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ANTIMICROBIAL ACTIVITY OF UNCOMBINED AND COMBINED EXTRACTS OF *DOLIOCARPUS DENTATUS* AND *MONTRICARDIA ARBORESCENS*

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Keywords:

Antimicrobial activity, *Doliocarpus dentatus, Montricardia arborescens*, Agar Diffusion method, microbial potency

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ABSTRACT: The antimicrobial activity of the individual extracts of *Doliocarpus dentatus* and *Montricardia arborescens* and combined extracts of both plants were investigated against bacterial strains *E. coli*, *S. aureus* and *K. pneumoniae* using the Disc/ Agar Diffusion method under asceptic conditions. The magnitude of the diameter of the zone of inhibition was used as an indication for antimicrobial susceptibility or potency. Aqueous and C_2H_5OH extract of *Doliocarpus dentatus* showed insignificant antimicrobial activities whereas the CH_2Cl_2 and EtOAC extract of *Doliocarpus dentatus* were antimicrobial. The area of zone of inhibition ranging from 68.3 mm^2 to 89.4 mm^2. However, all the extracts of *Montricardia arborescens* were antimicrobial against all three bacterial strains. The area of zone of inhibition ranging from 68.3 mm^2 to 172.6 mm^2. The combined extract also showed variation in potency against the three microbial strains with the area of zone of inhibition ranging from 180 mm^2 to 368.6 mm^2.

INTRODUCTION: There is an urgent need to intensify research in herbal medicines and synthetic drug discovery, considering the presence of incurable diseases such as HIV AIDS and the threat of new emerging disease such as SARS, bird flu etc. Over the years, plants extracts and fractionated plant extracts have been a good source of herbal medicines and natural products/ phytochemicals [1-9]. Guyana has a rich biodiversified flora whose crude extracts, both organic and aqueous are currently been screened for their antimicrobial activity [10-13].

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Also, the specified plants parts fractionated or screened for natural products whose antimicrobial activity can also be investigated and compared with the crude extracts [14]. Crude plant extracts can be chromatographed, leading to the isolation and purification of new and known bioactive natural products/phytochemicals, whose medicinal activity can also be investigated [15-19].

Following this, clinical trials of crude extracts or fractionated natural products can lead to the formulation of an herbal plant cream or herbal medicine. A few herbal medicine shops are established in Guyana and the "bush" medicine man is still an important figure in Guyana's culture. Isolated natural products with good biological activity that correlates with structure have also been the source of knowledge for the synthesis of many drugs [1, 20-22]. There are a large number of plants in Guyana whose antimicrobial

activity need urgent investigations as there is a need for the development of new antimicrobials, considering that bacteria develop resistance to antimicrobials over the years.

Doliocarpus dentatus, commonly called (Dilleniaceae), is a forested climber to 16m from the diverse flora of Guyana. The stems and bark are used as a tea and have aphrodisiac properties. The plant is often used as a tonic with other plants. A liqueur is produced from these plants and sold in market. The watery sap from a freshly cut stem is drunk for the relief of stricture or simply as a thirst quencher [23].

Montricardia arborescens(Araceae) is an erect little branched arborescent herb to 3 m high. The stem has numerous nodes and is sometimes prickly. Medicinally, the succulent stem is pounded and made unto a poultice which is applied to cuts and bruises to stop bleeding. Juice from the fresh stem is used for sore eyes. A decoction of dried roots and leaves is taken daily to relieve hypertension [23].

The isolation and biological activities of some other constituents from *Doliocarpus dentatus* are noted. For example, cytotoxic metabolites such as betulinic acid, nataloe-emodin, bisnordihydro-toxyferine, 2',4'-dihydroxy-6'-methoxy-3, 4-methylene dioxy dihydrochalcone, 2',4'-dihydroxy-4,6'-dimethoxydihydrochalcone and 12- O-tetradecanoyl phorbol-13-acetate were isolated from Peruvian *Doliocarpus dentatus* [24].

