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Research Article

EVALUATION OF ANTIOXIDANT, ANTIBACTERIAL ACTIVITY OF ETHANOLIC EXTRACT IN THE LEAVES OF *COMBRETUM ALBIDUM* AND GAS CHROMATOGRAPHY-MASS SEPTROMETRY ANALYSIS

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ABSTRACT

Objective: This study evaluates chemical composition, antioxidant, and antibacterial activity of ethanol extract from leaves of *Combretum albidum*.

Methods: An evaluation of antibacterial activity was carried out by the disc-diffusion method and resazurin microplate assay to determine minimum inhibitory concentration (MIC). The anti-oxidant activity was done using 1,1-diphenyl-2-picrylhydrazyl (DPPH) method and superoxide assay. The gas chromatography-mass septrometry evaluation of *C. albidum* was done to analyze the phytochemical constituents that are responsible for the antimicrobial and antioxidant property. The results were compared with NIST library.

Results: The disc-diffusion method showed zone of inhibition that ranged from 19.8±0.57 (*Bacillus subtilis*) to 9.3±0.57 (metallo-beta-lactamase producing *Escherichia coli* [MBLe]), and the MIC was least for *B. subtilis* at 0.08 mg/ml. It was found that *C. albidum* exhibited antibacterial activity against all the 8 Gram-negative and Gram-positive bacteria including multidrug-resistant bacteria, MBL producing bacteria, and methicilin-resistant *Staphylococcus aureus*. The IC_{50} value for the antioxidant activity was 156.26 µg/ml and 69.03 µg/ml for DPPH and superoxide assay, respectively. This study yielded a total of 23 bioactive compounds with potent biological activity.

Conclusion: The study proves the potential ability of *C. albidum*, suggesting its exploitation in bioactive compounds for the antimicrobial and antioxidant property.

Keywords: *Combretum albidum*, Antioxidant activity, Antibacterial activity, Bioactive compounds.

INTRODUCTION

Drug resistance to pathogenic bacteria has lead to an alarming situation worldwide due to the development of antibitotic resistance genes against current antibitotics [1,2]. Misuse and overuse of antibiotics have led to new resistance patterns in several bacteria and the WHO focuses on control and prevention of such antibiotics. Multi-drug resistant (MRD) such as metallo-beta-lactamase (MBL) and methicillin-resistant *Staphylococcus aureus* (MRSA) bacteria is rapidly evolving causing an emerging threat to the community [3,4]. The discovery and development of newer antibiotics are limited with the increase in resistance of such MRD bacteria [5] creating the need for alternative new therapeutic agents.

Phytochemical from medicinal plants provide new drugs due to their diverse chemical structures present as secondary metabolites. The several medicinal plants are explored for bioactive compounds to cure various diseases and infections. Steroids, terpenoids, alkaloids, glycosides, tannins, flavonoids, and phenolic compounds are reported to have inhibitory effects on a variety of micro-organisms [6]. These bioactive compounds overcome the disadvantage of chemically derived drugs and decrease the development of resistance in bacteria [7]. The treatment of bacterial infections by plant-based drugs may reduce the adaptability nature of bacteria to evolve into resistant forms. Oxidative damage to the human body may be reduced by antioxidants, which are abundant in medicinal plants and fruits [8,9]. The presence of various antioxidant compounds, especially, phenolics, which are the most reactive compounds may neutralize free radicals by donating an electron [10].

India comprises a large number of diverse medicinal plants. It is anticipated that phytochemicals present in these plants can be used for the treatment of bacterial infections [11]. *Combretum albidum* is commonly known as Manjakody or "buffalo calf" in English. The plant is used as an effective remedy for jaundice, diarrhea, dysentery and skin diseases by Muthuvans, Chinnar tribe of Idukki District, Kerala and Paliyan tribes in Sirumalai Hills of Eastern Ghats [12-15]. The bioactivity of *C. albidum* is not much explored, and such medicinal plants are of interest because of their availability for phytoconstituents with therapeutic value. This study was undertaken to investigate the activity of ethanolic extracts from *C. albidum* that may have a large number of potential antibacterial and antioxidant compounds. The evaluation of ethanol extract of *C. albidum* was done against Gram-negative and Gram-positive pathogens. The constituents responsible for the bioactivity of extract was studied and identified using gas chromatography-mass spectrometry (GC-MS).

METHODS

Collection and identification of plant material

The fresh leaves of from *C. albidum* were collected from Kancheepuram district, Tamil Nadu, India. The collected plant was authenticated by a taxonomist and further confirmed by DNA barcoding.

