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ANTIMICROBIAL ACTIVITY OF TANNINS AND EXTRACTS OF DIFFERENT PARTS OF *CONOCARPUS ERECTUS* L.

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ABSTRACT

In this study, the antimicrobial activity of crude extracts of flower, fruit, stem and leaf of Conocarpus erectus and purified tannins were evaluated against three fungi and seven Gram-positive, Gram-negative and acid-fast bacteria. While, by disc diffusion method, extracts of different parts of C.erectus were active only against Saccharomyces cerevisiae, purified tannins were active against Sac. cerevisiae, Aspergillus niger and Penicillium chrysogenum. Disc diffusion method was also used to evaluate the antibacterial activity of the crude methanolic extracts. The extracts had broad spectrum activity and their descending order of activity was flower, fruit, stem and root. Gram-positive bacteria were more sensitive to extracts than other bacteria. Methanol extracts of different parts of C. erectus were fractionated by chloroform, ethyl acetate, and n-butanol and their MICs and MBCs were determined against the tested bacteria. Generally speaking ethyl acetate extracts were more active than other fractionated extracts. The two tested Gram-positive bacteria, Staphylococcus aureus and Bacillus subtilis were the most sensitive, Mycobacterium phlei and Pseudomonas aeruginosa were intermediate, and Escherichia coli, Salmonella typhimurium and Klebsiella pneumonia were the least sensitive to the extracted fractions. Compared to crude methanol extract, tannins of C. erectus were significantly ($p \leq 0.01$) more active than alcohol extracts against the tested bacteria and had 6 to 12 times lower MICs and MBCs. Therefore, it may be concluded that C. erectus extracts have broad spectrum antimicrobial activity and their activity could be attributed, to a great extent, to their contents of tannins.

KEY WORDS

Antibacterial, antifungal, Conocarpus erectus, tannins

INTRODUCTION

Although, the use of plants to treat various maladies including common infectious diseases is an art as old as mankind, plant-based, traditional medicine continues to play an essential role in health care. About 80% of the world's inhabitants are relying mainly on traditional medicines for their primary health care [1]. Medicinal plants represent a rich source of antimicrobial agents and a source of many potent and powerful drugs [2, 3]. In spite of the recent domination of the synthetic chemistry as a method to discover and produce drugs, the potential of bioactive plants or their extracts to provide new and novel products for disease treatment and prevention is still enormous [4-6]. Therefore, in recent years, there appears to be a revival in the use of traditional medicinal plants and pharmaceutical companies have spent a lot of money in developing natural products extracted from plants, to produce more safe and cost effective remedies [7]. More than 130 drugs, extracted from higher plants, or modified further synthetically,

are currently in use, though some of them are now made synthetically for economic reasons [2, 8]. Although, hundreds of plant species have been tested for their medicinal properties, the vast majority of plants have not been adequately evaluated [9].

Conocarpus erectus L., family *Combretaceae*, which is commonly called button wood or button mangrove, is a tropical and subtropical evergreen tree. It is widely cultivated as an ornamental tree in many countries around the world, including Saudi Arabia. The tree is 6m tall with spreading crown, grey or brown bark, glaucous medium-green leaves and greenish flowers in dense cone-like heads in terminal panicles [10]. In folk medicine *C. erectus* is used as a remedy for several ailments like anemia, catarrh, conjunctivitis, diabetes, diarrhea, fever, hemorrhage, orchitis, skin ulcers and syphilis [11-13]. *C. erectus* extracts were found to possess antioxidant and anticancer capacities [14, 15].

The increasing incidence of resistance of pathogenic bacteria to antibiotics emphasizes the need to screen plants for their potential antimicrobial activity [16, 17]. In a preliminary study in our laboratory, we demonstrated that *C. erectus* has antibacterial activity [15]. In another study, amongst 50 South Florida medicinal plants extracts tested for anti-quorum sensing activity using *Chromobacterium violaceum* 12427, six plant extracts including *C. erectus*, had anti-quorum sensing activity [18]. In this study, we report to the first time the antimicrobial activity of extracts of different parts of *C. erectus* and purified tannins against different types of bacteria and fungi.

METHODS

Plant and chemicals used:

Different parts of *C. erectus* (leaves, fruits, flowers and stems) were collected from trees growing in Taif City. The collected parts of the plant were dried on the shade away from light and ground to powder using an electric blender. Chemicals and media used were of analytical grade and were purchased from Sigma-Aldrich and Oxoid.

