

ANTIBACTERIAL ACTIVITIES, PHYTOCHEMICAL SCREENING AND CHROMATOGRAPHIC STUDIES OF *MITRACARPUS SCABER* (GIRDLEPOD) LEAF EXTRACTS

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Abstract - Plants have been used as valuable sources of natural products for maintaining animal and human health. The study investigated the phytochemical and antibacterial properties of the leaf extract of *Mitracarpus scaber*. The antibacterial effect of *Mitracarpus scaber* extracts was done using Agar well diffusion method with Mueller Hinton agar. Phytochemical composition of aqueous and ethanol extracts of *Mitracarpus scaber* leaves were determined using Gas chromatography-mass spectrometry. The study revealed that the concentration of phenols and tannins were more compared to other phytochemicals in both extracts when determined quantitatively. The observed phenolic content was 19.34±1.04 mg/100 g and 44.48±0.96 mg/100 g for the aqueous and ethanol extracts respectively. While tannin content was 14.32±1.04 mg/100 g and 32.94±1.28 mg/100 g for the aqueous and ethanol extracts respectively. Both extracts inhibited the growth of *Escherichia coli* (15±2.00 mm and 23±1.00 mm), *Klebsiella oxytoca* (17±1.00 mm and 20 ± 2.00 mm), *Pseudomonas aeruginosa* (13±2.50 mm and 17±1.00 mm), *Proteus mirabilis* (16±1.00 mm and 22±1.00 mm), *Enterococcus faecalis* (18±1.00 mm and 24±1.00 mm) and *Staphylococcus aureus* (14±2.00 mm and 21±2.00 mm) at 100 mg/ml. Gas Chromatography-Mass Spectrometry confirmed the presence of 5-Nonanol (7.11%), 5-hydroxy-2-(hydroxyl methyl)- 4H-pyran-4-one (6.17%), 2-octenoic acid (16.70%), 1-hydroxy-2,2,6,6-tetramethyl-3- (4-nitroso-1-(piperazinylmethyl)- piperidin-4-one (8.03%), Tetradecanoic acid (3.72%), Pentadecanoic acid (4.27%), 1, 2-Epoxyhexadecane (10.73%), Phytol (1.61%) and 11-bromoundecanoic acid (32.91%) in the aqueous extract. While the ethanol extract contained 5-Nonanol (29.71%), 5-hydroxy-2-(hydroxyl methyl)- 4H-pyran-4-one (24.95%), 2-octenoic acid (0.34%), 1-hydroxy-2,2,6,6-tetramethyl-3- (4-nitroso-1-(piperazinylmethyl)- piperidin-4-one (12.72%), Pentadecanoic acid (6.37%), 1, 2-epoxyhexadecane (0.07%), Phytol (2.53%), 6-octadecenoic acid (7.23%), 1,2-Benzenedicarboxylic acid (8.60%) and 11-bromoundecanoic acid (1.10%). The results of this study revealed that *Mitracarpus scaber* leaves possesses antimicrobial effects.

Keywords: antimicrobial, aqueous, ethanolic, phenol, phytochemical

INTRODUCTION

For many years now, plants have been used as an important source of natural component for maintaining animal and human health. Plants have been reported to contain a great variety of chemical substances that possess important preventive and curative therapies (Chuang *et al.*, 2007). A great percentage of individuals from developing countries use traditional medicines which have compounds derived from medicinal plants (Barrett, 2004). Despite the presence of various approaches to drug discovery, plants still remain the main reservoir of natural medicines (Eloff *et al.*, 2005).

Antimicrobial resistance has revived the interest in plants with antimicrobial properties. This resistance could be associated to indiscriminate use of commercial drugs or not taking an antibiotic prescription

according to the instruction, for example not taking all the prescription in the treatment of infectious diseases (Fortadar *et al.*, 2005). In addition, certain antibiotics present undesirable side effects such as nausea, depression of bone marrow, thrombocytopenic purpura and agranulocytosis leading to the emergence of previously uncommon diseases (Poole, 2001; Graden *et al.*, 2002). This has given scientists the reason to search for newer and alternative antimicrobial compounds from medicinal plants (Aliero and Afolayan, 2006). Besides the high cost of conventional drugs, particularly in resource limited communities has led to the increased use of plants as an alternative for treatment of infectious diseases. Plant extracts and phytochemicals with antimicrobial properties are of great significance in therapeutic treatments. Their antimicrobial properties are due to compounds synthesized in the secondary metabolism of the plant. The screening of plant extracts and plant products for antimicrobial activity has shown that plants represent a potential source of novel antibiotic prototypes (Afolayan, 2003).

