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Research Article
Bioactive Compounds and *in vitro* Antimicrobial Activities of Ethanol Stem Bark Extract of *Trilepisium madagascariense* DC.

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Abstract
Background and Objective: *Trilepisium madagascariense* DC. is a medicinal plant used in the treatment of gastrointestinal infections in Nigeria. The aim of the study was to investigate the antimicrobial activities and determine the pharmacologically bioactive compounds present in this plant to justify its traditional folkloric use in treating diarrhoea. **Materials and Methods:** The pharmacologically bioactive compounds in the ethanol stem back extract of *Trilepisium madagascariense* were determined using gas chromatography-mass spectrometry and its antimicrobial activities were assayed *in vitro* by agar well diffusion and macrobroth dilution techniques against different microbial isolates. **Results:** The mass spectra of the identified compounds in the extract at different retention time showed the presence of 1-Heptatriacotanol, Betulin, ethyl ester of hexadecanoic acid, Desulphosinigrin, N-methyl-N-[4-(3-hydroxypyrrolidinyl) -2-butynyl]-acetamine, Paromomycin, 5-hydroxymethylfurfural, 1-4-Hydroxylysine lactone and 5-amino-1H-imidazole-4-carboxamide, amongst other bioactive compounds of therapeutic potentials. The antibacterial activities showed that the inhibition zones ranged between 20 and 30±1.0 mm at the highest concentration and the antifungal activities against the fungal isolates ranged between 15 and 25±1.0 mm. Although the Minimum Inhibitory Concentrations (MICs) ranged between 1.25 and 5.0 mg mL^{-1} and the minimum bactericidal concentrations (MBCs) was between 5.0 and 10.0 mg mL^{-1} against the bacterial isolates, the MICs and the minimum fungicidal concentrations (MFCs) ranged from 1.563-6.25 mg mL^{-1} against the fungal isolates. **Conclusion:** Although the clinical isolates were more inhibited by the extract than the bacterial strains used as control, this study showed that the pharmacological effects of *Trilepisium madagascariense* depended on bioactive compounds identified. The presence of paromomycin with antidiarrhoeal activity in the extract justifies the use of this plant in the folkloric treatment of gastrointestinal infections. This plant is, therefore, a significant source for isolating novel drugs having significant therapeutic potentials.

Key words: Antimicrobial, bioactive compounds, pharmacological potentials, *Trilepisium madagascariense*, paromomycin

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Microbial infections occur when pathogenic microorganisms invade and multiply in a host by evading and overcoming the host's natural defense mechanisms. Microbial infections can also be influenced by virulence factors of the invading organism, microbial adherence, biofilm formation, antimicrobial resistance and defects in host defense mechanisms[1]. Bacterial infections can be caused by a wide range of bacteria leading from mild to life threatening illnesses while hospitalized patients and those with chronic diseases are more at risk[2], because immune function is impacted by age, sex, pre-existing diseases and severity[3]. Neonates whose immune systems are underdeveloped are, also, more susceptible to infections caused by various pathogens[4,5]. Neonates may be affected by diarrhoea resulting from an imbalance of absorption and secretion of ions and solute across the gut epithelium, followed by the movement of water in an attempt to restore the appropriate ions concentration as a result of toxin producing bacteria disturbing the organization of the epithelium[6]. Also, while mycosis affects approximately 1-2% of the world's population and *Cryptococcus*, *Histoplasma*, *Coccidioides* and *Blastomyces* associated with certain environmental conditions, can infect humans after heavy exposure[7], it is estimated that candidiasis infects more than 30% of the world population, especially females over the age of twelve[8] and constituted a health crisis since 1980s in, at least, one-third of the population including those with cancer, HIV/AIDS and severe allergies[8,9].

Medicinal plants have certain pharmacological and therapeutic values which are of great importance in the pharmaceutical world[10]. It is estimated that about 75% of useful bioactive plant derive pharmaceuticals used globally are discovered by systematic investigation of leads from traditional medicines[11]. While over one and a half million traditional medicine practitioners use medicinal plants in preventive and curative applications[12], the growing demand for herbal products has led to a quantum jump in volume of plant materials traded within and across the countries[13]. Phenolic compounds, ranging from simple molecules such as phenolic acids to complex compounds such as tannins[14] are one of the most numerous groups of medicinal importance in the plant kingdom. Daily dietary intake of flavonoids from fruits, vegetables, wine tea, chocolate and other cocoa products and similar polyphenols exceeds that of antioxidative vitamins and provitamins[15]. Alkaloids, used as anaesthetic agents are found in medicinal plants[16]. These secondary metabolites of medicinal importance[17]constituted a reservoir of new natural antimicrobial therapeutic substances to be discovered. Therefore, in agreement with previous studies[18-22], quantitative determination of the phytoconstituents and therapeutic values of *Trilepisium madagascariense* (*T. madagascariense*) to justify its ethnomedicinal importance becomes necessary.

