomonas aeruginosa* (NCIB 950), *Pseudomonas fluorescens* (NCIB 3756), *Shigella dysenteriae* (LIO) for the antibacterial tests. *Candida albicans* was used for the antifungal test. The organisms were obtained from the Department of Microbioilogy, Obafemi Awolowo University, Ile-Ife, Nigeria.

Nutrient broth (Oxoid Ltd) and Nutrient agar (Oxoid Ltd) were used for subculturing the bacterial isolates, malt extract agar (Oxoid Ltd) was used for subculturing the fungal isolate, while diagnostic sensitivity test agar (Biotech Ltd) was used for sensitivity testing.

Antimicrobial screening

The sensitivity testing of the plant extracts, fractions and the sesquiterpene lactone were determined using the agar-well diffusion method (Irobi et al., 1996; Russell and Furr, 1977). The bacterial isolates were first grown in nutrient broth (Oxoid Ltd) for 18 h before use. The inoculum suspensions were standardized and then tested against the effect of the crude plant extracts and fractions at a concentration of 2 mg/mL each. The plates were observed for zones of inhibition after 24 h incubation at 37°C. Controls were maintained with DMSO and streptomycin (standard antibiotic, at 1 mg/mL, for the bacteria).

Determination of minimum inhibitory concentrations

The minimum inhibitory concentrations (MIC) were determined by two-fold dilutions method (Russel and Furr, 1977). The plant extracts, fractions and the sesquiterpenes lactone (**1**) were dissolved in dimethylsulfoxide and different concentrations ranging between 0.0078 and 1 mg/mL were prepared. 2 mL of the concentrate from each dilution was added to 18 mL of molten sterile nutrient agar (Oxoid Ltd) aseptically, and thoroughly mixed together in a sterile Petri dish. This was then allowed to set. The surface of the nutrient agar was allowed to dry properly before streaking with the appropriate bacterial isolate. The plates were then incubated at 37°C for up to 72 h. The lowest concentration preventing all visible growth was taken as the minimum inhibitory concentration.

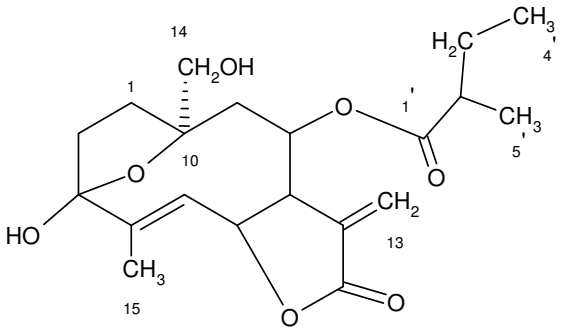
RESULTS AND DISCUSSION

Purification of the ethyl acetate extract of *T. diversifolia* by column chromatography on silica gel gave several fractions (TDE 1- TDE 11). Further purification of TDE 11 afforded the germacranolide type sesquiterpene lactone **1.**

The infrared spectrum of **1** showed two strong, distinct absorption bands at 3545 and 3508 cm^{-1}, indicating the presence of two different alcoholic OH groups. Other bands appeared at 2979, 2943, 2876 (C-H), 1764 (C = O of lactone), 1727 (C = O of ester), 1460, 1387, (CH) and 1198, 1076 and 1023 (C – O of ester and alcohol) cm^{-1}.

The molecular ion in the mass spectrum of **1** was found at m/z 380 (19.0%). Other ions include (m/z, (rel. int.): 363 (11, $[M - OH]^+$), 362 (9, $[M - H_2O]^+$), 349 (5, $[M– CH_2OH]^+$), 277 (14), 260 (17), 247 (100), 229 (58), 214 (32), 205 (67), 187 (51), 179 (88), 133 (38), 91 (24), 79 (18).

The structure of **1** was elucidated by its ^{1}H and ^{13}C NMR spectra. The ^{1}H NMR spectra of compound **1** (Figure 1) indicated structural similarity with the germacranolides tirotundin (**2**) and woodhousin (**3**) (Baruah et al., 1979).



