GC-MS Analysis and Antimicrobial Effects of Methanol Stem Bark Extract of *Trilepisium madagascriense* DC

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ABSTRACT: The therapeutic potentials of methanol stem bark extract of *Trilepisium madagascriense* was determined using Gas Chromatography-Mass Spectrometry to identify its bioactive compounds of pharmaceutical importance while the antimicrobial activities were assayed *in vitro* by agar well diffusion and macrobroth dilution techniques against different microbial isolates. The mass spectra of the identified compounds in the extract at different retention time showed the presence of ethyl iso-allocholate, (3β,5Z,7E)-9,10-Secocholesta-5,7,10(10)-triene-3,24,25-triol, 2,6-Dimethoxyamphetamine, 4-Hexenoic acid, 4-methyl-6-(fluorodimethylsilyl)-6-trimethysily-, 2-methoxy-4-(methoxymethyl)-Phenol, 2-methoxy-1,4-Benzenediol, 2,4-Dimethoxyphenol, Indole, Paromomycin, Hydroquinone and Tetrahydro-N-[(tetrahydro-2-furanyl)methyl-2-Furanmethanamine amongst other bioactive compounds of therapeutic potentials. This extract showed antimicrobial activities. At the lowest concentration of 25 mg/ml, 100 μl of the extract produced inhibition zones ranging between 14 and 18 ± 1.0 mm and inhibition zones ranging between 18 and 28 ± 1.0 mm in all the isolates at the highest concentration of 100 mg/ml. While the bacterial MICs ranged between 1.25 and 5 mg/ml and the MBCs ranged between 2.5 and 10 mg/ml, the fungal MICs ranged between 0.098 and 12.5 mg/ml while the MFCs ranged between 0.781 and <25 mg/ml. With exception of MIC_{index} of *Klebsiella pneumoniae* ATCC 10031 which was equal to 4, the MIC_{index} of other isolates ranged between 1 and 2. *Klesbiella pneumoniae* ATCC 10031 and *Proteus vulgaris* CSIR 0030 had the highest MICs of 1.25 mg/ml, followed by *B. cereus* ATCC 10702, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 19582, *Enterococcus faecalis* ATCC 29212 and *Bacillus subtilis* KZN with MICs of 2.5 mg/ml while *Escherichia coli* ATCC 25922, *Enterococcus cloacae* ATCC 13047, *Enterococcus faecalis* KZN, *Shigella sonnei* ATCC 29930, *Klebsiella pneumoniae* KpFa, *Staphylococcus aureus* SaFa, *Escherichia coli* EcFa and *Pseudomonas aeruginosa* PmFa had the least MICs of 5.0 mg/ml. *Enterococcus faecalis* KZN, *Bacillus subtilis* KZN and *Proteus vulgaris* CSIR 0030 had the highest MBCs of 2.5 mg/ml. Although *Candida albicans* had MICs ranging between 0.098 mg/ml and *Candida tropicalis* had the least MICs of 12.5 mg/ml, the MFCs were 0.781 mg/ml and 25 mg/ml. This study shows that the pharmacological effects of *Trilepisium madagascriense* depends on bioactive compounds identified while this plant is a source for isolating novel drugs having significant therapeutic potentials.

Keywords: Antimicrobial, bioactive phytoconstituents, betulin, paromomycin, pharmacological effects

INTRODUCTION

Before the introduction of chemical medicines, man relied on the healing properties of medicinal plants (Ahvazia *et al.,* 2012), which took the form of crude drugs such as tea, poultices, powders and other herbal formulations (Samuelsson and Bohlin, 2004). The particular plant to be used and the mode of application for particular ailments were passed down through oral history (Balunas and Kinghorn, 2005). An estimated 80 percent (4.8 billion people) of the world's population depend on medicinal plants for health care because they are generally free or readily available at very affordable cost (Listorti and Doumani, 2001). The medicinal qualities of plants are of course due to chemicals. Plants synthesize many compounds called primary metabolites that are critical to their existence. These includes proteins, fats and carbohydrates that serve a variety of purposes indispensable for sustenance and reproduction, not only for the plants themselves, but

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also for animals that feed on them. Plants also synthesize a dazzling array of additional components, called secondary metabolites, whose function has been debated. Many secondary metabolites are "antibiotic" in a broad sense, protecting the plants against fungi, bacteria, animals and even other plants (Small and Catling, 1999). Drug discovery from medicinal plants led to the isolation of early drugs such as cocaine, codeine, morphine, digitoxin and quinine from *Erytheoxylon coca* leaves, *Opium poppy* latex and *Digitalis* leaves respectively (Balunas and Kinghorn, 2005).

In recent years, antimicrobial drug resistance in human pathogenic bacteria has been commonly reported from all over the world (Doughari, 2012). In developing and developed countries, this situation is alarming due to prolong and indiscriminate use of antibiotics. This has resulted in the development of multidrug resistance (Alp, 2007; Tiwari *et al.,* 2014) in microbes especially bacteria such as methicillin resistant *Staphylococcus aureus*, *Helicobacter pylori* and MDR *Klebsiela pneumoniae* (Upadhyay *et al.,* 2014). Therefore, alternative antimicrobial strategies are urgently needed as the efficacy of antibiotics is now significantly limited and, thus, has led to a re-evaluation of the therapeutic use of ancient remedies such as plants (Chowdhury et al., 2013).