Trilepisium madagascariense, also known as false-fig or urn-fig tree, is a medicinal plant growing extensively in the tropical and subtropical West and Central Africa and occurs Southwards to Zimbabwe, Mozambique and South Africa, Madagascar and Annóbon Island[23]. It belongs to the Moraceae family. The tree is rarely cultivated but grows to a medium or large size in primary or secondary forests or in forest patches. It grows in evergreen or deciduous forests, flooded forests or forest patches often growing along rivers and streams, extending toward to the borders of Savannah[24]. It varies from rare to locally abundance. The wood is suitable for furniture and the sap yields red dye. The seeds can be roasted and used as food. Traditionally, the stem bark is used to treat venereal diseases, arthritis, rheumatism, diarrhea and dysentery while the roots are used against cutaneous and subcutaneous parasitic infections[25]. The leaves of *T. madagascariense* have also been shown to have antidiabetic and anti-hyperglycemic activities[26]. To scientifically justify the rationale behind its ethnopharmacological importance, this study aimed at investigating the antimicrobial properties *in vitro* and quantitatively determined the pharmacologically bioactive compounds present in the ethanol extract of *Trilepisium madagascariense*.

MATERIALS AND METHODS

Collection of plant material: The bark materials of *Trilepisium madagascariense* were collected in June, 2014, from the plant growing in a jungle in Ilisan Remo, Ogun State, Nigeria. The plant was authenticated ethnobotanically while the voucher specimen (BUSP/OOLAJ/01) is being prepared and kept at Babcock University. The study was carried out partly in Babcock University and partly in the Mangosuthu University of Technology, South African between June and September, 2016.

Extract preparation: The bark sample was air-dried at room temperature, pulverized with a milling machine and extracted as described by Olajuyigbe and Afolayan[27]. Briefly, exactly 200 g of the pulverized sample was extracted with 1000 mL of ethanol 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, Heidolph, city, Germany). Ethanol was used as

the extracting solvent, in this study, because it is the solvent commonly used traditionally in the preparation of herbal medicine while methanol was used in authors previous study[28] because it was known as junk extractor extracting more phytochemicals than ethanol[29]. The crude extract collected was allowed to dry at room temperature to a constant weight. The extract was reconstituted in the extracting solvent to the required concentrations for the bioassay. The reconstituted extract solution was sterilized by filtering 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 ethanol 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 (30 m×0.25 mm 1D×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 of 1 mL min^{-1} and an injection volume of 2 μL was employed (Split ratio of 10:1) injector temperature -250°C and ion-source temperature 280°C. The oven temperature was programmed from 110°C (isothermal for 2 min) with an increase of 10-200°C min^{-1} then 5-280°C min^{-1}, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV, a scan interval of 0.5 sec and fragments from 40-550 Da. 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[30].

Test organisms and inoculum preparation: The bacteria used in this study included *Enterococcus faecalis* ATCC 29212, *Klebsiella pneumoniae* (*K. pneumoniae*) ATCC 10031, *Bacillus subtilis* (*B. subtilis*) KZN, *Staphylococcus aureus* (*S. aureus*) ATCC 6538, *Bacillus cereus* (*B. cereus*) ATCC 10702, *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC 19582, *Escherichia coli* (*E. coli*) ATCC 25922, *Proteus vulgaris* (*P. vulgaris*) CSIR 0030, *Enterobacter cloacae* (*E. cloacae*) ATCC 13047 and *Shigella sonnei* (*S. 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 Sabouraud dextrose agar slants and was recovered by growth in Sabouraud dextrose broth for 72 h. Bacteriologically, each of the clinical strains was streaked on nutrient agar which was incubated overnight at 37°C for 24-48 h[31]. These isolates were subjected to Gram staining, microscopic appearance, colony morphology and biochemical tests according to standard protocols[32-34]. The inoculum of the test isolates were prepared using the colony suspension method[35]. 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 0.1 mL of the bacterial suspension and thoroughly agitated before being used.