Leishmanicidal triterpenes (betulinic acid, betulin and betulinaaldehyde) and lignans (as (+) pinoresinol, (+) medioresinol and (−) lirioresinol B were also isolated. The antiproliferative bioassay-guided fractionation of *Doliocarpus dentatus* is also reported [24]. Literature review revealed that little or no work has been done on the antimicrobial activity of both plants

Thus, this paper focuses on the antimicrobial (antibacterial and antifungal) properties of leaf extract of *Montricardia arborescens* and stems of *Doliocarpus dentatus* from the coastal plain of the Guyana flora with a view to develop the possible use as herbal medicines. Also, the spectroscopic analyses of the extract is reported.

Antimicrobial properties were investigated against three pathogenic bacteria, *S. aureus* (gram positive), *E. coli* (gram negative), *Klebsiella pneumonia* (gram negative) [25]. To date, no work has been reported on the antimicrobial properties of neither *Doliocarpus dentatus* nor *Montricardia arborescens.*

MATERIALS AND METHODS:

Materials and Equipment: n - C_6H_{14}, CH_2Cl_2, EtOAc, CH_3CH_2OH were purchased from Sigma Aldrich Company (USA) and were redistilled prior to use. Na_2SO_4 was used to dry each solvent type extract after filtration. Solvents were removed in *vacuo* from the dried extracts using a Buchii rotavapor, R-124 with Laboport vacuum pump.

Filter paper of size 150 mm and 240 mm diameter were used for filtration. Analytical TLC analyses were done on precoated Kiesegel 60 F254 (Merck) plates and were 0.25 mm thick. TLC plates were viewed under a UV lamp, (Spectroline Longlife Filter) and developed chromatograms were visualized via initial spraying with iodine. Extracts were weighed using a Metler Citizen type balance.

Plant Material: *Doliocarpus dentatus* stems were collected from a commercial planter in the interior (remote) area of Guyana, whereas the leaves of *Montricardia arborescens* were collected along the East Coast Demerara. Both Stems and leaves were freed of visible fungal and bacterial infection via inspection. Products of microbial synthesis might result in added chemical composition of the plant material and inspection was important.

Stems of *Doliocarpus dentatus* were stored in plastic bags, sealed, labelled and transported to the University of Guyana. It was identified by a Taxonomist at the Bio Diversity Centre of the University. A voucher specimen (#037656) is deposited in the herbarium of the Bio Diversity centre.

A voucher specimen of the leaves of *Montricardia arborescens* is also deposited at the Bio Diversity Centre of the University. Stems and leaves respectively were subjected to aerial drying for a week and were later ground into a fine powder (250g) using a grinding mill, Arthur H. Thomas Co. model (# 750611).

Plant extracts: Extractions were done in extraction jars at ambient temperature using sequentially freshly distilled n-C_6H_{12}, CH_2Cl_2, EtOAc and CH_3CH_2OH. For each solvent type, three extractions were done and each extract was filtered and dried over anhydrous Na_2SO_4 and solvents were removed *in vacuo* to yield viscous oils and semi solids. The physical nature of the C_6H_{14}, CH_2Cl_2, EtOAc and CH_3CH_2OH extract of *Doliocarpus dentatus* bean oil, white solid, red brown solid and black gummy material, whereas those for *Montricardia arborescens* been greenish brown, black, brown solid and gummy black respectively.

Elemental analyses: 1.0g of the dried solvent type extracts was dissolved in 10ml of the requisite solvent. This was followed with the addition of 1ml of 10% HNO_3. It was shaken to ensure a uniform mixture and then made up to 100ml mark. Appropriate working standard solutions were prepared for each element. The levels of requisite metal elements (K, Cu, Fe, Mn, Co and Zn) were determined using Atomic Absorption Spectrophotometry (AAS) method on a Varian AAS 50 spectrophotometer. First the sample solution was aspirated by a pneumatic nebulizer, transformed into an aerosol, which was introduced into a spray chamber, where it was mixed with the flame gases and conditioned in a way that only the finest aerosol droplets (< 10μm) entered the flame.