Preparation of extract

The leaves were rinsed thoroughly with running water followed by 70% ethanol and dried at 36°C±2°C for 48-72 h. The dried plant leaves were grind into a coarse powder in a laboratory blender, labeled and stored in airtight containers at room temperature until used for extraction. The coarse powder (10 g) was soaked in 100 mL of alcohol. The mixture was macerated in a shaker incubator at 150 rpm, and 37°C for 24 h and the process was repeated thrice for completion of extraction. The macerated samples were filtered through Whatman No. 1 filter

paper, and the solvent was removed using rotary evaporator under reduced pressure, and dried extracts were stored in the refrigerator for further use.

Zone of inhibition

Confirmation of antibacterial activity of plant extract was carried out using a disc diffusion method [16,17,19,20]. The density of the test cultures was standardized using McFarland 0.5 turbidity standard (1×10^8 CFU/mL), were swabbed on the top of the Muller-Hinton agar media and allowed to dry for 10 minutes. The disks loaded with ethanolic extract of *C. albidum* (2 mg) and placed on the surface of the medium. Colistin (10 µg/disc) was used as positive control, and the negative control was prepared using dimethyl sulphoxide (DMSO) (20 µL/disc). The culture plates were incubated for 24 h at 37°C and zone of inhibition was measured for each plant in triplicate.

Minimum inhibitory concentration (MIC)

MIC was determined using Resazurin Microplate Assay, described by Palomino *et al*. [18] with slight modification. A stock solution of plants extract (500 µg/µL) was prepared in DMSO, and subsequent serial dilutions (10.24-0.02 mg/mL) were made by micro broth dilution technique in Muller-Hinton broth. The culture concentration was grown to 0.5 McFarland, and 3 µL of inoculum was added to 100 µL reaction mixture. Colistin was used as positive control, and DMSO was used as negative control. Sealed microtitre plates were incubated overnight at 37°C. After the incubation period, 3 µL of resazurin solution was added to the wells and incubated overnight at 37°C for color change (blue to pink).

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

A stable radical DPPH was used to measure antioxidant activity in terms of hydrogen donating or radical scavenging ability. The experiments were done according to the method of Blois [19] with a slight modification. A volume of 2 mL of an ethanolic plant extracts stock solution with 2 mL of 1 mM DPPH solution was added into test tubes. The tubes were covered with parafilm and kept again in the dark for 30 minutes. Absorbance at 517 nm was measured, and each assay was carried out in triplicate. Percentage inhibition was calculated using:

$$\%I=[(A_0-A_1)\div A_0]\times I=d$$

Where A_0 was the absorbance of the control, and A_1 was the absorbance of the extract or standard.

Superoxide radical (O_2) scavenging activity

The assay was based on the capacity of the aqueous extract to inhibit formazan formation by scavenging the superoxide radicals generated in a riboflavin-light-nitrobluetetrazolium (NBT) system [20]. The reaction mixture contained 20 µg riboflavin, 12 mM ethylenediaminetetraacetic acid, NBT, 0.1 mg/3 mL, and 50 mM phosphate buffer pH 7.6 added in that sequence [21]. The absorbance was measured at 590 nm after illumination. Similar tubes with reaction mixture kept in the dark were used as blanks. The superoxide anion generation was calculated using percentage inhibition formula.

GC-MS conditions

The analysis of the constituents was run on an Agilent Technologies (GC-7890B: MS 5977AMSB). The fused-silica HP-5 MS HP-5MS (5% Phenyl methyl siloxane) capillary column (30 m × 250 µm × 0.25 µm) was directly coupled to the MS. The carrier gas used was helium with a flow rate of 1 mL/minute. Oven temperature was programed (50°C for 2 minutes, then 50-250°C at a rate of 10 C/minute) and, held isothermally for 15 minutes. Injector Port: 250°C, split ratio 1:50. The volume injected: 1 µl, solvent delay 2 minutes. Electron energy 70 eV; mass range 40-700 amu and total run time was 120 minutes. The software used to handle mass spectra, and chromatograms were MassHunter. The spectrum of the compounds was compared with the spectrum of the known components in the NIST library.