Secondary metabolites which are low molecular weight compounds have provided numerous pharmaceutical agents. While their synthesis is not normally required for growth, development or reproduction of plants (Harborne, 1984; Rosenthal, 1991), they protect plants from any possible harm in the ecological environment (Stamp, 2003) and other interspecies predation (Samuni-Blank *et al.,* 2012). They, also, protect plants from disease and damages as well as contributing to the plant's colour, aroma and flavour (Saxena *et al.,* 2013). Phytochemicals which are primary and secondary compounds include terpenoid, alkaloids, tannins and phenolic compounds exhibiting various important pharmacological activities (Wadood *et al.,* 2013). Though phytochemical screening is a process of tracing plant constituents, it is of paramount importance to qualitatively and quantitatively identify new source of therapeutically and industrially valuable compounds having medicinal values (Geetha and Geetha, 2104). Thus, various phytochemical analyses have been used for the identification of specific phytochemicals such as furfural, catechol, indole and benzoic acid (Doughari, 2012; Gracelin *et al.,* 2013). Of the quantitative phytochemical analyses, Gas Chromatography-Mass Spectrometry (GC-MS) analysis is of importance. Gas chromatography separates the components of a mixture and mass spectrometry characterizes each of the components

individually (Bull, 2008). Literature search reveals that most of the predominant compounds identified in such analysis in different crude extracts are biologically active molecules (Kar *et al.,* 2007; Cock *et al.,* 2009; Lucantoni *et al.,* 2010; Hossain and Nagooru, 2011).

Trilepisium madagascriense D.C. Leeuwenberg (Moraceae), also known as Urnfig or False-fig is a forest tree belonging to the Moraceae family that grows to a height of about 30 m and is usually found in riverine ground, lowlands and sub-mountainous forest in Tropical Africa. It is propagated by bud grafting and its leaves are domestically consumed as vegetable (Ampa *et al.,* 2013). The stem bark is traditionally used to treat venereal diseases, arthritis, rheumatism, diarrhoea and dysentery, while the roots are used against cutaneous and subcutaneous parasitic infections (Teke *et al.,* 2010). Pharmacologically, while the methanol extract from the leaves of *T. madagascariense* was reported to inhibit the growth of *Staphylococcus aureus* (Sabrina *et al.,* 2006) and the ethanol extract of the leaves showed antidiabetics properties (Ampa *et al.,* 2013), isoliquiritigenin, a compound isolated from ethyl-acetate fraction of the methanol stem bark extract of this plant was indicated to possess antidiarrheal activities (Teke *et al.,* 2010). Also, trilepisflavan, dihydrokaemferol and 8-C-glucopyranosylapigenin compounds isolated from this plant have been reported to possess significant antimicrobial properties (Ango *et al.,* 2012). The methanol extract of the stem bark was also reported to possess antifungal activity against *Candida guillermondi* (Kuiate, 2011). To identify and enumerate the bioactive compounds of therapeutic value of *T. madagascriense*, this study was aimed at investigating the phytochemical constituents and antimicrobial properties of the stem bark methanol extract of *T. madagascriense* D.C.

MATERIALS AND METHODS
Collection and authentication of plant sample
Stem bark of *Trilepisium madagascriense* was collected from Ilishan, Ogun state, Nigeria, on the 17th of April, 2015. The samples collected were identified and authenticated ethnobotanically and confirmed by Dr. O.O. Olajuyigbe at the Department of Microbiology, Babcock University, Nigeria while the specimen voucher is being prepared.

Extract preparation
The bark sample was air-dried at room temperature, pulverized with a milling machine and extracted as described by Olajuyigbe and Afolayan, (2012). Briefly, exactly 200 g of the pulverized sample was extracted with 1000 ml of methanol for 72 h with continuous agitation (Stuart Scientific Orbital Shaker,

Staffordshire, UK). The extract was filtered through Whatman No. 1 filter paper and concentrated under reduced pressure at 40°C using a rotary evaporator (Laborota 4000–efficient, Heldolph, city, Germany). The extraction was done for two more consecutive times. The crude extract collected was dried at room temperature to a constant weight. The extract was later dissolved in methanol to the required concentrations for bioassay analysis. The reconstituted extract solution was filtered through 0.45 μm membrane filter and tested for sterility after membrane filtration by introducing 2 ml of the extract in 10 ml of sterile nutrient broth before being incubated at 37°C for 24 h. A sterile extract was indicated by the absence of turbidity in the broth after the incubation period.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis of methanol extract
The GC-MS analysis was carried out using GC-MS-QP 2010 Plus Shimadzu system and Gas chromatograph interfaced to a mass spectrometer instrument employing the following conditions: Column Elite-1 fused silica capillary column (30m x 0.25mm 1D x μl df, composed of 100% dimethyl polysiloxane). For GC-MS operation, an electron ionization system with ionization energy of 70eV was used. Helium gas (99.99%) was used as the carrier gas at constant flow rate 1 ml/min and an injection volume of 2 μl was employed (Split ratio of 10:1) injector temperature - 250°C; ion-source temperature 280°C. The oven temperature was programmed from 110°C (Isothermal for 2 min) with an increase of 10°C/min to 200°C then 5°C/min to 280°C/min, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 550Da. Total GC running time was 60 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total area. Software adopted to handle mass spectra and chromatogram was a Turbo-Mass-OCPTVS-Demo SPL software. The identification of the compounds was based on the comparisons of their mass spectra with NIST Ver. 2.0 year 2008 library WILEY8, FAME (Sriranmsridharan, 2011).