Antibacterial assay by agar diffusion (inhibition zones) methods: For the initial determination of the antibacterial activity of the ethanol extract of *T. madagascariense*, the susceptibility screening of the test bacteria to the extract was determined by using the modified Kirby-Bauer diffusion technique[33] involving swabbing Mueller-Hinton agar (MHA) (Lab M Ltd, Quest Park, Lancashire, UK) plates with the resultant saline suspension of each adjusted bacterial strain. For the antifungal assay, 1 cm^2 of 7 days old fungal cultures was dropped in sterile distilled water and vortexed for 2 min to release the fungal spores. Sabouraud dextrose agar plates were seeded with 0.2 mL of the fungal spore solutions, allowed to stand for 1 h on the laboratory bench. Wells, later filled with 0.1 mL of different concentrations of the extract (20, 40, 60, 80 and 100 mg mL^{-1}) 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 bacterial cultures were incubated at 37°C for 24 h and fungal cultures were incubated at 27°C for 72 h. Ceftriaxone (30 μg) antibiotic disk was used as control antibacterial agent. Wells in blank Mueller Hinton agar containing 10% ethanol representing the final concentration of the ethanol 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 millimetre[36] and interpreted using the Clinical and Laboratory Standard Institute CLSI Zone Diameter Interpretative Standards[37].

Macrobroth dilution for determining Minimum Inhibitory Concentrations (MICs): Minimum Inhibitory Concentrations (MICs) defined as the lowest concentration which resulted in maintenance or reduction of inoculums' viability determined by serial tube dilution technique[38,39] for each of the test isolates. Different concentrations ranging from 0.039-20 mg mL^{-1} of the extract were prepared by serial dilutions in double strength Mueller Hinton broth for the bacterial isolates while concentrations for the determination of the minimum inhibitory concentrations of the fungal isolates were serially diluted in Sabouraud dextrose broth. Each tube was then inoculated with 0.1 mL of each of the adjusted bacterial and fungal strains. Two blank Mueller Hinton broth tubes and two blank Sabouraud dextrose broth tubes, with and without bacterial 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 and fungicidal concentrations (MBCs and MFCs): 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 0.1 mL aliquot was removed. Each aliquot was placed on a single antibiotic-free nutrient agar plate in a single streak down the centre of the plate, for the bacterial isolates and Sabouraud dextrose agar was used for the fungal isolates, in accordance with the method of Shanholtzer *et al.*[40]. 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 carry over from the 0.1 mL subcultured volume[41,42]. 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 MFCs determining plates were incubated at 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 growth on the solid medium were regarded as the MBC and minimum fungicidal concentration (MFC) values for this extract[43]. This observation was matched with the MIC test tubes 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.*[44] to elucidate whether the observed antibacterial effects were microbicidal (bactericidal/fungicidal) or microbiostatic (bacteriostatic/fungistatic). When the ratio of MBC/MIC or MFC/MIC was $\leq$2.0, the extract was considered bactericidal/fungicidal or otherwise bacteriostatic/fungistatic. If the ratio is $\geq$16.0, the extract was considered ineffective.

RESULTS

In this study, there are 43 pharmacologically bioactive compounds of therapeutic values in the ethanol stem back extract of *T. madagascariense* DC. as presented in Table 1 while the chromatogram for the Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the extract is presented in Fig. 1. The most prominent of these compounds include 5-Hydroxymethylfurfural (13.18%), 1-(2,3-Dimethyl-phenyl)-pyrrolidin-2-ylidene amine (9.51%), catechol (7.65%), phenol (6.91%), benzene (4.88%), 4H-pyran-4-one (3.60%), 1H-1,5-benzodiazepine (2.77%), 1,3,5-triazine (2.72%), supraene (2.56%), phenethyl alcohol (2.50%), 2-Methoxy-4-vinylphenol (2.28%), 4-(4-Methyl-piperazin-1-yl)-1,5,dihydro-imidazol-2-one (2.25%), hexadecanoic acid (2.06%) and indole (1.65%). 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 important therapeutic compounds in each retention time, the presence of 5-Amino-1H-imidazole-4-carboxamide, paromomycin, desulphosinigrin and betulin in the ethanol extract were identified.