Mitracarpus scaber commonly known as “Girdlepod” is called “Ogwungwo or Obuobwa” in Igbo, “Gududal” in Hausa and “Irawo Ile” in Yoruba. It belongs to the family of Rubiaceae and commonly found in the Tropics and Orient (Oghenejobo *et al.*, 2013). It is an annual plant with erect stems of about 53 cm high and branches that are about 8 cm long. The plant has lanceolate leaves of about 4.5 cm long with an upper scabrous surface. Its inflorescence consists of clusters of small white flowers which turn yellowish as the plant matures. It also has dehiscent capsulate fruits of about 1 mm long. The tap root grows to about 9.5 cm from the surface of the soil down. It is perennial and grows where there is much water during the rainy season (Irodi and Daramola, 2009).

The plant is widely used traditionally in the treatment of skin diseases, particularly infectious dermatitis, eczema, ringworm and scabies (Shinkafi, 2013). *Mitracarpus scaber* has been reported to show antimicrobial efficacy against disease causing microorganisms such as *Salmonella enteritidis*, *Staphylococcus aureus*, *Campylobacter sp.*, *Escherichia coli*, *Candida albicans* and *Aspergillus* species. It has also been reported to cure urinary tract infections, gastrointestinal disorders, respiratory diseases and contagious infections (Irodi and Daramola, 2009; Shinkafi, 2013).

This research work focuses on the phytochemical and antibacterial properties of the aqueous and ethanolic extracts of *Mitracarpus scaber* leaves.

MATERIALS AND METHODS

Leave Collection

The leaves of *Mitracarpus scaber* were collected from Ugbiokho Community in Egor Local Government Area of Edo State, Nigeria. The samples were identified by the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Nigeria, where voucher specimen was deposited with the number, UBH-M458.

Preparation of Extracts

The leaves were washed with distilled water to remove dirt and other contaminants, shade dried and pulverized into powder using electric blender. The powder was further sieved to obtain finer particles. In the aqueous extraction process, fifty (50) grams of the powdered sample was soaked in 500 ml of distilled water. The mixture was allowed to stand for 24 hours with occasional stirring. The mixture was then double-filtered using cheese cloth and collected in a conical flask. The filtrate was dried in hot-air oven at 70°C. For the ethanol extraction process, fifty (50) grams of the powdered sample was soaked in 500 ml of absolute ethanol and allowed to stand for 24 hours. The mixture was stirred occasionally. After 24 hours, the sample was double-filtered using cheese cloth and collected in a conical flask. The filtrate was dried in hot-air oven at 45°C (Handa, 2008).

Bacterial Isolates

The pure cultures of *Escherichia coli*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Streptococcus faecalis* and *Staphylococcus aureus* were collected from Medical Microbiology Laboratory, University of Benin Teaching Hospital (UBTH). These viable isolates were maintained on agar slants at 4 °C and sub-cultured for 24 hours after which the various morphological, cultural and biochemical tests were carried out for confirmation before use (Cheesbrough, 2006).

Phytochemical Investigation

Quantitative phytochemical investigation was carried out on the crude extracts using standard methods as described by Edeoga and Gomina (2005). To determine phenols, tannins, flavonoids, alkaloids, saponins, steroids and glycoside.

Bacterial Susceptibility Testing

Antibacterial activity of *Mitracarpus scaber* extracts was done using Agar well diffusion method with Mueller Hinton agar. The surface of the agar plates were inoculated with standardized inocula of the test bacterial isolates and adjusted to 0.5 McFarlad turbidity standards. Thereafter, a sterilized 6 mm cork borer was used to bore holes on the agar plates, and filled with 0.2 ml of the respective plant extracts. Plates were incubated at 37°C for 24 hours, after which it was examined for zone of inhibition. Each zone of inhibition was thereafter measured with a transparent metre rule (Harley *et al.*, 2005).