Test organisms and inocula preparation
The bacteria used in this study included *Enterococcus faecalis* ATCC 29212, *Klebsiella pneumoniae* ATCC 10031, *Bacillus subtilis* KZN, *Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 10702, *Pseudomonas aeruginosa* ATCC 19582, *Escherichia coli* ATCC 25922, *Proteus vulgaris* CSIR 0030, *Enterobacter cloacae* ATCC 13047 and *Shigella sonnei* ATCC 29930. The clinical bacterial isolates include *Enterococcus faecalis* KZN, *Klebsiella pneumoniae* KpFa, *Staphylococcus aureus* SaFa,

Escherichia coli EcFa, *and Pseudomonas aeruginosa* PmFa. The clinical fungal isolates used include *Candida albicans* CA4, *Candida tropicalis* CT4, *Candida albicans* CA15, *Candida albicans* CA6 and *Candida albicans* CA23. Each bacterial isolate was maintained on nutrient agar slants and was recovered for testing by growth in nutrient broth for 24 h while the fungal isolates were maintained on Saboraud dextrose agar slants and was recovered by growth in Saboraud dextrose broth for 72h. Bacteriologically, each of the clinical strains was streaked on nutrient agar which was incubated overnight at 37°C for 24 – 48 h (Forbes *et al.,* 2007). These isolates were subjected to Gram staining, microscopic appearance, colony morphology and biochemical tests such as tube coagulase test according to standard protocols (Holt *et al.,* 1994; Cheesbrough, 2002; Cheesbrough, 2009). The inocula of the test isolates were prepared using the colony suspension method (EUCAST, 2000). Colonies picked from 24 h old cultures grown on nutrient agar were used to make suspensions of the test organisms in saline solution to give an optical density of approximately 0.1 at 600 nm. The suspension was then diluted 1:100 by inoculating 9.9 ml of sterile nutrient broth with 100 μl of the bacterial suspension and thoroughly agitated before being used.

Antimicrobial assay by agar diffusion (inhibition zones) methods
For the initial determination of the antibacterial activity of the crude methanol extract of *Trilepisium madagascriense*, the susceptibility screening of the test bacteria to the extract was determined by using the modified Kirby-Bauer diffusion technique (Cheesbrough, 2002). This method involves swabbing Mueller-Hinton agar (Lab M Ltd, Quest Park, Lancashire, UK) plates with the resultant saline suspension of each adjusted bacterial strain. For the antifungal assay, 1 cm² of seven day old fungal cultures was dropped in sterile distilled water and vortexed for 2 min to release the fungal spores. Saboraud dextrose agar plates were seeded with 200 μl of the fungal spore solutions, allowed to stand for 1 h on the laboratory bench. Wells, later filled with 100 μl of different concentrations of the extract (20 mg/ml, 40 mg/ml, 60 mg/ml, 80 mg/ml, and 100 mg/ml) taking care not to allow spillage of the solutions onto the agar surface, were then bored into the agar medium with a heat sterilized 6 mm cork borer. The culture plates were allowed to stand on the laboratory bench for 1 h to allow proper diffusion of these solutions before being incubated at 37°C for 24 h and 27°C for 72 h for fungal cultures. Wells in blank Mueller Hinton agar containing 10% methanol representing the final concentration of the methanol in the test plates without the extract served as positive control. The determinations were done in duplicates.

After 24 h of incubation, the plates were examined for the presence of inhibition zones. The diameters of the inhibition zones produced by each concentration of each of the extract were measured in millimetres (Wikler, 2007) and interpreted using the Clinical and Laboratory Standard Institute Zone diameter interpretative standards (Wikler, 2008).

Macrobroth dilution for determining minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) defined as the lowest concentration which resulted in maintenance or reduction of inoculums' viability was determined by serial tube dilution technique (Iwaki *et al.,* 2006; Khan *et al.,* 2007) for each of the test isolates. Different concentrations ranging from 0.039 mg/ml to 20 mg/ml of the extract were prepared by serial dilutions in double strength Mueller Hinton broth medium for the bacterial isolates while concentrations for the determination of the minimum inhibitory concentrations of the fungal isolates were serially diluted in Saboraud dextrose broth. Each tube was then inoculated with 100 μl of each of the adjusted bacterial and fungal strains. Two blank Mueller Hinton broth tubes and two blank Saboraud dextrose broth tubes, with and without bacterial and fungal inoculation, were used as the growth and sterility controls. The bacteria containing tubes were incubated at 37°C for 24 h. The fungal containing tubes were incubated at 27°C for 72 – 96 h. After the incubation period, the tubes were observed for the MICs by checking the concentration of the first tube in the series of tubes that showed no visible trace of growth. The first tubes in the series with no visible growth after the incubation period were taken as the MICs.

Determination of minimum bactericidal concentrations (MBC)

Since the clinical occurrences of tolerance usually necessitate bactericidal testing, the MBC was determined by sampling all the macroscopically clear tubes and the first turbid tube in the MIC series. Before being sampled, the tubes were gently mixed by flushing them with a sterile pipette, and a 100 μl aliquot was removed. Each aliquot of the bacterial isolates was placed on a single antibiotic-free nutrient agar plate in a single streak down the centre of the plate and Saboraud dextrose agar was used for the fungal isolates, in accordance with the method of Shanholtzer *et al.* (1984). The samples were allowed to be absorbed into the agar until the plate surface appeared dry (after 30 min). The aliquot was then spread over the plate by making a lawn of the bacterial and fungal culture with sterile cotton swab. In many studies on microbial susceptibility, this subculturing method has been found satisfactory in

eliminating the problem of antimicrobial agent carryover from the 100 μl subcultured volume (Bamberger *et al.,* 1986; Moody *et al.,* 1987; Fasching *et al.,* 1990). The growth and sterility controls were sampled in the same manner. The MBC determining lawned plates were incubated for 24 h at 37°C for bacterial isolates and 27°C for 72 – 96 h for the fungal isolates. After the incubation periods, the lowest concentrations of the extract that did not produce any bacterial or fungal growth on the solid medium were regarded as the MBC and minimum fungicidal concentration (MFC) values for this extract (Irkin and Korukluoglu, 2007). This observation was matched with the MIC test tube that did not show evidence of growth after 48 h of incubation.