The ethanol stem bark extract of *T. madagascariense* showed concentration dependent antibacterial activities against all the test bacterial isolates. In Table 2, the extract

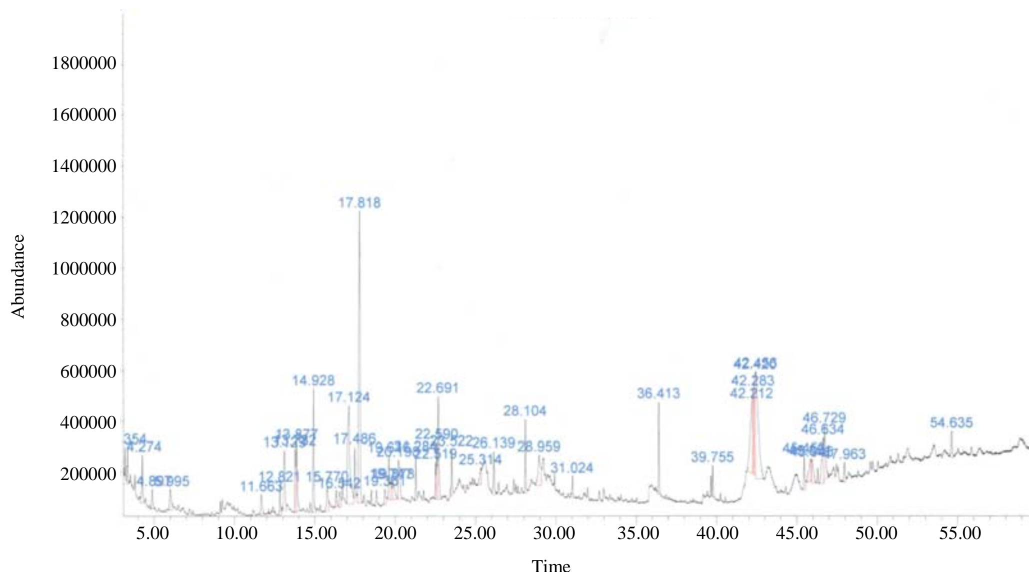

Fig. 1: Chromatograms of phytoconstituents in ethanol stem bark extract *T. madagascariense*

Table 1: Phytochemical analysis of ethanol extract of *T. madagascariense*

Retention time	Area (%)	Name of compound	Quality	Activity or therapeutic potential
3.354	0.98	Ethene	64	Antioxidant, antiurolithiatic
4.274	1.62	Furfural	87	Antibacterial, antifungal, antityrosinase, anti-inflammatory, anti-cancer
4.897	0.73	2-furanmethanol	97	Antioxidant
5.995	1.09	1,3- Dihydroxyacetone dimer	59	Antibacterial
11.663	0.95	2-pyrrolidinemethanol	43	Antitumor
12.821	0.93	2-Furancarboxylic acid	50	Hypolipidemic antibacterial, antifungal, antitumor
13.129	2.72	1,3,5-triazine	47	Antiproliferative, antitumor, antibacterial, antiviral, anti HIV, antiangiogenic
13.782	2.25	4- (4-Methyl-piperazin-1-yl)-1,5, dihydro-imidazol-2-one	42	Antibacterial, herbicidal, antitumor
13.877	2.50	Phenylethyl alcohol	86	Antibacterial, antifungal, neuroprotective
14.928	3.60	4H-Pyran-4-one	91	Antidiabetic, antioxidant, antibacterial, anti-inflammatory, antifungal
15.770	1.37	2 (3H) –Furanone	52	Antibacterial, anti-inflammatory, antifungal, antioxidant, cytotoxic, anti-convulsant, antiviral
16.542	0.85	2 (1H)- Pyridinone	89	Antitumor, anti-inflammatory, antibacterial, anti-fibrotic
17.124	7.65	Catechol	94	Antioxidant, antiplatelet, antimicrobial
17.486	1.28	Catecholborane	49	Anti HIV, antibacterial, antifungal, anti-inflammatory, antioxidant, antitumor, analgesic, antidepressant, antidiabetic, antitumor, anticonvulsant
17.818	13.18	5- Hydroxymethylfurfural	97	Antioxidant, antiproliferative, antibacterial, hepatoprotective
19.361	0.71	Hydroquinone	64	Antineoplastic, anti-tyrosinase, antibacterial
19.616	1.65	Indole	94	Anti-inflammatory and antipyretic
19.747	0.74	1,2-Benzenediol	72	Antibacterial, antioxidant, antidermatic, antifungal, antiseptic, pesticide
19.878	1.19	N,N-Dimethyl-1-propanamine	22	Antiepileptic, proteasome inhibitor, anti-inflammatory
20.198	2.28	2-Methoxy-4-vinylphenol	76	Antibacterial, anti-inflammatory, antioxidant, antitumor
21.284	1.16	Phenol	96	Antioxidant, anti-cancer, antidepressant, anti-bacterial
22.519	0.80	Decanoic acid	98	Anti-bacterial, anti-inflammatory, antioxidant, antitumor
22.590	1.71	Vanillin	95	Anti-inflammatory, antioxidant, antitumor, anti-cancer,
22.691	3.43	1,4-Benzenediol	95	Antibacterial
23.522	1.31	Phenol	98	Antioxidant, anticancer, antidepressant, antibacterial
25.314	0.86	Acetaldehyde semicarbazone	53	Antibacterial, antifungal, analgesic, antipeptic ulcer, anticonvulsant, Antiviral
26.139	1.49	Homovanillyl alcohol	95	Antioxidant, antiplatelet
28.104	2.02	Phenol	95	Antioxidant, anticancer, antidepressant, antibacterial
28.959	2.52	Phenol	30	Antioxidant, anticancer, antidepressant, antibacterial