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

One milliliter (1 ml) of each extract was transferred to a test tube, 1 ml nutrient broth was added and then a loopful of the test organism previously diluted to 0.5 McFarlad turbidity standard was introduced to the tubes and observed for turbidity after 24 hours of incubation at a temperature of 37°C. The minimum bactericidal concentration (MBC) was an offshoot of the previously determined MIC. The plant extract in the test tubes with no turbidity was taken as the Minimum Inhibitory Concentration (MIC). Subsequently, those tubes that showed no turbidity were plated out on sterile nutrient agar plates and incubated at 37°C for 24 hours, absence of growth after incubation period were considered as the MBC (Ajaiyeoba *et al.*, 2003; Andrews, 2004; Etebong and Nwafor, 2009;).

Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis of the extract of *Mitracarpus scaber* leaves was performed using a Thermo GC-Trace ultra version 5.0. The oven temperature was maintained at 220°C at a rate of 6°C/min; the carrier gas with a flow rate of 1 ml/min. The split sampling technique was used to inject the sample in the ratio of 1:10. Retention indices (RI) of the compounds were determined by comparing the retention times of a series and identification of each component was confirmed by comparison of its retention index with data in the literature. The spectrum of the unknown components were compared and subsequently

interpreted using the spectrum of known components already stored in the National Institute of Standards and Technology (NIST) library, which have in it more than 62,000 spectrum patterns. The molecular weight, name, chemical structure and molecular formula of the components of the test materials were ascertained (Shinkafi, 2013).

Statistical analysis

Data obtained for the different parameters were subjected to statistical analysis using the analysis of variance (ANOVA) technique and the least significance difference (LSD) test was used to compare the means, according to recommended method (Ogbeibu, 2014).

RESULTS AND DISCUSSION

Table 1 shows that *Mitracarpus scaber* leaves (ethanol and aqueous extracts) contained an appreciable amount of phytochemicals. The presence of a variety of phytochemicals in plants usually indicates its efficacy as an antibacterial agent. Aqueous extract of *Mitracarpus scaber* leaves contained phenols (19.34±1.04 mg/100 g), tannins (14.32±1.04 mg/100 g), flavonoids (13.66±1.38 mg/100 g), saponins (6.54±1.33 mg/100 g) and glycoside (2.58 ±0.23 mg/100 g); while ethanol extract revealed phenols (44.48±0.96 mg/100 g), tannins (32.94±1.28 mg/100 g), flavonoids (31.42± 2.84 mg/100 g), alkaloids (3.17±0.59 mg/100 g), saponins (2.26±0.39 mg/100 g), steroids (0.25±0.00 mg/100 g) and glycoside (0.73±0.00 mg/100 g).

Table 1: Quantitative phytochemical screening

Phytochemicals	Aqueous Extract (mg/100 g)	Ethanol Extract (mg/100 g)	P- Value
Phenols	19.34 ±1.04 ^a	44.48 ±0.96 ^b	0.036
Tannins	14.32 ±1.04 ^a	32.94 ±1.28 ^b	0.013
Flavonoids	13.66 ±1.38 ^a	31.42 ± 2.84 ^b	0.035
Alkaloids	ND	3.17 ±0.59	-
Saponins	6.54 ±1.33 ^a	2.26 ±0.39 ^a	0.059
Steroids	ND	0.25 ±0.00	-
Glycoside	2.58 ± 0.23 ^a	0.73 ± 0.00 ^a	0.057

Values are expressed as Mean±Standard Error of triplicate experiments. Mean values with similar superscript on the same row are not significantly different from each other ($p>0.05$).

ND= Not Detected.

Figures 1 and 2 shows the GC/MS Chromatogram of *Mitracarpus scaber* Aqueous and ethanol leaf extracts respectively, indicating the various compounds identified with their various peak area and retention time.