On top of the spray chamber, a burner head produces a flame that is laterally long (usually 5–10cm) and only a few mm deep. The radiation beam passed through this flame at its longest axis, and the flame gas flow rates was adjusted to produce the highest concentration of free atoms. The burner height was adjusted, so that the radiation beam passed through the zone of highest atom cloud density in the flame, resulting in the highest sensitivity. The concentration (C) of each analyte in the solutions aspirated was recorded [27].

Antimicrobial Assay: This was done in accordance with literature [28-30]. Molten agar was poured into 90 mm diameter sterile petri dishes to a depth of 4 mm (about 25 ml per plate) on a level surface so that the depth of the medium is uniform. Using Stokes Disc diffusion sensitivity technique, an inoculum containing bacterial or yeast cells was applied onto nutrient agar plates.

On each plate, four discs were applied. One of these disc was a reference one on which the positive control was applied. The positive control is Ampicillin and Nystatin for the bacterial and fungal strains respectively. The reference antibiotic disc usually contains about 200mg antibiotic/ml.

The discs (5-6mm) were punched out from a filter paper using a perforator or are commercially sold. Each disc was impregnated with the anticipated antimicrobial plant extract at appropriate concentration of 200 mg/ml using a microlitre syringe.

The antimicrobial compound is expected to diffuse from the disc into the medium. Following overnight incubation, the culture was examined for areas of no growth around the disc (zone of inhibition). The radius of the inhibition zone was measured from the edge of the disc to the edge of the zone.

The end point of inhibition is where growth starts. Larger the diameter of zone inhibition, greater is the antimicrobial activities. The inhibition zones produced by different antimicrobials against the same organism vary in size due to differences in antimicrobial molecular structures.

A larger zone of inhibition is produced by an antimicrobial that diffuses rapidly and a smaller zone by one that diffuses more slowly. It is anticipated through the antimicrobial activity of plant extract, no area of growth will be induced around the disc.

Bacteria or fungal strains sensitive to the antimicrobial are inhibited at a distance from the disc whereas resistant strains grow up to the edge of the disc. Discs applied to the plates already streaked with bacteria and the fungus. In other disc techniques, including the Kirby-Bauer method, the control strain is inoculated on a separate plate.

Source of microorganisms: For the bacterial organisms, gram negative bacteria used were *Staphylococcus aureus* (ATCC 25923). For the fungi, yeast of the *Candida albicans* (ATCC 1023) species was investigated. These microorganisms were stored in a refrigerator at the microbiology laboratory at St. Joseph Mercy Hospital.

Reference and Control: The references were antibiotic in nature. *Ampicillin* was chosen as the reference for all bacterial species used: *E. coli* and *S. aureus*. The Control experiment consists of a plate of solidifying agar onto which was inoculated pure solvent with microorganism mixed in a 1:1 portion.

RESULTS:

TABLE 1: REFERENCE EXPERIMENT (POSITIVE CONTROL): ANTIMICROBIAL ACTIVITY OF AMPICILLIN

Microorganism Type	Diameter of zone of inhibition (mm)	Mean Diameter of Zone of inhibition with SD	Area of zone of inhibition $(mm)^2$	Variance, $(SD)^2$
E. coli	34, 35, 35	34.67 ± 0.57	943.58	0.32
S. aureus	36, 35, 36	35.67 ± 0.57	998.79	0.32
Klebsiella pneumoniae	38, 39, 37	38.0 ± 1.0	1133.54	1.0