Extraction of the plant:

Known weights of the dry powdered plant parts, were extracted by methanol and the extracts were dried using a rotary vacuum evaporator. The alcoholic extracts were defatted with petroleum ether and were, then successively fractionated with organic solvents of different polarities; chloroform, ethyl acetate and n-butanol.

Bacterial and fungal strains

Standard and clinical isolates of bacteria and fungi used in this study and their sources are summarized in **Table 1**.

Table 1: Bacteria and fungi used in the study and their source

Bacteria	*Characteristic*	*Source*
Bacillus subtilis	Standard 168 strain	Faculty of Pharmacy, Tanta University, Egypt
Staphylococcus aureus	Ap, Cf, Sm, Gn*	Clinical isolate, Taif University, KSA
Mycobacterium phlei	Standard strain (ATCC 6841)	Tanta University, Egypt
Pseudomonas aeruginosa	Ap, Pp, Cp, Tc, Cm, Sx, Sm*	Clinical isolate, Taif University, KSA
Escherichia coli	Ap, Tc, Cm, Sx, Sm*	Clinical isolate, Tanta University, Egypt
Klebsiella pneumoniae	Ap, Pp, Cp, Tc, Cm, Sx*	Clinical isolate, Tanta University, Egypt
Salmonella typhimurium	Ap, Tc, Sx, Sm*	Clinical isolate, Taif University, KSA
Aspergillus niger	Standard strain (ATCC-13794)	Faculty of Pharmacy, Tanta University, Egypt
Penicillium chrysogenum	Standard strain (ATCC-18226)	Faculty of Pharmacy, Tanta University, Egypt
Saccharomyces cerevisiae	Standard strain (ATCC-9080)	Faculty of Pharmacy, Tanta University, Egypt

* Clinical isolate resistance to: Ap, ampicillin; Cf, cephalexin; Cp, cephoperazone; Pp, piperacillin; Tc, tetracycline; Sm, streptomycin; Cm, chloramphenicol; Sx, sulfamethoxazole; Gn, gentamicin.

Assessment of antimicrobial activity by agar well diffusion method

This was done as previously described by Halawani and Shohayeb [19], with some modifications. Briefly, suspensions (10^7CFU/ml, in case of bactria) of each tested microorganism in physiological saline were spread onto the surface of Muller-Hinton agar and Sabouraud's dextrose agar plates for bacteria and fungi respectively. Six mm cork borer was used to punch wells into the plates and 50 µl of each extract dissolved in DMSO (10 mg/ml) was applied to each well. The plates were incubated for 18 h at 37°C in case of bacteria and for 48 h at 27°C in case of fungi. The diameter of inhibition zones for each extract was measured.

Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

MIC and MBC were performed as previously described [20]. Briefly extracts were serially diluted with nutrient broth to give series of concentrations in sterile microtitre plates. Each series of dilutions was inoculated with 10^4 CFU/ml of the tested bacteria and incubated at 37°C for 18 hours before determining the least concentration that inhibited the appearance of visible growth. The minimum bactericidal concentrations (MBCs) were determined from broth microdilution assays by subculturing 10 µl volumes from inhibitory concentrations onto Muller-Hinton agar plates.

Preparation of tannins

Dry powdered leaves, were extracted with methanol and the extract was defatted with petroleum ether and dried using a rotary vacuum evaporator. Tannins were separated from methanol extract, using XAD-16 resin as described by Seeram, *et al.,*[21]. Briefly, the resin was packaged into a glass column, washed with methanol and equilibrated with water. Vacuum was applied to remove water from the resin. Dried methanol extract of leaves was dissolved in water and applied to the column. The column was washed with water which was removed from the column by vacuum aspiration and the adsorbed tannins were eluted by methanol. The collected dark brown solution was evaporated at 50 °C in a rotary vacuum evaporator.

Test for tannins [22]

Small amount of the crude methanol extract or purified tannins were dissolved in water separately and warmed up. A few drops of 5% ferric chloride solution were added and observed for the formation of green or blue colour.

Statistical analysis

All determinations were carried out in triplicates and the statistical analyses were carried out using SPSS 13.0.