Determination of mechanisms of antibiosis (bactericidal or bacteriostatic)

The mechanism of antibiosis of the extract was calculated using the ratio of MBC/MIC or MFC/MIC or MIC_{index} as described by Shanmughapriya *et al.* (2008) to elucidate whether the observed antibacterial effects were bactericidal or bacteriostatic. When the ratio of MBC/MIC or MFC/MIC was ≤ 2.0, the extract was considered bactericidal/fungicidal or otherwise bacteriostatic/fungistatic. If the ratio is ≥16.0, the extract was considered ineffective.

In this study, Gas Chromatography-Mass Spectrometry (GC-MS) analysis was used to identify the bioactive compounds of pharmaceutical importance in methanol stem back extract of *T. madagascriense* and the phytoconstituents in the extract is as shown in Table 1. The chromatogram indicating different phytoconstituents of methanol extract is shown in Figure 1.

TABLE 1: Phytoconstituents of the methanol stem bark extract of *T. madagascriense*

PEAK	RETENTION TIME	AREA %	COMPOUND	QUALITY	ACTIVITY
1	3.401	0.69	Dimethylamine	53	Antitumor
2	4.339	0.82	1H-Imidazole	35	Antifungal
3	4.951	1.83	2 – Furanmethanol	64	Hepatoprotective and Cytotoxic
4	6.031	0.79	Dihydroxyacetone	40	Antioxidant and Anti-proliferative
5	6.060	0.63	Dihydroxyacetone	40	Antioxidant and Anti-proliferative
6	9.153	0.67	N- (n- Propyl) Acetamide	46	Anticancer, Anti-inflammatory
7	11.764	1.66	Acetamide	27	Anticancer and Anti-inflammatory
8	12.453	0.76	Cyclohexanol	27	Anticancer and Antidepressant
9	12.850	0.63	Methyl 2 –furoate	52	Anti-protozoal and Anti-inflammatory
10	13.147	6.01	3 – Methyl – 2 - Furoic acid	50	Cytotoxic and antitumor
11	3.901	8.96	4 – Chlorolysine	53	Analgesic and antiarrhythmic
12	14.720	0.82	Ethanamine	42	Antimicrobial
13	14.940	9.45	4H – Pyran - 4 – one	91	Antifungal, Anti-diabetic, Antioxidant, Antibacterial and Anti-inflammatory
14	15.628	0.97	2 (3H) – Furanone	64	Anti-inflammatory, Analgesic, Antiviral Anticonvulsant, Cytotoxic Antibacterial, and Antioxidant
15	16.368	1.15	Ethanedial	46	Antifungal, Anti-diabetic, Antioxidant, Antibacterial and Anti-inflammatory
16	16.584	0.92	2 (1H) – Pyridinone	81	Antitumor, Anti-inflammatory, Antibacterial and Anti-fibrotic
17	17.070	1.44	Catechol	64	Antioxidant, Anti-platelet, Antibacterial, Anti-helmithic and Antifungal
18	17.504	1.80	Catecholborane	49	Antibacterial, Antifungal, Anti-HIV, Analgesic, Antidepressant, Anticonvulsant, Anti-diabetes, Antitumor, Anti-tubercular, Antioxidant and Anti-inflammatory
19	17.765	12.26	5 - Hydroxymethylfurfural	96	Antioxidant, Anti-proliferative, Antibacterial, Antifungal and Hepatoprotective
20	18.168	1.37	Hexanoic acid	38	Antifungal
21	19.634	4.82	Indole	95	Anti-inflammatory, Antipyretic
22	19.836	0.69	N – Dimethylaminomethyl – N- methylform amide	38	Anti-inflammatory, Antiviral and Anti-malarial
23	20.210	3.38	Ethanone	46	Antioxidant, Antiepileptic, Anticonvulsant, Anti-inflammatory, Anticoagulant, Antiplasmodial and Antimalarial
24	21.296	3.10	Phenol	96	Antioxidant, Detoxifying agent, Antibacterial, Anti-inflammatory and Antifungal
25	21.439	0.90	Hexane	18	Antibacterial and Antifungal
26	22.596	2.36	Vanillin	90	Antioxidant, Antidepressant and Anti-inflammatory
27	22.685	7.62	1, 4 – benzenediol	94	Antioxidant, Antibacterial and Antifungal
28	23.439	0.89	Pyrrolidinium	43	Anti-bacteria
29	23.528	3.88	Phenol	95	Antioxidant, Detoxifying agent, Antibacterial, Anti-inflammatory and Antifungal
30	24.816	0.86	Benzonitrile	41	Antimicrobial, Anti-tubercular, antifungal, Antihistamine, Analgesic, Anti-helmintic and Antipsychotic
31	25.309	1.61	Acetaldehyde semicarbazone	50	Antifungal, Antibacterial, Antimalaria, Antiviral, Anticancer and Anticonvulsant
32	25.742	0.77	Benzoic acid	46	Antimicrobial
33	26.122	2.02	Homovanillyl alcohol	95	Antioxidant and Antiplatelet
34	26.413	0.67	Ethanone	50	Antioxidant, Antiepileptic, Anticonvulsant, Anti-inflammatory, Anti-malaria, Anti-plasmodial and Anticoagulant
35	27.374	1.07	Silane	58	Antioxidant and Antimicrobial
36	28.104	3.28	Phenol	95	Antioxidant, Detoxifying agent, Antibacterial, Anti-inflammatory and Antifungal
37	28.953	0.71	7 (3H, 7H) – dione	35	Antioxidant, Anticancer, Antibacterial and Antifungal
38	31.018	1.04	4 – ((1E) – 3 – Hydroxy – 1 – propenyl) – 2 -methoxyphenol	97	Antimicrobial, Anti-tubercular, Anti-inflammatory, Analgesic and Anticonvulsant
39	35.025	0.91	Hexadecanoic acid	95	Antifungal, Antibacterial, Antioxidant, Anti-androgenic, Nematicide, Haemolytic and Hypocholesterolemic
40	45.447	0.61	2, 6 – octadiene	35	Antiplatelet
41	46.296	0.63	Tricyclo [4. 3. 2. 1 (3, 8)] undecane – 1- carboxylic acid	50	Antiplatelet
42	47.085	0.93	Lanosterol	49	Antifungal and suppresses hepatic cholesterol
43	47.204	1.30	Azulene	50	Anti-inflammatory, Antibacterial, Antifungal and Antioxidant
44	47.952	1.09	Benzocyclododecene	47	Anti-inflammatory, Antimicrobial, Antitumor, Antiviral, Anti-hepatitis, and Anticoagulant
45	51.679	1.23	3 (2H) – dione	55	Antioxidant and Anticancer
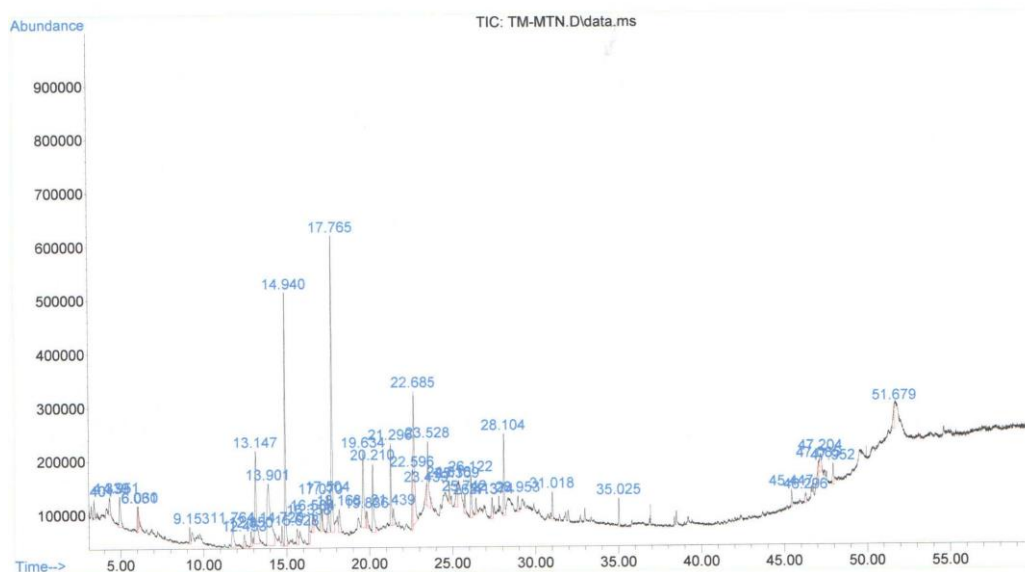
FIG. 1: The chromatograms of phytoconstituents in methanol stem bark extract