RESULTS

The identification of the collected plant was done using rbcL DNA barcode. The rbcL sequence was searched against NCBI database which showed 99.8% identity with *Combretum* sp. The ethanol extract obtained from *C. albidum* leaves was tested for antibacterial and antioxidant activity. Among the eight bacterial species that were tested against ethanolic extract of *C. albidum* leaves (Table 1), the zone of inhibition was the highest for *Bacillus subtilis* (19.8±0.57 mm) and lowest for MBL producing *Escherichia coli* (MBLe) (9.3±0.57 mm). Cefotaxime was used as positive control for all bacterial species. The resistant organisms, MRSA, and MBLe did not show zone of inhibition for the antibiotic. Clinical isolates showed resistance to the antibiotic used that varied from standard organism susceptibility. The MIC of organisms ranged from 0.08 mg/mL to 5.12 mg/mL for *B. subtilis* and *Klebsiella pneumonia*, respectively.

The ethanol extract of *C. albidum* showed a concentration dependent antiradical activity by inhibiting DPPH with an IC_{50} value of

Table 1: Antibacterial activity of ethanolic extract of leaves from *C. albidum*

Botanical name	Zone of Inhibition (mm)		MIC (mg/mL)
	C. albidum (2 mg)	Cefataxime (10 µg)	
E. coli	11±0.57	19.3±0.57	1.28
P. aeruginosa	10.3±0.57	16±1	2.56
B. subtilis	19.8±0.57	34±0.57	0.08
P. mirabilis (MDR)	18.1±0.57	34±0.57	1.28
E. coli (MBLe)	9.3±0.57	NZ*	2.56
K. pneumonia (MDR)	11.3±0.57	9±0.57	5.12
MRSA	15.2±0.57	NZ*	0.64
MSSA	17.3±0.57	18±0.57	0.32

*NZ: No zone of inhibition, values are expressed as mean±SD, n=3 ($p<0.05$). SD: Standard deviation, *C. albidum: Combretum albidum, E. coli: Escherichia coli, P. aeruginosa: Pseudomonas aeruginosa, B. subtilis: Bacillus subtilis, P. mirabilis: Proteus mirabilis, K. pneumonia: Klebsiella pneumonia, S. aureus: Staphylococcus aureus*, MDR: Multi-drug resistant, MBLe: Metallo-β-lactamase producing *Escherichia coli*, MRSA: Methicillin-resistant *Staphylococcus aureus*, MSSA: Methicillin-susceptible strain of *Staphylococcus aureus*, MIC: Minimum inhibitory concentration

Table 2: Antiradical activity of aqueous extract of *C. albidum* observed with DPPH

Sample concentration (µg/mL)	% Inhibition
100	10.28±0.86
120	15.88±1.25
140	30.37±1.81
160	51.77±0.51
180	73.2±0.29
200	80.11±0.92

Values are expressed as mean±SD, n=3 ($p>0.05$). SD: Standard deviation, *C. albidum: Combretum albidum*, DPPH: 1,1-diphenyl-2-picrylhydrazyl

Table 3: Superoxide anion-scavenging activity of aqueous extract of *C. albidum* observed with the riboflavin-light-NBT system

Sample concentration (µg/mL)	% Inhibition
20	20.96±0.55
40	39.11±1.38
60	48.79±0.91
80	54.2±0.47
100	68.34±0.22

Values are expressed as mean±SD, n=3 ($p>0.05$). SD: Standard deviation, *C. albidum: Combretum albidum*, NBT: Nitrobluetetrazolium

156.26 µg/mL (Table 2). The scavenging activity was more with increasing concentration of the extract, and the inhibition was 80.11±0.92% at 200 µg/mL. The scavenging activity of gallic acid (IC_{50}=2.38 µg/mL), used as a positive control and was relatively more than that of *C. albidum*.

The IC_{50} value of superoxide scavenging activity for ethanol extract of *C. albidum* was 69.03 µg/mL and was compared with ascorbic acid (IC_{50}=20.32 µg/mL) which was used as a positive control (Table 3). The percentage of inhibition was found to be 68.34±0.22% at 100 µg/mL.

The GC-MS analysis identified 23 constituents in the ethanol extract of *C. albidum* (Table 4) that includes major constituents such as phenol (10.216%), methyl 6-O-[1-methylpropyl]-β-d-galactopyranoside (1.404%), eicosane (1.181%), isopropyl myristate (1.049), heptadecane (2.367%), n-hexadecanoic acid (4.673) phthalic acid butyl tetradecyl ester indazol-4-one, 3,6,6-trimethyl-1-phthalazin-1-yl-(3.083%), 1,5,6,7-tetrahydro-, phytol (1.999%), 9,12,15-Octadecatrienoic acid (6.262%), L-ascorbic acid (2.388%), vitamin E (2.622%), octadecanoic acid (1.86%), 7,11b-dihydro-6H-indeno[2,1-c]chromene-3,4,6a,9,10-pentol pentakis (trimethylsilyl) ether (19.587%), squalene (1.213%), stigmasterol (3.253%), and 9,19-cyclolanostane-3,7-diol (4.178%). The GC-MS chromatogram for the ethanolic extract from *C. albidum* leaves showed retention time for various bioactive components (Fig. 1).