RESULTS AND DISCUSSION

Phytochemical constituents of medicinal plants are secondary metabolites that may act as antimicrobial agents [23]. In this study extracts of *C. erectus* and purified tannins were evaluated qualitatively and quantitatively for their antimicrobial activities.

Crude alcoholic extracts of leaf, stem, fruit and flower were evaluated against Gram-positive, Gram-negative, acid-fast bacteria and fungi by agar disc diffusion method. As shown in **Table 2**, while, tannins were active against the three tested fungi (*Sac. cerevisiae, A. niger*and *P. notatum*), extracts of flower, fruit, leaf and stem crude extracts were active only against *Sac. Cerevisiae*. The inhibition zones exerted by alcoholic extracts of flower, fruit, leaf and stem against *Sac. cerevisiae* were respectively 11.3±0.57, 13.3±0.57, 10.3 ±0.58 and 11.0 ±1.0mm. On the other hand inhibition zones of tannins against, *Sac. cerevisiae, A. niger* and *P. notatum* were 14.3±0.58, 12.5±1.29 and 13.3± 0.58 mm respectively (**Table 2**).

The inhibition zone diameters of alcoholic extracts of different parts of *C. erectus* against the seven tested bacteria ranged between 10.5 and 23mm (**Fig 1**). Generally speaking flower alcoholic extract was relatively more active than extracts of other parts of the plant. The descending order of activity of the extracted parts of the plant was flower, fruit, stem and leaf respectively (**Fig 1**). On the other hand Gram-positive bacteria, *S. aureus* and *B. subtilis*, were more sensitive than Gram-negative and acid-fast bacteria and their inhibition zones ranged between 21.0 and 23 mm. Inhibition zones of the acid-fast *M. phlei* and the tested Gram-negative bacteria (*E. coli*, *Sal. typhimurium, K. pneumonia* and *P. aeruginosa*) ranged between 11.0 and 18.0mm (**Fig 1**).

Because phytoconstituents have different solubility in different solvents, crude methanolic extracts of different parts of the plant, were partitioned by

chloroform, ethyl acetate and n-butanol. The antibacterial activity of these solvent extracts was quantitatively assessed by the determination of their MIC and MBC against the tested bacteria (**Tables 3 and 4**).

Table 2: Antifungal activity of methanol extracts of flower, fruit, and purified tannins of *Conocarpus erectus* and its tannins

Tested extract	*Fungus*		
	Saccharomyces cerevisiae	*Aspergillus niger*	*Penicillium notatum*
	Zone of inhibition in mm		
Flower ext.	11.3±0.57	-	-
Fruit ext.	13.3±0.57	-	-
Leaf ext.	10.3±0.58	-	-
Stem ext.	11.0±1.0	-	-
Tannins	14.3±0.58	12.5±1.29	13.3± 0.58

Table 3: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of fractionated extracts of *Conocarpus erectus* against Gram-positive ad acid-fast bacteria

Part of the plant	Solvent fraction	*Bacillus subtilis*		*Staphylococcus aureus*		*Mycobacterium phlei*	
		MIC	MBC	MIC	MBC	MIC	MBC
		mg/ml					
Fruit	Methanol	0.33±0.14	0.67±0.29	0.33±0.14	0.67±0.29	1.33± 0.58	2.33±0.58
	Chloroform	0.33±0.14	0.67±0.29	0.67±0.29	1.33± 0.58	2.33±0.58	2.33±0.58
	Ethyl acetate	0.21±0.07	0.42±1.4	0.33±0.14	0.67±0.29	0.33±0.14	0.67±0.29
	n-butanol	0.67±0.29	1.00 ±0.0	0.42±0.14	0.83±0.29	0.67±0.29	1.33±0.58
Flower	Methanol	0.42±0.14	0.67±0.29	0.42±0.14	0.67±0.29	0.67±0.29	1.33±0.58
	Chloroform	0.50 ±0.0	1.33± 0.58	1.33± 0.58	1.33± 0.58	0.67±0.29	1.67± 0.58
	Ethyl acetate	0.33±0.14	0.42±1.4	0.33±0.14	0.42±0.14	0.42±0.14	0.83± 0.29
	n-butanol	0.42±1.4	0.67±0.29	0.50 ± 0.0	1.0 ± 0.0	0.67±0.29	1.33±0.58
Stem	Methanol	1.00 ±0.0	2.33±0.58	1.33± 0.58	2.33±0.58	0.67±0.29	1.67± 0.58
	Chloroform	1.33± 0.58	2.33±0.58	1.33± 0.58	2.33±0.58	0.67±0.29	1.67± 0.58
	Ethyl acetate	0.33±0.14	0.67±0.29	0.33±0.14	0.83±0.29	0.42±0.14	0.83±0.29
	n-butanol	1.33± 0.58	2.33±0.58	0.83±0.29	1.33± 0.58	0.83±0.29	2.33±0.58
Leaf	Methanol	0.67±0.29	0.83±0.29	0.67±0.29	1.33±0.58	2.33±0.58	5.33±2.31
	Chloroform	0.67±0.29	1.00 ± 0.0	1.00 ±2.2	2.33±0.58	2.33±0.58	8.0 ± 0.0
	Ethyl acetate	0.33±0.14	0.67±0.29	0.42±0.14	0.83±0.29	0.67±0.29	2.33±0.58
	n-butanol	1.00 ±0.0	2.33±0.58	1.00 ± 0.0	2.33±0.58	2.33±0.58	3.7±0.58