Forty five phytoconstituents were identified as being present in the methanol stem bark extract of this plant. Some of these include 2 (1H) – pyridinone, catechol, 1H-imidazole, dihydroxyacetone, N- (n-propyl) acetamide, cyclohexane, catecholborane, 5 – hydroxymethylfurfural, indole, phenol, hexane, vanillin, pyrrolidinium, 4 – ((1E) – 3 – hydroxy – 1 – propenyl) – 2 –methoxyphenol, lanosterol and benzocyclododecene which are of various therapeutic importance. Comparing the mass spectra of the identified compounds in the extract with those in the GC-MS library and determining the mass spectra to identify some of the most significant compounds of therapeutic importance during each retention time showed the presence of ethyl iso-allocholate, paromomycin, indole, phenol and α-D-glucopyranoside in the methanol extract.

The methanolic extract of *T. madagascriense* stem bark obtained showed concentration dependent antibacterial activity against *E. faecalis* ATCC 29212, *K. pneumoniae* ATCC 10031, *B. subtilis* KZN, *S. aureus* ATCC 6538, *B. cereus* ATCC 10702, *P. aeruginosa* ATCC 19582, *E. coli* ATCC 25922, *P. vulgaris,* CSIR 0030, *E. cloacae* ATCC 13047 and *S. sonnei* ATCC 29930, *E. faecalis* KZN, *K. pneumoniae* KpFa, *S. aureus* SaFa, *E. coli* EcFa, *and P. aeruginosa* PmFa. As the concentration increases, the activity increases. At the lowest concentration of 25 mg/ml, 0.1 ml of the extract produced inhibition zones in all the isolates. The inhibition zones ranged between 14 and 18 ± 1.0 mm. At the highest concentration of 100 mg/ml, 0.1 ml of the extract produced inhibition zones ranging between 18 and 28 ± 1.0 mm as indicated in Table 2.