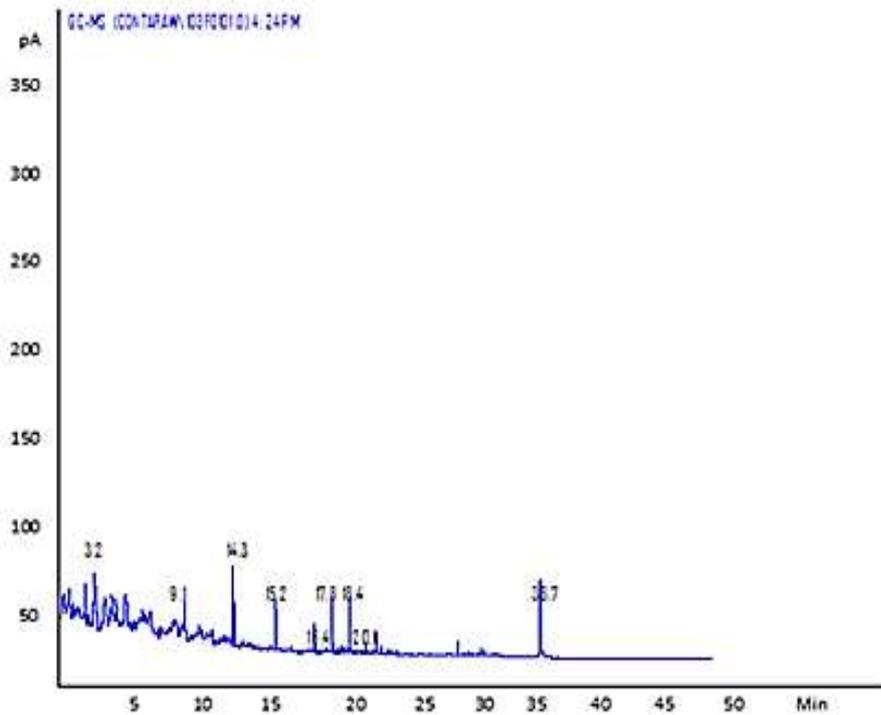


Figure 1: GC/MS Chromatogram of *Mitracarpus scaber* Aqueous Leaf Extract

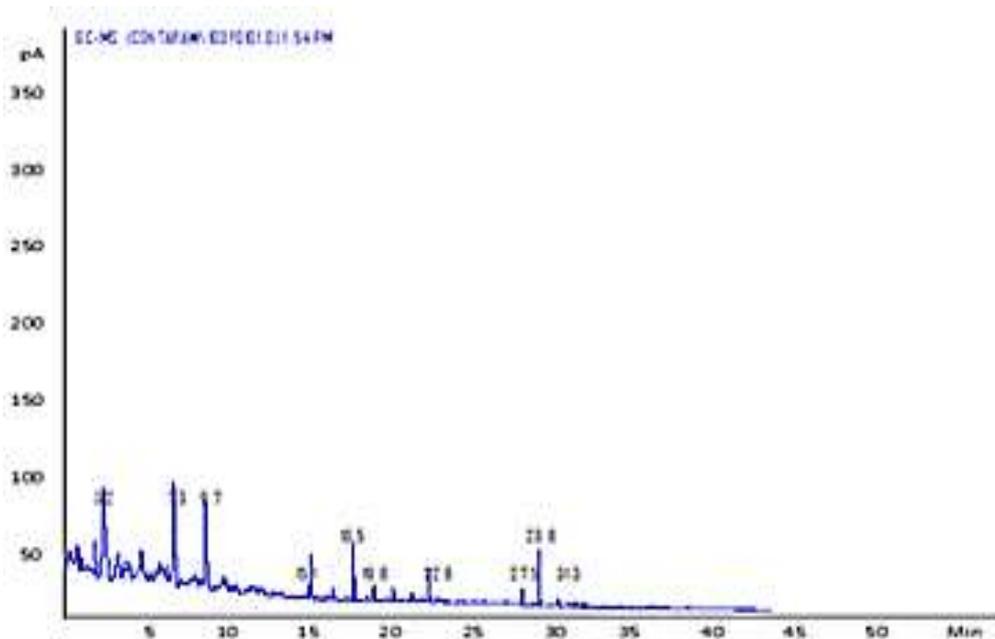


Figure 2: GC/MS Chromatogram of *Mitracarpus scaber* Ethanol Leaf Extract

Table 2 shows the compounds isolated from aqueous and ethanol extracts of *Mitracarpus scaber* leaves. The gas chromatography-mass spectrometry analysis revealed the presence of 5-nonanol (7.11%), 5-hydroxy-2-(hydroxyl methyl)- 4H-pyran-4-one (6.17%), 2-octenoic acid (16.70%), 1-hydroxy-2,2,6,6-tetramethyl-3- (4-nitroso-1-(piperazinylmethyl)- piperidin-4-one (8.03%), tetradecanoic acid (3.72%), pentadecanoic acid (4.27%), 1, 2-Epoxyhexadecane (10.73%), Phytol (1.61%) and 11-bromoundecanoic acid (32.91%) in the aqueous extract. While the ethanol extract contained 5-nonanol (29.71%), 5-hydroxy-2-(hydroxyl methyl)- 4H-pyran-4-one (24.95%), 2-octenoic acid (0.34%), 1-hydroxy-2,2,6,6-tetramethyl-3- (4-nitroso-1-(piperazinylmethyl)- piperidin-4-one (12.72%), pentadecanoic acid (6.37%), 1, 2-epoxyhexadecane (0.07%), phytol (2.53%), 6-octadecenoic acid (7.23%), 1,2-benzenedicarboxylic acid (8.60%) and 11-bromoundecanoic acid (1.10%).

Table 2. Phytocomponents identified in the different extracts of *Mitracarpus scaber* leaves by GC-MS Analysis