Table 4: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of fractionated extracts of *Conocarpus erectus* against Gram-negative bacteria

Part of the plant	Solvent fraction	*Pseudomonas aeruginosa*		*Escherichia coli*		*Salmonella typhimurium*		*Klebsiella pneumoniae*	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
		mg/ml							
Fruit	Methanol	0.42±0.14	0.83± 0.29	0.83± 0.29	2.33±0.58	1.67± 0.58	5.33±2.31	5.33±2.31	>8.0± 0.0
	Chloroform	0.83±0.29	1.67± 0.58	1.67± 0.58	4.0±0.00	2.0±0.0	5.33±2.31	6.7±0.58	>8.0± 0.0
	Ethyl acetate	0.42±0.14	0.67±0.29	0.83± 0.29	2.33±0.58	1.0± 0.0	2.33±0.58	3.7±0.58	5.33±2.31
	n-butanol	0.67±0.29	0.8±0.29	2.33±0.58	8.0± 0.0	2.33±0.58	5.33±2.31	8.0±0.00	>8.0± 0.0
Flower	Methanol	0.67±0.29	1.33± 0.58	2.33±0.58	5.33±2.31	2.33±0.58	6.7±0.58	5.33±2.31	8.0±0.00
	Chloroform	1.33± 0.58	2.0± 0.0	1.33± 0.58	4.0± 0.0	2.33±0.58	8.0±0.0	5.33±2.31	>8.0± 00
	Ethyl acetate	0.42±0.14	0.83± 0.29	0.83± 0.29	3.7±0.58	2.0±0.0	4.0±0.0	4.0±0.0	8.0± 0.0
	n-butanol	0.5±0.0.0	1.33± 0.58	1.67± 0.58	2.33±0.58	5.33±2.31	5.33±2.31	8.0±0.0	>8.0± 0.0
Stem	Methanol	3.7± 0.58	5.33± 0.23	3.7±0.58	8.0± 0.0	4.0±0.0	8.0±0.0	5.33±2.31	8.0±0.00
	Chloroform	6.7±2.31	>8.0±0.0	8.0± 0.0	>8.0± 0.0	8.0±0.0	>8.0±0.0	>8.0± 0.0	>8.0± 0.0
	Ethyl acetate	0.67±0.29	2.33±0.58	3.7±0.58	6.7±2.31	2.0±0.0	6.7±0.58	5.33±2.31	8.0± 0.0
	n-butanol	2.33±0.58	>8.0± 0.0	3.7±0.58	8.0± 0.0	5.33±2.31	>8.0±0.0	8.0±0.0	>8.0± 0.0
Leaf	Methanol	5.33±2.31	8.0± 0.0	5.33±2.31	8.0± 0.0	6.7±0.58	>8.0±0.0	8.0± 0.0	>8.0± 0.0
	Chloroform	5.33±2.31	>8.0± 0.0	5.33±2.31	>8.0± 0.0	4.0±0.0	>8.0±0.0	8.0±0.0	>8.0±0.0
	Ethyl acetate	2.33±0.58	5.33±2.31	3.7±0.58	8.0±0.00	3.7±0.58	8.0±0.0	5.33±2.31	8.00±0.0
	n-butanol	4.0±0.0	8.0± 0.0	8.0±0.0	>8.0± 0.0	8.0±0.0	>8.0±0.0	8.0± 0.0	>8.0±0.0