TABLE 2: Antibacterial effects of methanol stem bark extract of *T. madagascriense* by agar diffusion assay

Name of Organism	Inhibition zones produced by 0.1 ml of different concentrations of methanol extract (± 1.0 mm)			
	100 mg/ml	75 mg/ml	50 mg/ml	25 mg/ml
Enterococcus faecalis ATCC 29212	20 ± 1.00	18 ± 1.53	16 ± 0.00	15 ± 0.58
Klebsiella pneumoniae ATCC 10031	20 ± 0.58	18 ± 0.58	16 ± 0.0	15 ± 0.00
Bacillus subtilis KZN	22 ± 1.53	21 ± 1.00	20 ± 1.00	18 ± 0.58
Staphylococcus aureus ATCC 6538	21 ± 0.58	18 ± 0.00	17 ± 0.58	15 ± 0.00
Bacillus cereus ATCC 10702	18 ± 1.53	17 ± 1.00	16 ± 0.58	15 ± 0.00
Pseudomonas aeruginosa ATCC 19582	18 ± 0.00	17 ± 0.00	15 ± 0.58	14 ± 0.00
Escherichia coli ATCC 25922	21 ± 0.58	20 ± 0.00	18 ± 1.53	16 ± 1.00
Enterobacter cloacae ATCC 13047	18 ± 0.58	17 ± 0.58	16 ± 1.00	15 ± 1.00
Enterococcus faecalis KZN	20 ± 1.53	19 ± 1.00	17 ± 0.58	15 ± 1.53
Shigella sonnei ATC C 29930	19 ± 1.00	17 ± 1.00	16 ± 0.58	14 ± 0.00
Klebsiella pneumoniae KpFa	21 ± 0.00	19 ± 0.58	18 ± 1.00	15 ± 0.00
Staphylococcus aureus SaFa	23 ± 0.00	21 ± 1.53	18 ± 0.00	15 ± 1.00
Escherichia coli EcFa	21 ± 0.58	18 ± 1.53	15 ± 0.58	14 ± 0.00
Proteus vulgaris CSIR 0030	23 ± 0.58	20 ± 1.00	17 ± 0.00	15 ±0.00
Pseudomonas aeruginosa PmFa	28 ± 0.58	24 ± 1.53	20 ± 0.58	18 ± 0.58

The inhibitory and bactericidal activities of the extract of *T. madagascriense* against the different test bacterial strains are presented in Table 3 with the minimum inhibitory concentrations (MICs) ranging between 1.25 and 5 mg/ml while the MBCs ranged between 2.5 and 10 mg/ml. With exception of MIC_{index} of *K. pneumoniae* ATCC 10031 which was equal to 4, the MIC_{index} of other isolates ranged between 1 and 2. *K. pneumoniae* ATCC 10031 and *P. vulgaris* CSIR 0030 had the highest MICs of 1.25 mg/ml. These were followed by *E. faecalis* ATCC 29212, *B. subtilis* KZN and *P. vulgaris* CSIR 0030 with MICs of 2.5 mg/ml. While *E. coli* ATCC 25922,

E. cloacae ATCC 13047, *E. faecalis* KZN, *S. sonnei* ATCC 29930, *K. pneumoniae* KpFa, *S. aureus* SaFa, *E. coli* EcFa and *P. aeruginosa* PmFa had the least MICs of 5.0 mg/ml. *E. faecalis* KZN, *B. subtilis* KZN and *P. vulgaris* CSIR 0030 had the highest MBCs of 2.5mg/ml. These were followed by *K. pneumoniae* ATCC, *S. aureus* ATCC, *B. cereus* ATCC, *P. aeruginosa* ATCC, *S. aureus* SaFa and *P. aeruginosa* PmFa having 5.0 MBCs while *E. coli* ATCC, *E. cloacae* ATCC, *E. faecalis* KZN, *K. pneumoniae* KpFa, and *E. coli* EcFa had the least MBCs of 10 mg/ml.

TABLE 3: Minimum inhibitory & minimum bactericidal concentrations of methanol extract of *T. madagascriense* against different bacterial pathogens in dysentery

Name of Organisms	Inhibition and bactericidal concentrations of the methanolic extract			
	MIC (mg/ml)	MBC (mg/ml)	MBC / MIC	Remarks
Enterococcus faecalis ATCC 29212	2.5	2.5	1	Bactericidal
Klebsiella pneumoniae ATCC 10031	1.3	2.5	2	Bactericidal
Bacillus subtilis KZN	2.5	2.5	1	Bactericidal
Staphylococcus aureus ATCC 6538	2.5	5.0	2	Bactericidal
Bacillus cereus ATCC 10702	2.5	5.0	2	Bactericidal
Pseudomonas aeruginosa ATCC 19582	2.5	5.0	2	Bactericidal
Escherichia coli ATCC 25922	5.0	10.0	2	Bactericidal
Enterobacter cloacae ATCC 13047	5.0	10.0	2	Bactericidal
Enterococcus faecalis KZN	5.0	10.0	2	Bactericidal
Shigella sonnei ATCC 29930	5.0	10.0	2	Bactericidal
Klebsiella pneumoniae KpFa	5.0	10.0	2	Bactericidal
Staphylococcus aureus SaFa	5.0	5.0	1	Bactericidal
Escherichia coli EcFa	5.0	10.0	2	Bactericidal
Proteus vulgaris CSIR 0030	1.3	2.5	2	Bactericidal
Pseudomonas aeruginosa PmFa	5.0	5.0	1	Bactericidal

The methanolic extract of *T. madagascriense* stem bark obtained showed antifungal activity against *C. albicans* CA4, *C. tropicalis* CT4, *C. albicans* CA15, *C. albicans* CA6 and *C. albicans* CA23 as shown in Table 4. The antifungal activity of the methanolic extract is concentration dependent. As the concentration increases, the activity increases. At the lowest concentration all fungal specimen were susceptible with the exception of *C. albicans* CA15. However, fungal isolates inhibited at the different concentrations had inhibition zones ranging between 17 and 25 ± 1.0 mm at the highest concentration of 100 mg/ml and between 12 and 19 ± 1.0 mm at the lowest concentration of 25 mg/ml.