Peak	Name of the Compound	Molecular Formula	RT Aqueous Extract	Peak Area (%)	RT Ethanol Extract	Peak Area (%)
1	5-Nonanol	C ₉ H ₂₀ O	3.2	7.11	3.2	29.71
2	5-hydroxy-2-(hydroxylmethyl)-4H pyran-4-one	C ₆ H ₆ O ₄	9.1	6.17	7.3	24.95
3	2-octenoic acid	C ₈ H ₁₄ O ₂	14.3	16.70	9.7	0.34
4	1-hydroxy-2,2,6,6-tetramethyl-3- (4-nitroso-1-(piperazinylmethyl)- piperidin-4-one	C ₁₄ H ₂₆ N ₄ O ₃	15.2	8.03	15.1	12.72
5	Tetradecanoic acid	C ₁₄ H ₂₈ O ₂	17.8	3.72	ND	ND
6	Pentadecanoic acid	C ₁₅ H ₃₀ O ₂	16.4	4.27	19.6	6.37
7	1, 2-epoxyhexadecane	C ₁₆ H ₃₂ O	18.4	10.73	18.5	0.07
8	Phytol	C ₂₀ H ₄₀ O	20.1	1.61	27.5	2.53
9	6-octadecenoic acid	C ₁₈ H ₃₄ O ₂	ND	ND	22.9	7.23
10	1,2-Benzenedicarboxylic acid	C ₈ H ₆ O ₄	ND	ND	28.6	8.60
11	11-bromoundecanoic acid	C ₁₁ H ₂₁ BrO ₂	38.7	32.91	31.3	1.10

KEY:

RT: Retention Time

ND: Not Detected

The tested bacterial isolates showed varying degree of sensitivity by means of the various zones of inhibition observed for the aqueous and ethanol extracts (table 3). These zones of inhibition increased as concentration in both extracts increased. *Streptococcus faecalis* was observed to have the highest zone of inhibition (18 ± 1.00 mm and 24 ± 1.00 mm both in the aqueous and ethanol extracts respectively) at 100 mg/ml compared to other bacterial isolates tested.