Table 1: Continue

Retention time	Area (%)	Name of compound	Quality	Activity or therapeutic potential
31.024	0.66	4-[(1E)-3-hydroxyl-1-propenyl] -2-methoxyphenol	99	Antibacterial, cytogenetic
36.413	2.06	Hexadecanoic acid	97	Antitumour, anthelmintic
39.755	0.78	13-Octadecenal	66	Antioxidant, antimicrobial
42.212	4.64	1-(2,3-Dimethyl-phenyl) -pyrrolidin-2-ylidene amine	52	Antiviral, analgesic, anti-inflammatory, antibacterial, antitumor
42.283	2.77	1H-1,5-Benzodiazepine	50	Antioxidant, neuroleptic, anticonvulsant, antibacterial, antifungal
42.420	4.88	Benzene	64	Antidepressant, anti-cancer, anti-proliferative, antibacterial
42.456	9.51	1-(2,3-Dimethyl-phenyl) -pyrrolidin-2-ylidene amine	52	Antidepressant, anti-cancer, anti-proliferative, antibacterial
45.453	1.06	Morphine	25	Analgesic
45.850	1.34	Propanal	46	Antimicrobial, antitumor, CNS activitiy
45.945	1.43	Propanenitrile	60	Antimicrobial
46.634	2.95	Cholesta-5	90	Antimicrobial, antipyretic, analgesic
46.729	2.56	Supraene	35	Antioxidant, antilipidemic, anticancer
47.963	0.71	Estr-4-ene-3	42	Hormonal contraceptive
54.635	0.71	N-Methyl-adamantaneacetamide	38	Antifungal, anti-aflatoxigenic, antioxidant, antibacterial

Table 2: Antibacterial effects/inhibition zones (±1.0 mm) produced by 0.1 mL of different concentrations of the ethanol extract of *T. madagascariense* by agar diffusion assay

	Inhibition zones (±1.0 mm) from different concentrations of the extract				
Name of organism	100 mg mL^{-1}	75 mg mL^{-1}	50 mg mL^{-1}	25 mg mL^{-1}	CTR (30 µg)
Enterococcus faecalis ATCC 29212	20±1.00	18±0.58	16±1.00	14±1.53	18±1.53
Klebsiella pneumoniae ATCC 10031	23±1.00	20±1.53	19±0.00	16±0.00	16±0.00
Bacillus subtilis KZN	21±1.53	18±0.00	16±1.00	14±0.58	30±0.58
Staphylococcus aureus ATCC 6538	20±1.53	17±0.00	17±0.00	14±0.00	15±1.00
Bacillus cereus ATCC 10702	20±1.00	18±1.53	16±0.58	14±1.53	13±0.00
Pseudomonas aeruginosa ATCC 19582	20±0.58	18±1.00	16±0.58	14±0.58	14±0.00
Escherichia coli ATCC 25922	21±1.00	20±1.53	18±1.53	16±1.00	35±1.53
Enterobacter cloacae ATCC 13047	21±0.58	20±0.58	19±1.53	17±1.53	15±0.58
Enterococcus faecalis KZN	21±0.00	19±1.53	17±0.58	15±0.00	6±0.00
Shigella sonnei ATCC 29930	20±1.00	17±0.00	16±1.00	14±0.58	26±1.00
Klebsiella pneumoniae KpFa	23±0.58	20±0.58	17±0.00	15±0.00	14±0.00
Staphylococcus aureus SaFa	22±1.53	19±1.00	17±0.00	15±0.58	6±0.00
Escherichia coli EcFa	20±0.00	17±0.00	15±0.58	14±0.00	13±0.00
Proteus vulgaris CSIR 0030	22±0.00	20±0.58	18±1.53	17±1.53	6±0.00
Pseudomonas aeruginosa PmFa	30±1.53	25±1.00	23±1.53	21±0.58	30±1.53