TABLE 4: Antifungal effects of methanol extract of *T. madagascriense* by agar diffusion assay

Name of Organism	Inhibition zones produced by 100 μl of different concentrations of methanol extract (± 1.0 mm)			
	100 mg/ml	75 mg/ml	50 mg/ml	25 mg/ml
Candida albicans CA4	25	23	20	18
Candida tropicalis CT4	23	22	20	18
Candida albicans CA15	17	15	14	6
Candida albicans CA6	18	15	14	12
Candida albicans CA23	24	23	20	19

The minimum inhibitory concentrations (MICs) for the fungal isolates are shown in Table 5. The MICs of the fungal isolates ranged between 0.098 and 12.5 mg/ml while the MFCs ranged between 0.781 and <25 mg/ml for the methanolic extract of *T. madagascriense*. *C. albicans* CA23 had the highest MIC of 0.098 mg/ml, followed by *C. albicans* CA4 with MIC of 0.391 mg/ml and *C. albicans* CA6 with MIC of 1.562 mg/ml. *C. tropicalis* CT4 and *C.*

albicans CA15 had the least MICs of 12.5 mg/ml. *C. tropicalis* CT4 and *C. albicans* CA15 had the least MFCs of 25 and <25 mg/ ml while *C. albicans* CA23 had the highest MFC of 0.781 mg/ml. The MIC$_{index}$ was equal to 2 with the exception of *C. albicans* CA4, *C. tropicalis* CT4 and *C. albicans* CA23 for which MIC index was 15.985, <2 and 7.969 respectively.

TABLE 5: Minimum inhibitory & minimum fungicidal concentrations of the methanolic extract of *T. madagascriense* against different fungal pathogens

Name of Organisms	Inhibition and fungicidal concentrations of the methanolic extract			
	MIC (mg/ml)	MFC (mg/ml)	MFC/MIC	Remark
Candida albicans CA4	0.391	6.25	15.985	Ineffective
Candida tropicalis CT4	12.5	<25	<2	Fungicidal
Candida albicans CA15	12.5	25	2	Fungicidal
Candida albicans CA6	1.562	3.125	2	Fungicidal
Candida albicans CA23	0.098	0.781	7.969	Fungicidal

The plant used in this study, *T. madagascriense,* has been ethnobotanically indicated to be used in the treatment of various diseases in traditional medicine. The stem bark and the leaves are the preferred part for the preparation of traditional medicine (Ampa *et al.,* 2013; Teke *et al.,* 2010). The GC-MS analysis of the methanol stem bark extract of *T. madagascriense* indicated the presence of various phytochemicals, in this study, with antimicrobial, antiviral, antitumor, anticancer, anti-inflammatory, antidotal, antidepressant, anti-proliferative, antioxidant, anticonvultant, analgestic, cytotoxic, antifibrotic, antitubercular, antidiabetic, antiplatelet, antipyretic, antiplasmodial, hypercholesterolemic and anticoagulant activities. The antimicrobial activities of the extract recorded in this study can be attributed to paromomycin, indole, ethyl iso-allocholate, phenol and α-D-glucopyranoside which were part of the most significant therapeutic compounds identified during each retention time.

Paramomycin, indole, ethyl iso-allocholate and phenol have been reported to possess strong antimicrobial properties (Sundar and Chang, 1993; Meyer *et al.,* 1999; Huang *et al.,* 2011). Paramomycin which was originally isolated from the fermentation of *Streptomyces* in 1956 (Lu *et al.,*

1997) has been reported to possess broad spectrum activity against Gram-negative and Gram-positive bacteria (Stead, 2000) such as *E. coli*, *S. aureus*, *S. pyogenes*, *P. vulgaris* and *K. Pneumoniae* (Francois *et al.,* 2004) as well as some protozoa and cestodes (Davidson *et al.,* 2009). In humans, paromomycin has been used to treat leishmaniasis, cryptosporidiosis, and amebiasis (Flanigan *et al.,* 1996; Fahey, 2003; Murray *et al.,* 2005). Its presence in this plant is of significance. This is the first time it will be identified in a medicinal plant as it has not being reportedly found in medicinal plants prior now as evident from literature search. Indole which has been isolated from the stem bark ethanol extract of *Aspidosperma ramiflorum* (Tanaka *et al.,* 2006), stem bark methanol extract of *Aspidosperma olivaceum* (Chierrito *et al.,* 2014) and stem bark ethanol extract of *Winchia calophylla* (Zhu *et al.,* 2005) has been reported to have antibacterial effects against *S. aureus*, *E. coli* and *P. aeruginosa* (Medeiros *et al.,* 2011). Ethyl iso-allocholate which have been isolated from the ethanol extract of *Podophyllum hexandrum* rhizome (Li *et al.,* 2012), ethanol extract of *Kirganelia reticulate* aerial parts (Muthulakshmi *et al.,* 2012) and ethanol extract of *Feronia elephantum* stem bark (Muthulakshmi *et al.,* 2012 Sudha *et al.,* 2013) have been reported to possess antimicrobial activities

(Singariya *et al.,* 2012). Phenol has been isolated from the methanolic extract of *Ficus religiosa* stem bark (Manorenjitha *et al.,* 2013), and methanol extract of *Psidium guajava* root bark (Velmurugan *et al.,* 2012). Phenol bactericidal actions at high concentration include penetration and disruption of cell wall and precipitation of cell proteins while at low concentrations it causes bacteria death by inactivation of essential enzyme systems and leakage of essential metabolites from the cell wall (Prindle, 1983). It has been reported to possess antimicrobial activities against *E. coli, S. aureus, P. aerugimosa* and *C. albicans* (Cueva *et al.,* 2010). α-D-glucopyranoside, a glucopyranose which has been isolated from the aqueous extract of *Tulbaghia violacea* (Lyantagaye *et al.,* 2013), has been reported to possess antimicrobial activity against *B. cereus, S. sonnei, S. aureus, B. subtilis, S. typhi* and *Macrophomina phaseolina* (Kawsar *et al.,* 2014). The antimicrobial activities of *T. madagascriense* could, therefore, be attributed to the presence of these phytochemicals in its methanol stem bark extract.