TABLE 2: ANTIMICROBIAL ACTIVITY OF PLANT EXTRACTS VS *STAPHYLOCOCCUS AUREUS*

No. of Experiment	*Staphylococcus aureus*	Mean diameter (mm) with SD	Area of Zone of Inhibition $(mm)^2$	Variance
1	-	-	-	-
2	7, 7, 7	7.0 ± 0.0	38.47	0.00
3	9, 10, 10	9.67 ± 0.744	73.4	0.55
4	10, 11, 11	10.67 ± 0.33	89.4	0.11
5	15, 14, 14	14.33 ± 0.58	161.2	0.34
6	10, 9, 9	9.33 ± 0.58	68.3	0.34
7	20, 19 ,20	19.67 ± 0.33	303.7	0.11
8	16, 15, 16	15.67 ± 0.53	192.8	0.28
9	20, 19, 20	19.67 ± 0.58	303.7	0.28

TABLE 3: ANTIMICROBIAL ACTIVITY OF PLANT EXTRACTS VS *E. COLI*

No. of Experiment	*E. coli* (gram negative)	Mean diameter (mm) of Triplicates with SD	Area of Zone of Inhibition	Variance
1	-	-	-	-
2	7, 7, 8	7.33 ± 0.0	42.18	0.00
3	12, 10, 10	10.7 ± 3.65	89.37	13.32
4	13, 12, 12.5	12.5± 0.33	122.66	0.11
5	15.5,14, 14	14.5 ± 0.87	165.05	0.76
6	9, 10, 10	9.7 ± 0.56	73.40	0.31
7	18,17,18	17.7 ± 0.58	245.09	0.33
8	19, 18, 19	18.7 ± 0.58	368.63	0.33
9	16, 15, 15	15.3 ± 0.053	184.48	0.03

TABLE 4: ANTIMICROBIAL ACTIVITY OF PLANT EXTRACTS VS *KLEBSIELLA PNEUMONIAE*

No. of Experiment	*Klebsiella Pneumoniae* (gram negative)	Mean Diameter (mm) of Triplicates with SD	Area of Zone of Inhibition (mm^2)	Variance
1	-	-	-	-
2	7, 8, 8	7.67 ± 0.57	46.18	0.32
3	10, 10, 10	10.0 ± 0.0	78.5	0.00
4	10, 9,9	9.33± 0.33	68.3	0.11
5	15, 14.5, 15	± 0.288	172.6	0.08
6	12,12,11	11.7 ±0.5778	107.5	0.33
7	15.5,15,15	15.2 ± 0.26	180.7	0.07
8	22 21,22	21.7 ± 0.58	368.6	0.33
9	18, 17, 18	17.7 ± 0.58	245.1	0.33

Exp 1: Antimicrobial investigation of aqueous extract of (*Doliocarpus dentatus*); Exp 2: Antimicrobial investigation of CH_3CH_2OH extract of (*Montricardia arborescens*); Exp 3: Antimicrobial investigation of CH_2Cl_2 extract of (*Doliocarpus dentatus*); Exp 4: Antimicrobial investigation of EtOAc extract of (*Doliocarpus dentatus*); Exp5: Antimicrobial investigation of n-C_6H_{14} extract 5 (*Monticardia arborescens*); Exp 6: Antimicrobial investigation of CH_2Cl_2 extract 6 (*Monticardia arborescens*); Exp 7: CH_2Cl_2 extract of *Doliocarpus dentatus* + CH_2Cl_2 extract of *Monticardia arborescens*; Exp 8: CH_3CH_2OH extract of moko moko (*Montricardia arborescens*); Exp 9: Mixture of C_2H_5OH extract of *Doliocarpus dentatus* + moko moko (*Montricardia arborescens*). Extract.