CTR: Ceftriaxone, 6±0.00 = Resistance

produced varied degree of inhibition zones against all the organisms including control and clinical isolates. At the lowest concentration (25 mg mL^{-1}) of the ethanol extract, 0.1 mL produced inhibition zones that ranged between 14 and 21±1.0 mm while 0.1 mL of the highest concentration (100 mg mL^{-1}) produced inhibition zones that ranged between 20 and 30±1.0 mm. The clinical isolates including *K. pneumoniae* KpFa, *P. vulgaris* CSIR 0030, *S. aureus* SaFa, *E. coli* EcFa and *P. aeruginosa* PmFa were more inhibited by the extract than the typed bacterial isolates used as control. While *E. faecalis* KZN and *P. vulgaris* CSIR 0030 were resistant to ceftriaxone used as control antibacterial agent, other isolates had inhibition zones ranging between 13±1.0 and 35±1.0 mm.

From Table 3, the minimum inhibitory concentrations of the ethanol extract ranged between 1.25 and 5.0 mg mL^{-1} while its minimum bactericidal concentration ranged between 5.0 and 10.0 mg mL^{-1}. The *P. vulgaris* CSIR 0030 had the highest MIC and MBC (1.25 and 2.5 mg mL^{-1}, respectively). This was followed by *B. subtilis* KZN and *B. cereus* ATCC 10702 with MIC and MBC of 2.5 and 5.0 mg mL^{-1}, respectively. While all other bacterial isolates had MIC of 5.0 mg mL^{-1}, *E. faecalis* ATCC 29212, *K. pneumoniae* ATCC 10031, *S. aureus* SaFa and *E. coli* EcFa, had the least MBC of 10 mg mL^{-1}. The susceptibility of the isolates showed that the MBCs were equal to or one fold higher than the MICs with the exception of what was obtained from *P. vulgaris* CSIR 0030 where the MBC was four folds higher than the MIC. The activity of the extract was bactericidal against all the isolates except *P. vulgaris* CSIR 0030 to which it was bacteriostatic even though this isolate had the highest MIC of 1.25 mg mL^{-1}.

Table 3: Minimum inhibitory and minimum bactericidal concentrations of the ethanol extract of *T. madagascariense* against different bacterial pathogens

	MIC	MBC	MIC_{index}	Remarks
Test bacterial isolates	mg mL^{-1}			
Enterococcus faecalis ATCC 29212	5.0	10.0	2	Bactericidal
Klebsiella pneumoniae ATCC 10031	5.0	10.0	2	Bactericidal
Bacillus subtilis KZN	2.5	5.0	2	Bactericidal
Staphylococcus aureus ATCC 6538	5.0	5.0	1	Bactericidal
Bacillus cereus ATCC 10702	2.5	5.0	2	Bactericidal
Pseudomonas aeruginosa ATCC 19582	5.0	5.0	1	Bactericidal
Escherichia coli ATCC 25922	5.0	5.0	1	Bactericidal
Enterobacter cloacae ATCC 13047	5.0	5.0	1	Bactericidal
Enterococcus faecalis KZN	5.0	5.0	1	Bactericidal
Shigella sonnei ATCC 29930	5.0	5.0	1	Bactericidal
Klebsiella pneumoniae KpFa	5.0	5.0	1	Bactericidal
Staphylococcus aureus SaFa	5.0	10.0	2	Bactericidal
Escherichia coli EcFa	5.0	10.0	2	Bactericidal
Proteus vulgaris CSIR 0030	1.25	5.0	4	Bacteriostatic
Pseudomonas aeruginosa PmFa	5.0	5.0	1	Bactericidal

Table 4: Antifungal effects/inhibition zones (±1.0 mm) produced by 0.1 mL of the different concentrations of ethanol extract of *T. madagascariense* by agar well diffusion

	Inhibition zones (±1.0 mm) from different concentrations of the extract (mg mL^{-1})			
Test fungal isolates	100	75	50	25
Candida albicans CA4	25	23	21	20
Candida tropicalis CT4	25	23	20	17
Candida albicans CA15	20	18	15	14
Candida albicans CA6	15	13	0	0
Candida albicans CA23	24	21	17	15