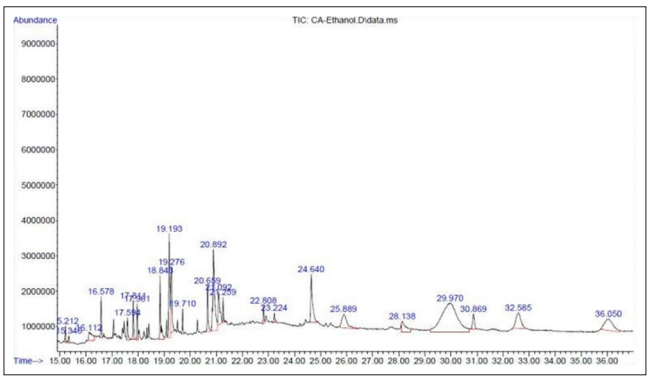

Fig. 1: Gas chromatography-mass spectrometry chromatogram of ethanolic extract of *Combretum albidum* leaves

DISCUSSION

The Combretaceae family is well-known for its antimicrobial activity especially, in the genus *Combretum* [22-25]. The species of *C. albidum* is less explored with few known compounds such as β-sitosterol, ursolic acid, betulin, betulinic acid, oleanolic acid, arjunolic acid, and gallic acid [26,27]. The previous study showed that methanol leaf extract of *C. albidum* exhibited antibacterial activity against MDR *Pseudomonas aeruginosa* [28], and stem bark showed activity against *E. coli*, *K. pneumonia*, *Proteus mirabilis*, *P. aeruginosa*, *B. subtilis*, and *Salmonella typhimurium* [29]. In this study, ethanol extract of *C. albidum* was examined against *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *B. subtilis* (MTCC 441), clinically isolated *P. mirabilis*, MDR *K. pneumonia*, MRSA, methicillin-susceptible strain of *S. aureus* (MSSA) and MBLe. The zone of inhibition of Gram-positive bacterium, *B. subtilis* (19.8±0.57), MSSA (17.3±0.57), and MRSA (15.2±0.57) were comparatively larger than the Gram-negative bacterium except *P. mirabilis* (18.1±0.57). The bacterial species such as *K. pneumonia, P. aeruginosa*, *E. coli,* and MBLe showed comparatively lower inhibition. The ethanolic extract of *C. albidum* showed activity against all the bacterial species tested. The MIC for Gram-positive bacterium such as MRSA, MSSA, *B. subtilis* was lower when compared with Gram-negative bacterium such as *K. pneumonia, P. aeruginosa*, and *E. coli*. The clinically isolated resistant organisms (MDRs, MBle) showed higher MIC values. Hence, the constituents present in the ethanol extract of *C. albidum* play a significant role, proving to be a potent plant extract for antibacterial activity.

Table 4: GC-MS analysis of bioactive compounds from an ethanolic extract of *C. albidum*