TABLE 5: METAL ION CONCENTRATION (mg/L) for n-C_6H_{14}, CH_2Cl_2 and CH_3CH_2OH EXTRACT OF *MONTRICARDIA ARBORESCENS* USING ATOMIC ABSORPTION SPECTROSCOPY (AAS)

Extract Type	K (mg/L)	Cu (mg/L)	Fe (mg/L)	Mn (mg/L)	Co (mg/L)	Zn (mg/L)	Pb (mg/kg)
n-C_6H_{14}	596	130	3378	85	3979	8784	3111
CH_2Cl_2	227	309	2619	328	8142	2292	2788
CH_3CH_2OH	106	40	ND	ND	72	ND	40

ND: Not detected

TABLE 6: R_f VALUES FOR n-C_6H_{14}, CH_2Cl_2 and CH_3CH_2OH EXTRACT OF *MONTRICARDIA ARBORESCENS*

n-C_6H_{14}	CH_2Cl_2	CH_3CH_2OH
0.93	0.97	0.64
0.78	0.78	0.73
0.56	0.32	0.81
0.49	0.22	0.9
0.41	0.12	
0.23		
0.15		

Graphs: **Graphs (1-9)** is a plot of the Area of Zone of Inhibition (AZOI) versus Pathogenic microorganisms for n-C_6H_{14}, EtOAc and CH_3CH_2OH extract of *Doliocarpus dentatus* and *Montricardia arborescens*