Table 5: Minimum inhibitory and minimum fungicidal concentrations of the ethanol extract of *T. madagascariense* against different fungal isolates

	MIC	MFC	MIC_{index}	Remarks
Test fungal isolates	mg mL^{-1}			
Candida albicans CA4	12.5	25.0	2	Fungicidal
Candida tropicalis CT4	6.25	6.25	1	Fungicidal
Candida albicans CA15	12.5	12.5	1	Fungicidal
Candida albicans CA6	6.25	12.5	2	Fungicidal
Candida albicans CA23	6.25	12.5	1	Fungicidal

The concentration dependent antifungal activity of the extract is shown in Table 4. While the inhibition zones produced by 0.1 mL of the lowest concentration (25 mg mL^{-1}) of the extract ranged between 0 and 20±1.0 mm, 0.1 mL of the highest concentration (100 mg mL^{-1}) produced inhibition zones that ranged between 15 and 25±1.0 mm. However, the MICs and MFCs for the ethanol extract ranged from 1.563-6.25 mg mL^{-1} for fungal isolates (Table 5). From the mechanism of antibiosis, the ethanol extract was considered bactericidal and fungicidal as the MBCs and MFCs were equal to or two folds higher than its MICs against the selected fungal isolates as shown in Table 5.

DISCUSSION

Medicinal plants have continued to gain attention and relevance in the pharmaceutical industry. The bioactive components of many medicinal plants have shown potency with fewer side effects as the synthesized antibiotics. The screening of these plants has led to the precise identification of some bioactive substances which are responsible for the therapeutic values possessed by the plant. The therapeutic effects of medicinal plants have been investigated *in vivo* and *in vitro*. These effects have been confirmed as various reports showed antibacterial and antifungal effects of medicinal[45-47].

From the determination of the phytoconstituents of therapeutic values in the ethanol extract of *T. madagascariense*, bioactive compounds with therapeutic values such as anticancer, anti-inflammatory, antioxidants, antiulcerogenic, anti-hyperglycemia, antitumor, antibacterial, antifungal and antiprotozoal were identified. Of these compounds identified, therapeutic compounds such as 5-amino-1H-imidazole-4-carboxamide, paromomycin, desulphosinigrin and betulin were identified from the mass

spectra. Paromomycin is a widely known antibiotic which shows an antimicrobial effect on susceptible bacteria and protozoa[48]. Although it is an aminoglycoside used to treat intestinal infections such as cryptosporidiosis, amoebiasis and leishmaniasis[49-51], it is non-inferior to amphotericin B in the treatment of visceral leishmaniasis[52]. Like other aminoglycosides, it demonstrates a broad spectrum activity against some Gram-negative and Gram-positive bacteria[53] which may be part of the reasons for the broad spectrum antimicrobial activities observed in this study. Though often produced by *Streptomyces rimosus var.* paromomycin[54] and was first isolated in 1956, there is a dearth of information on its presence or isolation from any medicinal plant unlike the other bioactive compounds. This is the first report identifying its presence in medicinal plant. On the other hand, betulin and its derivatives have been previously isolated from stem bark of birch trees[55]. Pharmacologically, it is effective against various tumors[56], ameliorate diet-induced obesity[57] and exhibits antimicrobial activities[58].

Phytochemical screening of *Vigna mungo* showed the presence of desulphosinigrin at its root nodules[59]. Desulphosinigrin was reported to bind to urease accessory proteins of *Proteus mirabilis*. This corroborated its *in vitro* antibacterial activity[60]. Basically, all the major identified phytoconstituents of this extract have been classified normal hydrocarbons, phenolic compounds, terpenoids, alkaloids and glycosides[61]. They are biologically active molecules[62-64] currently used as antioxidants, antimicrobial and in the formulation of different medicines[65-67]. For instance, indole alkaloids have a wide range of pharmacologically biological activities including cytotoxic, antiviral, antimicrobial, anti-inflammatory and enzyme inhibitory activities[68]. The β-carboline indole ring structure[69] is an important structure in drug discovery[70]. Its isolation as 1-Acetyl-β-carboline and combination with ampicillin has exhibited synergistic antibacterial activity against methicillin resistant *S. aureus*[71].