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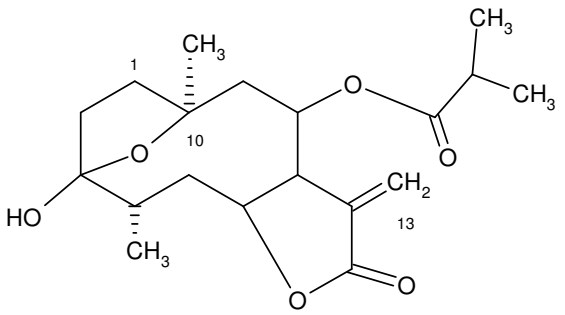

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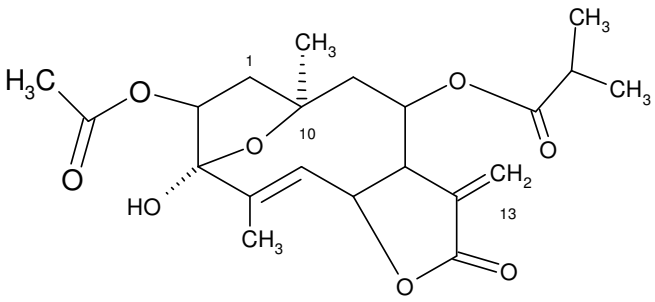

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Figure 1. Structure of sesquiterpene lactone **1** having structural similarity with the germacranolides tirotundin (**2**) and woodhousin (**3**) (Baruah et al., 1979).

The ^{1}H NMR spectrum included signals showing an exo-methylene protons (δ 5.60 and 6.27), an AB system at δ 3.49 and 3.65 indicating the presence of a hydroxymethyl group on a quaternary carbon atom, a methyl group on an sp^{2} carbon (δ 1.83) and a 2-butyl group (δ 0.80, 1.00, 1.35, 1.54 and 2.25). The 2-butyl group is part of the acyl group at C-8 of **1** and its chemical shift values are characteristic for a 2-methylbutanoic acid (Budesinsky and Saman, 1995). The ^{13}C NMR of **1** displayed signals typical of 2-methylbutanoyl group (δ 176.1, 41.5, 26.7, 169 and 11.8) (Passreiter et al., 1999), and hyroxymethyl group at δ 68.3.

^{1}H NMR (400 MHz, $CDCl_3$), δ: 0.80 (3H, t, J = 7.3 Hz, H-4'), 1.00 (3H, d, J = 7.0 Hz, H – 5'), 1.36 (^{1}H, m, H-3'a), 1.54 (^{1}H, m, H-3'b), 1.83 (3H, s, H-15), 2.00 (^{1}H, m, H-9a), 2.31 (^{1}H, m, H-9b), 2.05 (2H, m, H-7), 3.49 (^{1}H, d, J = 11.0 Hz, H-14a), 3.65 (1H, d, J = 11.0 Hz, H-14b), 5.40 (1H, m, H-8), 5.49 (1H, m, H-6), 5.55 (1H, m, H-5), 5.60 (1H, d, J = 2.56 Hz, H-13a), 6.27 (1H, d, J = 2.2 Hz, H-13b); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 175.9 (C – 1'), 170.1 (C – 12), 141.8 (C – 4), 137.2 (C – 11), 128.4 (C – 5), 123.5 (C – 13), 106.9 (C – 3), 85.8 (C – 10), 75.8 (C – 6), 73.4 (C – 8), 68.1 (C – 14), 49.7 (C – 7), 41.3 (C – 2'), 39.1 (C – 1), 37.5 (C – 2), 31.6 (C – 9), 26.6 (C – 3'), 21.4 (C – 15), 16.7 (C – 5'),, 11.7 (C – 4'). The NMR spectra were accomplished from the 2D-NMR experiments (^{1}H - ^{1}H COSY, ^{1}H – ^{13}C HETCOR).

The results for the antimicrobial sensitivity tests (inhibition zones (mm)) for the extracts, the fractions (TDE 2 – TDE 10) and the isolated sesquiterpene lactone from *T. diversifolia* (at 2 mg/ml), antibiotic standard, streptomycin (at 1 mg/ml) and DMSO (solvent) against the nine species of Gram +ve and five Gram -ve bacteria, along with one fungus are given in Table 1. The solvent used for the dissolution of the extracts showed no activity.

The ethyl acetate extract was the most active of the three extracts, showing activity against five Gram +ve and two Gram -ve bacterial strains. The hexane extract showed activity against three Gram +ve and two Gram -ve bacterial strains with the largest zone of inhibition on *Escherichia coli*, while the methanol extract was active against only two Gram +ve and two Gram -ve bacterial strains. The ethyl acetate fractions (TDE 2 – TDE 5, TDE 7, TDE 8 and TDE 10) showed varying degrees of activity with different zones of inhibition.