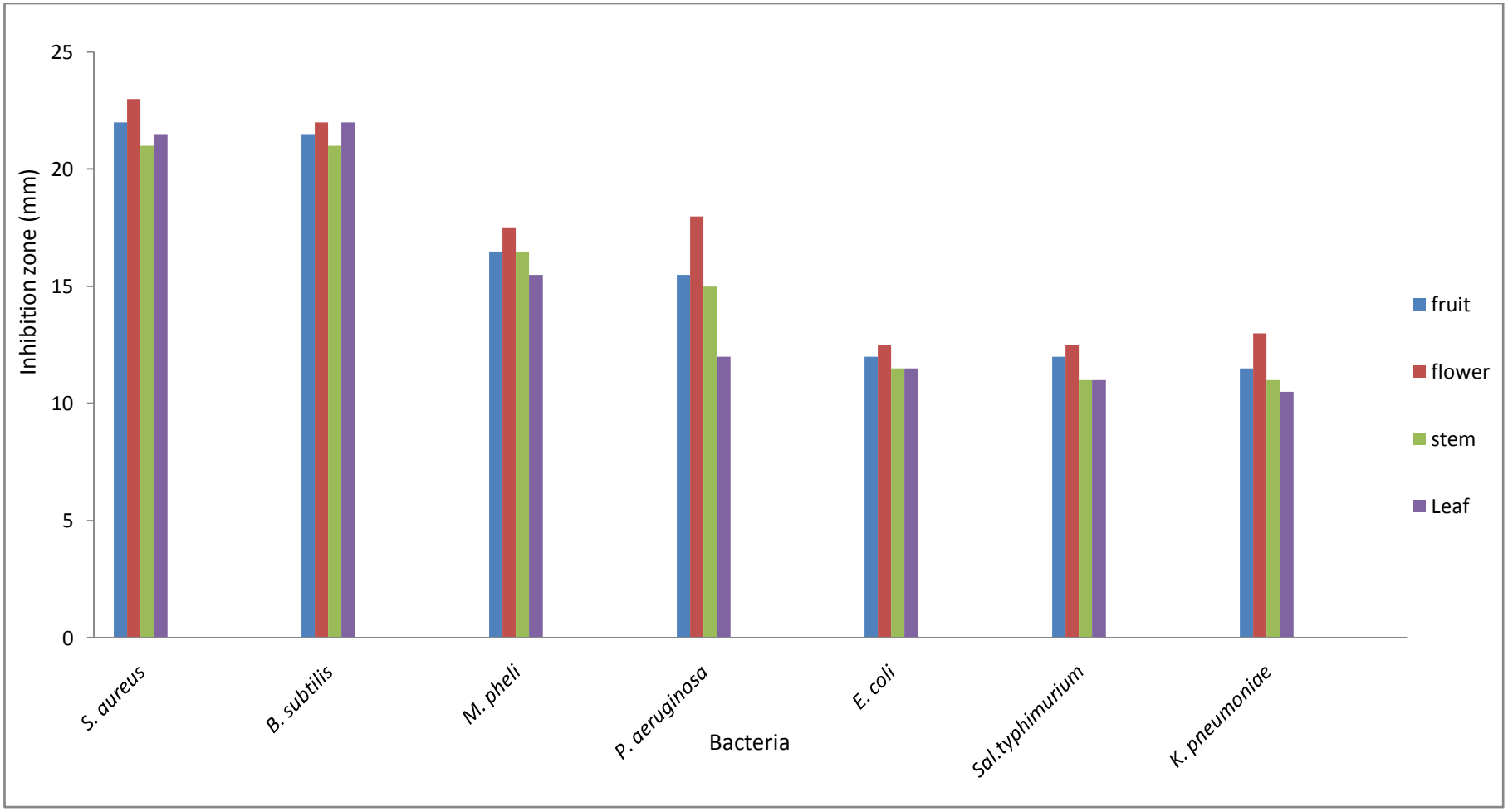

Figure 1: Mean zones of inhibition of methanol extract of different parts of *Conocarpus erectus* against different types of bacteria