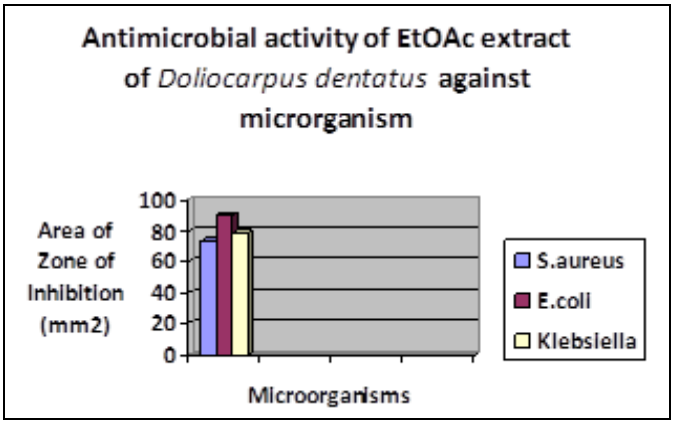

FIG. 1

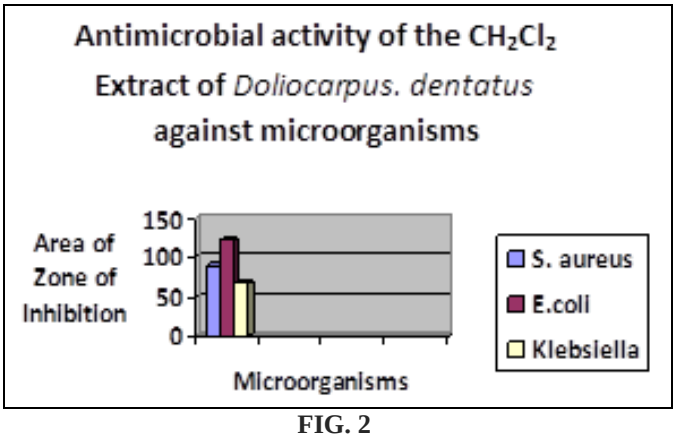

FIG. 2

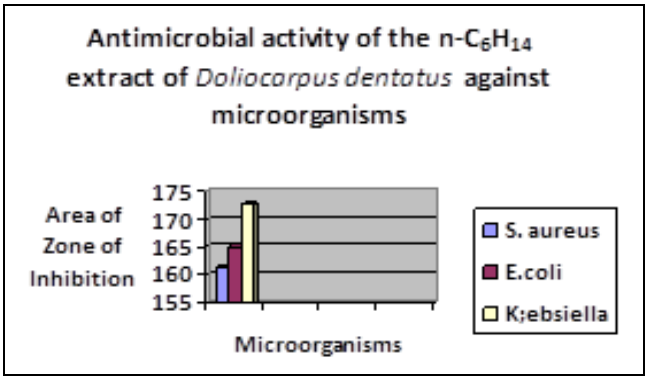

FIG. 3

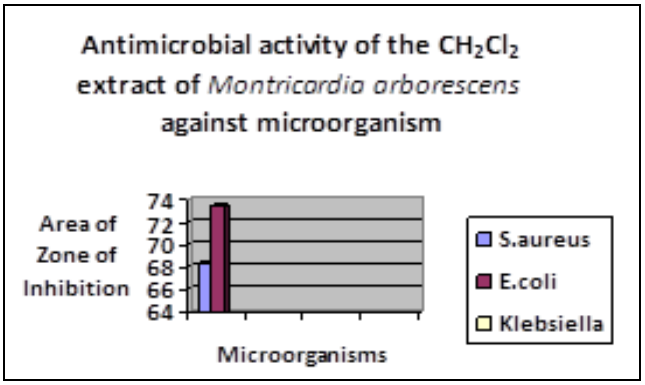

FIG. 4

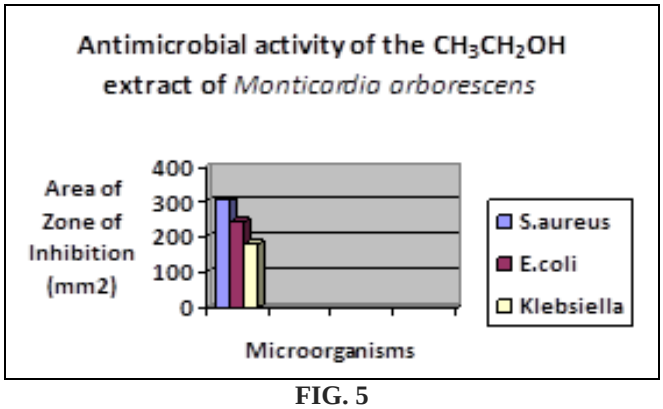

FIG. 5

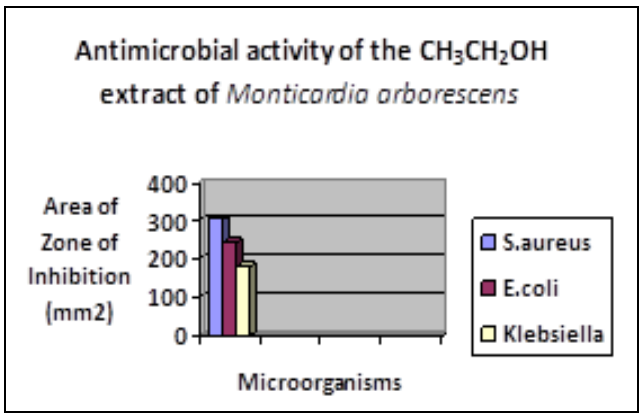

FIG. 6

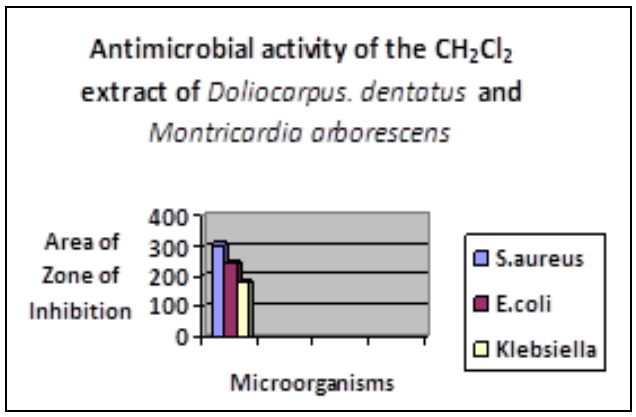

FIG. 7

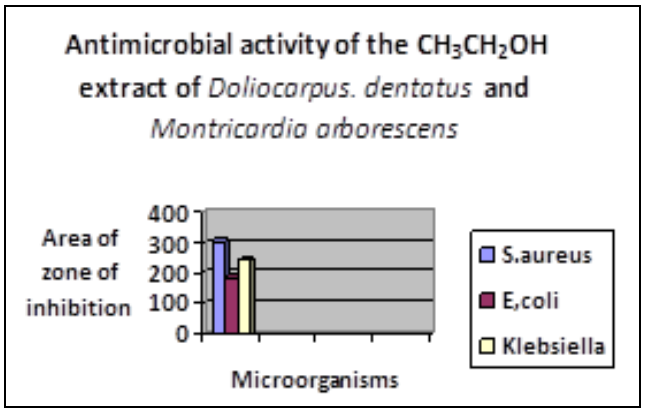

FIG. 8

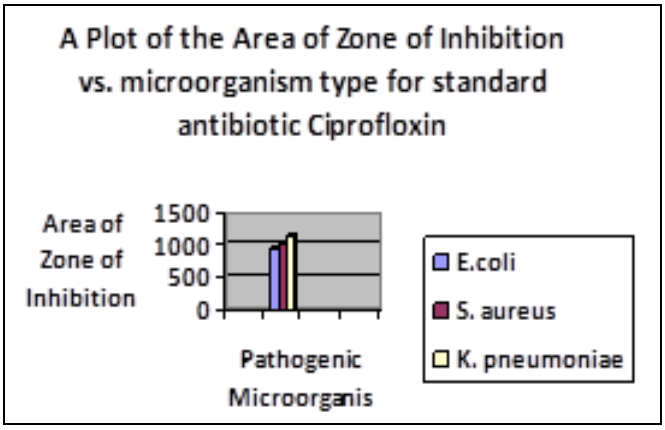

FIG. 9

DISCUSSION: Antimicrobial activity of Doliocarpus *dentatus* and *Moko moko*, *Monticardia arborescens* were investigated against bacterial strains: *S. aureus*, *E. coli* and *Klebsiella pneumonia* using Disc/Agar diffusion assay under aseptic conditions. The diameter of zone of inhibition measured in mm was used as an indication of the bacteria susceptibility to the two plant extracts. From the results, **Table 1-4** and graphical displays, **Fig. 1-9**, the following were noted. It was evident that the aqueous and CH_3CH_2OH extract of *Doliocarpus dentatus* were not antimicrobial as the diameter of zone of inhibition was less than 5 mm^2.

Interestingly, the CH_2Cl_2 and EtOAc extract of *Doliocarpus dentatus* induces significant zone of inhibition against all three bacterial strains, the area of zone of inhibition ranging from 68.3 mm^2 to 89.4 mm^2. The order of susceptibility of bacterial strains to the CH_2Cl_2 extract follows the sequence: *E.coli* > *Klebsiella pneumoniae* > *S.aureus*. For the EtOAc extract, the order of bacterial susceptibility follows the trend: *E.coli* > *S. aureus* > *Kblesiella pneumoniae*