The results further showed that the crude solvent extracts and the ethyl acetate fractions have no activity against two Gram +ve bacterial strains, *Staph. aureus* (except EA extract) and *B. cereus*. The sesquiterpene lactone **1** showed activity against all the tested microorganism except *Staph. aureus* and *B. cereus*. All the extracts, fractions and compound **1** are active against the fungus, *Candida albicans*.

The minimum inhibitory concentrations (MIC) (defined as the lowest concentration of drug that completely inhibited the growth of the organism) of compound **1** and streptomycin are shown in Table 2. The MICs of compound **1** varied between 15.6 and 125 µg/ml against all the bacterial strains under study, except against *Shigella dysenteriae* with MIC value of 1000 µg/ml. The MIC values of streptomycin varied between 7.8 µg/ml and 250 µg/ml but was not active against *E. coli* and *Klebsiella pneumoniae*. The results indicated that the sesquiterpene lactone **1** has a higher activity than streptomycin against most of the tested bacterial isolates,

Table 1. Antimicrobial screening of crude extracts of *Tithonia diversifolia* leaves, fractions of the ethyl acetate extract and compound 1.

Micro-organisms	Zones of inhibition (mm)*												
	Hx Extract	**EA Extract**	**M Extract**	**TDE 2**	**TDE 3**	**TDE 4**	**TDE 5**	**TDE 7**	**TDE 8**	**TDE 10**	**Compd. 1**	**S***	**DMSO**
Gram positive													
Bacillus subtilis	27	22	25	25	24	18	17	18	20	28	23	22	0
Bacillus stearothermophilus	26	15	0	28	30	0	23	22	25	22	28	23	0
Bacillus polymyxa	0	13	0	0	10	0	0	20	10	12	12	15	0
Clostridium sporogenes	10	18	0	11	12	18	18	26	10	10	13	28	0
Bacillus anthracis	0	0	11	15	12	0	11	15	12	13	14	20	0
Streptococcus faecalis	0	0	0	16	10	0	0	22	0	13	10	24	0
Corynebacterium pyogenes	0	0	0	16	0	0	0	0	12	0	12	19	0
Staphylococcus aureus	0	16	0	0	0	0	0	0	0	0	0	21	0
Bacillus cereus	0	0	0	0	0	0	0	0	0	0	0	N.D	0
Gram negative													
Escherichia coli	30	22	20	30	25	18	25	20	26	28	26	0	0
Pseudomonas aeruginosa	0	0	0	18	15	12	0	10	13	14	17	N.D	0
Pseudomonas fluorescens	0	0	0	0	10	0	0	0	0	12	14	N.D	0
Klebsiella pneumoniae	16	22	14	13	14	18	18	25	10	11	16	0	0
Shigella dysenteriae	0	0	0	12	13	12	0	15	0	10	15	22	0
Fungus													
Candida albicans	25	25	22	20	18	20	18	22	25	16	18	N.D	0

Screening conc. = 2 mg/mL
mm* = mean diameter of three readings
S* = Streptomycin (at 1 mg/mL)
DMSO = Dimethylsulfoxide
N.D = Not determined
Hx = hexane
EA = ethyl acetate
M = methanol

Table 2. Minimum inhibitory concentration (MIC, µg/ml) values for compound 1 compared to Streptomycin.

Micro-organism	Compound 1 (µg/ml)	Streptomycin (µg/ml)
Gram Positive Bacteria		
Bacillus subtilis	15.6	62.5
Bacillus stearothermophilus	15.6	62.5
Bacillus polymyxa	62.5	125
Clostridium sporogenes	62.5	7.8
Bacillus anthracis	15.6	31.3
Streptococcus faecalis	15.6	62.5
Corynebacterium pyogenes	125	31.3
Gram Negative Bacteria		
Escherichia coli	15.6	N.A
Pseudomonas aeruginosa	15.6	250
Pseudomonas fluorescens	15.6	250
Klebsiella pneumoniae	15.6	N.A
Shigella dysenteriae	1000	250

N.A = Not Active.

except for *Clostridium sporogens*, *Corynebacterium pyogenes* (Gram +ve) and *Shigella dysenteriae* (Gram -ve).

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