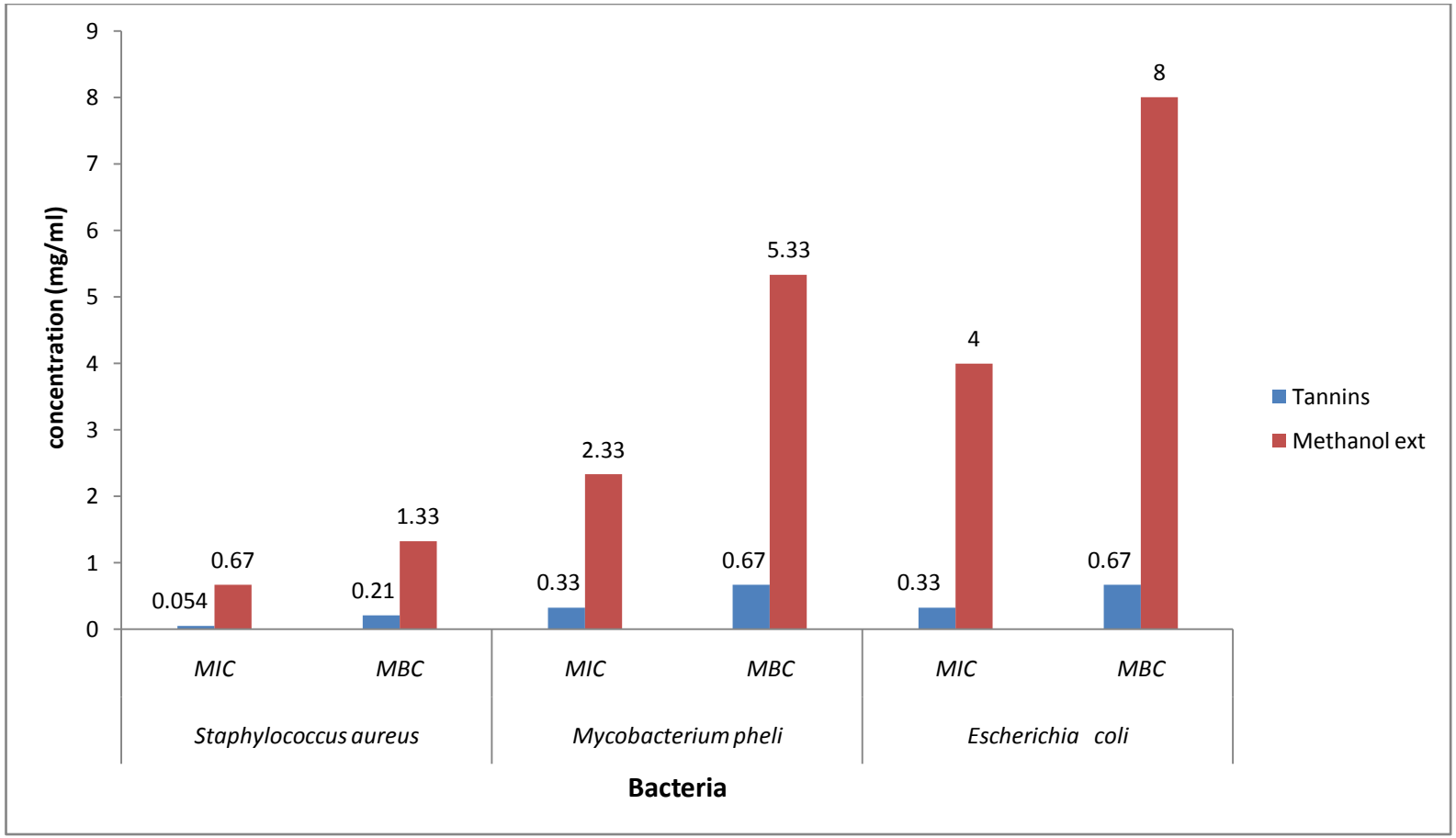

Figure 2: Mean minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of methanol extract of leaf of *Conocarpus erectus* and purified tannins against three types of bacteria.