The n-C_6H_{14}, CH_2Cl_2 and CH_3CH_2OH extract of *Montricardia arborescens* are indeed antimicrobial, Table 2-4. Significant zone of inhibition were observed. The area of zone of inhibition (AZOI) ranging from 161.2 mm^2 to 368.6 mm^2. The order of microbial potency of the extract been: C_2H_5OH > n-C_6H_{14} > CH_2Cl_2, suggesting that the plant antimicrobials are localized primarily in the CH_3CH_2OH extract. The order of bacterial susceptibility for the n-C_6H_{14} and CH_3CH_2OH extract is: *E. coli* > *Kblesiella pneumoniae* > *S. aureus*. For the CH_2Cl_2 extract, the susceptibility trend is: *E. coli* > *Kblesiella pneumoniae* > *S. aureus*

In an effort to amplify the antimicrobial potency, the CH_2Cl_2 mixture of *Doliocarpus dentatus* and *Montricardia arbtorescens* and that of a mixture of CH_3CH_2OH extract of *Doliocarpus dentatus* and *Montricardia arborescens* were investigated, Table 2-4. It was observed that the CH_2Cl_2 mixture of *Doliocarpus dentatus* and *Montricardia arboresecens* (50:50, v/v) gave significance higher zone of inhibition than either was observed for the mixture of *Doliocarpus. dentatus* and *Montricardia arborescens* extract against *S. aureus* and *E. coli* respectively.