Previous study on the methanol stem bark extract of this plant indicated the isolation of isoliquiritigenin, a flavonoid and vanillin (Teke *et al.,* 2011), trilepisflavan, trilepisuimic acid, dihydrokaempferol and 8-C-glucopyranosylapigenin (Ango *et al.,* 2012). These phytoconstituents, except vanillin which was indicated in the GC report of the methanol extract, were all not identified in the GC-MS analysis while α-D-glucopyranoside, which is a glucopyranose like 8-C-glucopyranosylapigenin, was identified in the mass spectrogram of the methanol extract of *T. madagascriense* in this study. This variation could be as a result of differences in geographical locations, maturity and time of harvesting as well as methods of identification while the former was carried out on plant harvested in Dschang, Cameroun.

Anti-diarrheal and anti-dysenteric activities of medicinal plants have been attributed to the presence of tannins, alkaloids, saponins, flavonoids, steroids and terpenoids (Havagiray *et al.,* 2004). Alkaloids, flavonoids, tannins and saponins were previously detected in the phytochemical analysis of the methanol extract of this plant (Teke *et al.,* 2010), this explains the activity of the plant extract against diarrhoea and dysentery causing organisms used in this study. All selected bacterial strains used in this study were susceptible to the methanol extract of the plant. This may be due to the extraction ability of methanol as it is known to be a junk extractor and whose plant extracts have been observed to be very effective against most bacteria strains . It could also be due to the amount of phytochemicals being extracted. The degree of susceptibility did not depend on the fungi and bacteria class as both Gram-positive and Gram-negative strains were susceptible to the methanol extract. This indicated that the plant extract has broad spectrum of antimicrobial capabilities. *E. coli* EcFA, *E. faecalis* KZN, *K. pneumoniae* and *P. aeruginosa*, though multidrug resistant strains were susceptible to all concentrations of this extract. The ability of this extract to inhibit the growth of the multidrug resistant clinical isolates showed the importance of this plant in treating multi-drug resistant infections and nosocomial infections. Iinuma *et al.* 1994, Kim *et al.* 1995, Aburjai *et al.* 2001 and Darwish *et al.* 2002 indicated that many plants are known for their action as resistance-modifying agents.

Using turbidity as a measure of growth, the MIC and MBC showed that the methanol extract of this plant was both bacteriostatic and bactericidal. The MIC and MBC values are often close or hooked values, however, MBC values can either be the same or higher than the corresponding MIC values (Olajuyigbe and Afolayan, 2011). The MIC$_{index}$ showed that the methanol extract was bacteriostatic at lower concentration and bactericidal at higher concentration. The MICs of the methanol extract of *Thuja orientalis* was higher (2.00 mg/ml and 1 mg/ml) than the methanol extract of *T. madagascriense* against *P. aeruginosa* and *S. aureus,* 2.5 mg/ml and 2.5 mg/ml respectively, while *T. madagascriense* MIC was higher against *K. pneumonia* (1.25 mg/ml) than that of *Thuja orientalis* (1.5 mg/ml) (Duhan *et al.,* 2013). This shows that methanol extract of *T. madagascriense* could be more effective than those of other medicinal plants in the treatment of infections caused by the susceptible bacterial isolates.

All the fungal strains used in this study were susceptible to the extract of this plant at varied concentration and indicated that the extract has strong antifungal properties. The MIC and MFC showed that the methanol extract of the plant was both fungistatic and fungicidal. The MIC$_{index}$ showed that the extract was fungistatic at lower concentration and fungicidal at higher concentration. The antifungal (anticandidiasis) activities of the methanol extract of *T. madagascariense* (0.098 – 12.5 mg/ml) were higher than that of the methanol extract of *Lupinus varius* (32 mg/ml) against *Candida albicans*. The antifungal (anticandidiasis) activities of the extract of *T. madagascariense* producing inhibition zones ranging between 17 and 25 ± 1.0 mm was higher than that of the methanol extract of *Ecbalium elaterium* (16 ± 1.0 mm) and *Mandragora autumnalis* (18.7 ± 1.0 mm) at 100 mg/ml of each extract (Obeidat *et al.,* 2012).

In conclusion, this study identifying phytoconstituents of therapeutic importance and antimicrobial activities of *T. madagascriense*, used in the treatment of various ailments by the local populations, indicated the therapeutic potentials of this plant scientifically and justify its ethnomedicinal use in the treatment of diarrhoea and dysentery. Of the bioactive compounds identified through GC-MS analysis, the mass spectrogram showed the presence of paramomycin not yet reportedly present in any medicinal plant. Further research on the isolation and characterization of these bioactive compounds of economic, pharmaceutical and therapeutic values from *T. madagascriense* is ongoing in our research laboratory.

CONFLICTS OF INTEREST
The authors declared no conflict of interest.

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