Table 3: Zone of inhibition of aqueous and ethanol extracts of *Mitracarpus scaber* leaves

Extract concentration (mg/ml)	Solvent	Diameter of inhibition zone (mm)					
		<i>E. coli</i>	<i>K. oxytoca</i>	<i>P. aeruginosa</i>	<i>P. mirabilis</i>	<i>E. faecalis</i>	<i>S. aureus</i>
100	Aqueous	15±2.00	17±1.00 20 ± 2.00	13±2.50	16±1.00	18±1.00	14±2.00
	Ethanol	23±1.00	2.00	17 ±1.00	22±1.00	24±1.00	21± 2.00
50	Aqueous	12±2.00	13±1.00	10±2.00	14±1.00	15±1.00	12±1.00
	Ethanol	17±1.00	15±1.00	14±2.00	18±1.00	22±1.00	17±0.00
25	Aqueous	9±0.00	10±1.00	8±1.00	10±2.00	12±1.00	9±2.00
	Ethanol	14±2.00	13±0.00	9±1.00	15±1.00	17±2.00	13±1.00
12.5	Aqueous	8±1.00	8±0.00	8±1.00	9±1.00	9±2.00	8±1.00
	Ethanol	13±2.00	9±2.00	8±1.00	11±2.00	11±1.00	10±1.00
6.25	Aqueous	8±1.00	8±1.00	8±0.00	8±0.00	8±1.00	8±1.00
	Ethanol	8±1.00	8±2.00	8±0.00	9±2.00	10±1.00	8±1.00
Distilled water		0.00	0.00	0.00	0.00	0.00	0.00

Inhibition zone diameters are expressed as Mean ± Standard Error of triplicate experiments.

The minimum inhibitory concentration of the ethanol extract ranged from 12.5-50 mg/ml and minimum bactericidal concentration ranging from 50-100 mg/ml for the tested organisms. The aqueous extract had its minimum inhibitory concentration ranging from 25-100 mg/ml and minimum bactericidal concentration at 100 mg/ml (table 4). The result clearly indicates that the ethanol extract has more inhibitory activities than the aqueous extract.

Table 4: Minimum inhibitory and minimum bactericidal concentration of aqueous and ethanol extracts of *Mitracarpus scaber* leaves

Bacterial isolates		<i>E. coli</i>	<i>K. oxytoca</i>	<i>P. aeruginosa</i>	<i>P. mirabilis</i>	<i>E. faecalis</i>	<i>S. aureus</i>
Aqueous extract (mg/ml)	MIC	50	50	100	50	25	50
	MBC	100	100	100	100	100	100
Ethanol extract (mg/ml)	MIC	12.5	12.5	50	25	25	25
	MBC	50	100	100	50	50	100

MIC= Minimum Inhibitory Concentration. MBC= Minimum Bactericidal Concentration.

DISCUSSION

Plant extracts are potential sources of antimicrobial compounds especially against bacterial pathogens (Sahu *et al.*, 2011). Ethanol and distilled water were the solvents used in the extraction of *Mitracarpus scaber* leaves in this study. The presence of secondary metabolites (table 1) in the various leaf extracts of *Mitracarpus scaber* could be responsible for the antibacterial activities observed during the antibacterial susceptibility test as shown in table 3.

Tannins, alkaloids, saponins and glycosides had earlier been reported to be present in *Mitracarpus scaber* (Abere *et al.*, 2007). The aqueous extract was also earlier reported to contain tannins, flavonoids, alkaloids, saponins, steroids and glycoside (Shinkafi, 2013) which was in agreement with the result of this study.

Saponins and glycosides widely exist in plants and it is believed to contain antibacterial compounds (Soetan *et al.*, 2006). Alkaloids are a large, diverse group of secondary metabolites, reported as antimicrobial because of its ability to intercalate with the microbial DNA thereby inhibiting DNA replication which leads to the distortion of microbial growth and survival (Garba and Okeniyi, 2012). Flavonoids are hydroxylated phenolic compounds produced by many plants to combat bacterial infections by interacting with the bacteria cell wall and proteins (Bansode and Chavan, 2014). Hence, the presence of secondary metabolites such as saponins, flavonoid, phenol, glycosides and alkaloid in *Mitracarpus scaber* leaves of the present study may be responsible for the antibacterial activity of the

extracts. However, the exact active agent responsible for the antibacterial efficacy has to be screened further.