All four fractions exerted broad spectrum antibacterial activity against Gram-positive, Gram-negative and acid-fast bacteria. Extracts of fruits and flowers of the plant were, generally speaking, relatively more active as antibacterial than the other parts of the plant as previously demonstrated by disc diffusion method. With regard to the fractions, extracts partitioned with ethyl-acetate were relatively more active than most other extracts of *C. erectus* (**Tables 3 and 4**). Based on the MICs and MBCs of different extraction fractions of *C. erectus* against the tested organisms, the two tested Gram-positive bacteria, *S. aureus* and *B. subtilis* were relatively, but not significantly ($p \geq 0.05$), the most sensitive and their MICs and MBCs were 0.21-1.33 mg/ml and 0.42-2.33 respectively. *P. aeruginosa,* was relatively more sensitive than the other Gram-negative bacteria and *K. pneumoniae* was the least sensitive one. The MICs of both *B. subtilis* and *S. aureus* for different fractions were in most cases significantly ($p \geq 0.05$) less than those of *K. pneumoniae. M. phlei*, on the other hand, was intermediate in its sensitivity to the extracted fractions compared to Gram-positive and Gram-negative bacteria.

The higher susceptibility of the tested Gram-positive bacteria than Gram-negative bacteria to *C. erectus* extracts is consistent with previous studies on the antibacterial activity of natural products [20, 24-27]. A possible explanation for this observation may lie on the fact that Gram-negative bacteria possess an outer membrane which acts as a barrier which prevents or decreases the penetration of numerous antimicrobials [28-30]. Because Gram-positive bacteria lack the outer membrane, and possess only a cytoplasmic membrane, it is more vulnerable to damaging molecules and this leads to the leakage of their cytoplasm contents [31].

P. aeruginosa and *M. phlei* were found to be more sensitive to different extracts of *C. erectus* compared to the other tested Gram-negative bacteria. This is rather interesting because both organisms are known to be less susceptible to antimicrobials including antibiotics, preservatives, antiseptics and disinfectants [32, 33]. This resistance is attributed to the less permeable outer membrane of *P. aeruginosa*[33]. On the hand, *M. phlei* is characterized by a cell wall which is highly hydrophobic with a mycolyl-arabinogalactan-peptidoglycan skeleton that leads to its impermeability to antimicrobials [34].

The least sensitive Gram-negative bacterium was *K. pneumonia*. This bacterium was also reported previously to have a lower susceptibility to the antibacterial effect of *Nigella sativa* extracts [27]. This lower susceptibility may be attributed to the surrounding capsule which is made of acidic polysaccharides [35]. The large partially negatively charged macromolecular structure of the capsule was reported to decrease the uptake of antimicrobials like peptides [36]. It seems from this study that the capsule might also decrease the uptake of the antibacterial phytoconstituents of *C. erectus* extracts presumably, as a result of their repulsion or attraction to its charged polysaccharide.

Tannins are water-soluble polyphenols that are commonly found in higher herbaceous and woody plants [37]. They have been reported to possess both bacteriostatic and bactericidal activities [37-39]. Because *C. erectus* contains large amounts of tannins [15, 40], in this study, tannins were purified from methanolic extracts of leaves.

The MICs and MBCs of tannins against *S. aureus, M. phlei* and *E. coli* were determined and compared with their corresponding values for methanol extract of leaves (**Fig 2**). The MICs and MBC were at least 6 to 12 times lower than those of alcoholic extracts. Both MICs and MBC were significantly ($p \leq 0.05$) more active than methanol extracts of leaves against the three tested organisms. The mean MICs of tannins against *S. aureus, M. phlei* and *E. coli* were 0.054, 0.33 and 0.33 mg/ml, respectively and their respective MBCs were 0.21, 0.67 and 0.67 mg/ml (Fig 2). On the other hand the MICs and MBCs in case of methanol extract were 0.67-4.0 mg and 1.33-8.0 mg/ml respectively (**Fig 2**).

The five clinical Gram-positive and Gram-negative isolates included in this study were multidrug-resistant (Table 1), and this did not affect their susceptibility to *C. carpus* extracts. The phenomenon of irrelevance of resistance to chemotherapeutic agents and the antibacterial activity of natural products has been previously reported [27, 41]. This is likely because the mechanisms of action of the phytochconstituents of *C. erectus* are different to those of antibiotics.

CONCLUSION

Extracts of different parts of *C. erectus* investigated in this study possessed broad-spectrum antimicrobial activity against Gram-positive, Gram-negative, acid-fast bacteria and fungi. The broad-spectrum antibacterial activity of the plant extracts, confirms its use as a health remedy in folklore medicine. The higher antibacterial activity of *C. erectus* purified tannins against bacteria compared to different extracts suggests that tannins are responsible to a great extent for the antimicrobial activity *C. erectus*.

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