For the CH_2Cl_2 extract of *Doliocarpus dentatus* and *Montricardia arborescens*, area of zone of inhibition of (73.4 mm^2, 60.3 mm^2) and (89.37 mm^2, 73.40 mm^2) were observed for *Doliocarpus dentatus* CH_2Cl_2 and *Montricardia arborescens* respectively. The order of bacterial susceptibility to the combined CH_2Cl_2 extract been: *S. aureus* > *E. coli* > *Kblesiella pneumonia.*

However, there was a variation in the potency of the mixed CH_3CH_2OH extract compared with the potency of the individual C_2H_5OH extract. The mixed CH_3CH_2OH extract showed a smaller zone of inhibition (184.48 mm^2) against *E. coli and Klebsiella pneumoniae* compared with the C_2H_5OH extract of either plant. However, the mixed C_2H_5OH extract showed a larger area of zone of inhibition (303.7 mm^2) against *S. aureus* compared with the C_2H_5OH extract of either plant. For the CH_3CH_2OH mixture, the order of bacterial susceptibility been: *S. aureus* > *K. pneumoniae* > *E. coli*

The antimicrobial activity of standard antibiotics such as Ciprofloxin was also investigated against the above three bacterial strains, Table 1. The diameter and area of zone of inhibition was found to be greater than that induced by the antimicrobial activity of either plant extracts or the combined plant extracts.

The n-C_6H_{14}, CH_2Cl_2 and CH_3CH_2OH extract of *Montricardia arborescens* were subjected to atomic spectrophotometric analyses, Table 5. Recently, the atomic spectrophotometric analysis of *Doliocarpus dentatus* was reported. Elements tested for were K, Cu, Fe, Co, Zn and Pb. Atomic spectrophotometric analyses revealed selective presence of metal ions. For example, the n-C_6H_{12} extract shows a high concentration for Zn (8,784 mg/L) but a low concentration for manganese (Mn = 85 mg/L). Likewise the CH_2Cl_2 extract showed a high concentration for cobalt, Co (8,142 mg/L) but a low concentration for potassium, K.

Zinc, micronutrients is essential for optimum crop growth. Their deficiency causes various adverse effects on growth and yield of crops. It is also involved in the formation of chlorophyll, carbohydrates, in several dehydrogenises, proteinese and peptidase enzymes. It promotes growth hormones (auxin) and starch formation.

For the C_2H_5OH extract, there was no detection of Fe, Mn and Zn. Table, 6 shows the R_f values for the components of the n-C_6H_{14}, CH_2Cl_2, and CH_3CH_2OH extract of *Montricardia arborescens*. As indicated seven, five and four components were detected for these extracts respectively, each presumably due to a pure natural product.

CONCLUSION: The CH_2Cl_2 and EtOAC extract of *Doliocarpus dentatus* have been shown to be antimicrobial, whereas the CH_3CH_2OH and aqueous are not. However, all the solvent type extract of *Montricardia arborescens* are antimicrobial. In addition, the combined extract of *Doliocarpus dentatus* and *Montricardia arborescens* have shown enhanced antimicrobial activity in comparison to the individual solvent type extract. Atomic absorption spectroscopic analyses of the three extracts reveal selective presence of the seven metals: K, Cu, Fe, Mn, Co, Zn and Pb in the solvent type extract.

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