Though all the bacteria tested in the present investigation (table 3) were sensitive to all the plant extracts, their effectiveness varied in the different extracts. The difference in the antibacterial efficacy of the plant extracts is suggested to be depended on the variation in their phytochemicals. Less effectiveness against the bacteria may be due to the absence or insufficient concentrations of the antibacterial constituents (Sahu *et al.*, 2011).

Generally, the result as shown in table 1 therefore suggests that ethanol can extract a wider range and quantity of phytochemicals from *Mitracarpus scaber* leaves than water. Hence having a greater antibacterial potential.

GC-MS analysis of the plant extracts can be an interesting tool for testing the amount of some active compounds in herbs used in cosmetic, drugs, pharmaceutical or food industry.

Of the compounds detected by GC-MS in the medicinal *Mitracarpus scaber* leaves extracts, several compounds have been reported to have medicinally important bioactivities and also used for the production of various bioactive products. 1, 2- benzenedicarboxylic acid which was present only in the ethanolic extract is a plasticizer compound and acts as antimicrobial and antifouling agent (Heinonen *et al.*, 1998). Phytol is reported to have antioxidant, antiallergic (Santos *et al.*, 2013).

The compound 5-hydroxy-2-(hydroxyl methyl)-4H-pyran-4-one have antibacterial and antifungal properties and inhibits melanin production (Elizabeth *et al.*, 2013).

Other compounds such as 5-nonanol a fatty alcohol is used as Pheromones in the form of 4- Methyl-5-nonanol. Pentadecanoic acid a fatty acid is used as Food additive (Usually flavouring agent) while 2-octenoic acid a fatty acid is used as Surfactant and Emulsifier in the industry and also as flavouring agent (Elizabeth *et al.*, 2013). 1, 2-epoxyhexadecane is well known as preservative in food, drugs and cosmetics. It is also antifungal against dermatophytes; anti-tumor, analgesic, antibacterial, anti-inflammatory; anticoagulant properties; reduces liver damage; effective in killing cancer cells and treating rheumatoid arthritis (Igwe *et al.*, 2015). The presence of these compounds may have contributed to the bioactivities of *Mitracarpus scaber* leaves.

In the present study, the antibacterial activity of the different extracts of *M. scaber* was tested against six bacteria (*Klebsiella oxytoca*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Escherichia coli*, *Enterococcus faecalis* and *Staphylococcus aureus*). The aqueous extract showed maximum zone of inhibition against *Enterococcus faecalis* (18 ± 1.00 mm) at 100 mg/ml. The ethanol extract showed a maximum zone of inhibition against *Enterococcus faecalis* (24 ± 1.00 mm) at 100 mg/ml. It was clear from the present results, that ethanol extract exhibited pronounced activity against all the tested bacteria, making the ethanol extract having a significantly higher antibacterial activity than the aqueous extract. This difference is attributed to the solubility of the active component in the different solvents (Karou *et al.*, 2007).

The antibacterial activities of *Mitracarpus scaber* reported in this study was in agreement with the report of Madziga *et al.* (2010), who reported that the ethanol extract of *Mitracarpus scaber* had inhibitory effect against *Enterococcus faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*.

The results obtained for minimum inhibitory concentration and minimum bactericidal concentration (table 4) revealed that the ethanol extract of *Mitracarpus scaber* leaves can inhibit and cause bacteria death at a much lower concentration than the aqueous extract.

It is also worthy of note that MBC values obtained for the extracts against the clinical isolates are higher than MIC, indicating that the extracts are bacteriostatic at lower concentrations and bactericidal at higher concentrations. This suggests that the plant extracts, when used traditionally as antimicrobial agent, inhibit bacteria growth without necessarily killing the bacteria and since most of the traditional preparations lack specific concentrations, this may thus account for the use of large quantity of the extracts by traditional medical practitioners for the treatment of their patients. The results of this study showed that *Mitracarpus scaber* leaf extracts has antimicrobial effects. The presence of bioactive chemicals in the extracts may have been responsible for this antimicrobial effects and the ethnopharmacological usage in traditional medicine. This study also provides data on the selection of an appropriate solvent concentration and indicates that ethanolic extract of *Mitracarpus scaber* leaves can offer significant potential for the development of antibacterial therapies.

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