In this study, the ethanol extract of *T. madagascariense* at different concentrations showed inhibitory effects against bacterial and fungal isolates indicating its broad spectrum of antimicrobial activity. The extract had MICs of 5.0 mg mL^{-1} for *E. coli*, *P. aeruginosa*, *K. pneumoniae* and *E. faecalis.* This is in agreement with Tabassum *et al.*[72], who reported that the MICs of the ethanol stem bark extract of *Cinnamomum cassia* was 2.5 mg mL^{-1} for *E. coli* and 5.0 mg mL^{-1} for *P. aeruginosa*, *K. pneumoniae* and *E. faecalis* and better than MICs of 5.0, 6.25, 10.0 and 100.0 mg mL^{-1} of the ethanol extract of *Psidium guajava* reported against *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa*, respectively[73]. Although Baker and Silverton[74] indicated that an organism is considered sensitive to a chemical agent only when the inhibition zone is either equal to the control, more than or not more than 3 mm smaller than the control, the control or typed isolates and the clinical isolates in this study had wider concentration dependent inhibition zones which are mostly above the reference range of the Clinical Laboratory Standard Institute[75].

Considering the degree of the antimicrobial activity of the extract, the MIC values of the extract against bacteria isolates were higher compared to its MIC values against fungal isolates indicating that the bacterial isolates were more susceptible to the extract. This may be due to the fact that the phytoconstituents in the extract could penetrate the bacterial isolates more than the fungal isolates as well as differences in the composition of their cell walls. The difference in the effectiveness of the extract against bacteria and fungi may also be attributed to the variations in the chemical composition of the secondary metabolites in the extract[76].

The MIC/MBC and the MIC/MFC showed that at lower concentrations, the extracts are bacteriostatic and fungi static but at higher concentrations the extracts are bactericidal and fungicidal. In cases where MIC is equal to MBC or MFC, bactericidal and fungicidal effects are affirmed[77]. Although Rios and Recio[78] suggested that MIC greater than 1 mg mL^{-1} of crude extracts should be disregarded as being ineffective antimicrobials and Simoes *et al.*[79] reported that phytochemicals are routinely classified as antimicrobials when susceptibility tests had MICs in the range of 100-1000 µg mL^{-1}, Fabry *et al.*[80] defined active crude extracts as those having MIC values <8 mg mL^{-1} and Van Vuuren[81] indicated that medicinal plants are active when the MIC is $\leq$2.0 mg mL^{-1}. By inference, since lower MIC and MBC values indicate higher efficacy[82], the extract is considered having significantly effective antimicrobial agents as their MICs ranged between 1.25 and 5 mg mL^{-1} against all the bacterial isolates and those of the fungal isolates ranged between 6.25 and 12.5 mg mL^{-1}. In consensus with other reports on the antimicrobial effects of medicinal plants[27,83], the antimicrobial activity of this plant may be accounted for by the presence of desulphosinigrin[60,84], betulin[85,86], paromomycin[52,87], 5-amino-1H-imidazole-4-carboxamide[88] amongst others. This ethanol extract is very effective based on the degree of antimicrobial activities obtained resulting in significant range of inhibition zones and lower MIC_{index} and MFC_{index} values. Although this could be explained by the fact that antibacterial actions are related to the chemical components in the crude extracts[89,90], the effectiveness may also be due to the synergistic effect of the bioactive compounds present in the extracts[91,92].

CONCLUSION

Traditional knowledge of medicinal plants and their uses by indigenous cultures are not only useful for conservation of cultural traditions and biodiversity but also for pharmaceutical purposes and drug development in the present and future time. It is concluded that, this study indicated that *T. madagascariense* is a potential plant species from which novel antimicrobial compounds of therapeutic importance could be isolated and structurally elucidated. Hence, further investigation of ethnopharmacology is worthwhile to affirm the therapeutic potential of *T. madagascariense*, isolate the plants' active chemical compounds identified and decipher their modes of action. The present study, therefore, justifies the rationale for the traditional use of the ethanol stem bark extract of *T. madagascariense* in the treatment of microbial infections by rural communities in Southwestern part of Nigeria.

SIGNIFICANCE STATEMENT

This study discovers that stem bark of *Trilepisium madagascariense* is highly medicinal and could be used in the treatment of infections in ethnomedicine and identified bioactive compounds of therapeutic values. This study, therefore, indicated the potential effectiveness of this plant in the treatment of microbial infections and showed the presence of paromomycin and 5-amino-1H-imidazole-4-carboxamide which are essential drug agents for the treatment of diarrhoea and protozoa infections. Paromomycin and 5-amino-1H-imidazole-4-carboxamide have not been reported as being present in plants. This study will help researchers to form the basis for selecting this plant species for further investigation in the discovery of new natural bioactive compounds and isolation of the identified drug compounds.

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