Serial number	RT time	Compound name	Activity	Area (%)	Mol. formula	Mol. wt
1	14.2800	Phenol	Antibacterial [32]	10.216	$C_{14}H_{22}O$	206
2	14.4960	Benzoic acid	Antibacterial [32]	0.758	$C_{11}H_{14}O_3$	194
3	15.2120	3-Octadecene	anticancer, antioxidant and antimicrobial activity [33]	0.378	$C_{18}H_{36}$	846
4	15.3460	Diethyl phthalate	Antibacterial [35-37]	0.258	$C_{12}H_{14}O_4$	222
5	16.1120	Methyl 6-O-[1-methylpropyl]-β-d-galactopyranoside		1.404	$C_{11}H_{22}O_6$	250
6	16.5780	Eicosane	Antibacterial [34]	1.181	$C_{21}H_{44}$	296
7	17.5940	3-Hexadecanol	Antibacterial [43]	0.864	$C_{16}H_{34}O$	242
8	17.8110	Isopropyl myristate	Antioxidant and antibacterial activity [47]	1.049	$C_{17}H_{34}O_2$	270
9	18.8430	Heptadecane	Anticancer, antioxidant and antimicrobial activity [33,34]	2.367	$C_{25}H_{52}$	352
10	19.1930	n-Hexadecanoic acid	Antimicrobial [44]	4.673	$C_{16}H_{32}O_2$	256
11	19.2760	Phthalic acid, butyl tetradecyl ester	Antibacterial [35-37]	3.083	$C_{26}H_{42}O_4$	418
12	19.7100	Indazol-4-one, 3,6,6-trimethyl-1-phthalazin-1-yl-1,5,6,7-tetrahydro-	Antibacterial [35-37]	0.791	$C_{18}H_{18}N_4O$	306
13	20.6590	Phytol	Antibacterial [42]	1.999	$C_{20}H_{40}O$	296
14	20.8920	9,12,15-Octadecatrienoic acid	Antibacterial [44]	6.262	$C_{21}H_{36}O_4$	352
15	21.0920	L-Ascorbic acid	Antibacterial and antioxidant[45]	2.388	$C_{24}H_{42}O_7$	442
16	21.2590	Tetracosane	Anticancer, antioxidant and antimicrobial activity [33,34]	0.723	$C_{24}H_{50}$	338
17	23.2240	Octadecane	Anticancer, antioxidant and antimicrobial activity [33,34]	0.337	$C_{26}H_{54}$	366
18	25.8890	Vitamin E		2.622	$C_{29}H_{50}O_2$	430
19	28.1380	Octadecanoic acid	Antibacterial [44]	1.86	$C_{21}H_{42}O_4$	358
20	29.9700	7,11b-Dihydro-6H-indeno[2,1-c] chromene-3,4,6a, 9,10-pentol pentakis (trimethylsilyl) ether		19.587	$C_{31}H_{54}O_6Si_5$	405
21	30.8690	Squalene	Antioxidant [41]	1.213	$C_{30}H_{50}$	410
22	32.5850	Stigmasterol	Antibacterial and Antioxidant [46]	3.253	$C_{29}H_{48}O$	725
23	36.0500	9,19-Cyclolanostane-3,7-diol	Antibacterial [48]	4.178	$C_{30}H_{52}O_2$	444

C. albidum: Combretum albidum, GC-MS: Gas chromatography-mass spectrometry

The nature of secondary metabolites in a plant extract may show variation in free radical scavenging activity. The DPPH assay is commonly used to test the free radical scavenging activity of plants. The ethanolic extract of *C. albidum* was able to reduce and decolorize the stable radical DPPH to the yellow-colored diphenyl picrylhydrazine [19]. Although the IC_{50} value of the control gallic acid was 2.38 µg/mL and the activity was significantly higher than that of the plant extract, the percentage inhibition was 80.11±0.92% at 200 µg/mL for ethanol leaf extract of *C. albidum*. The superoxide scavenging activity was also done to check the reduction of flavins that generates O_2^- and reduces NBT to form blue formazan [20]. The inhibition of ethanol extract from *C. albidum* at concentration 80 and 100 µg/mL was 54.2±0.47 and 68.34±0.22%, respectively. The activity of ascorbic acid (IC_{50}=20.32 µg/mL) was significantly greater than *C. albidum* leaf extract (IC_{50}=69.03 µg/mL). Our study used both DPPH method and superoxide scavenging activity to evaluate their antioxidant capacities. The results indicated that the *C. albidum* leaf extract had a significant effect on scavenging of superoxide at lower concentration as compared to DPPH assay.

GC-MS analysis was done to identify the components responsible for antibacterial and antioxidant activity. We identified 23 constituents from the ethanol leaf extract of *C. albidum*. The compound 9,19-Cyclolanostane-3,7-diol (4.178%) was reported for the first time in the leaf extract of *C. albidum*. Previously, GC-MS analysis from *C. albidum* leaf oil identified only 18 constituents [25]. The presence of acetic acid [30,31], phenol and benzoic acid [32] was reported for antibacterial activity. The compounds such as 1-Octadecene and 1-Heptadeceane possess anticancer, antioxidant and antimicrobial activity [33,34]. Phthalic acid and its derivatives from *Nauclea latifolia, Streptomyces bangladeshiensis* have shown antimicrobial activities and considered to be non-toxic [35-38]. GC-MS analysis has also demonstrated that *C. albidum* is not only antibacterial but is also a rich source of vitamin E (2.622%) that can be a potent source of nutrition. Compounds such as squalene, tetracosane, ascorbic acid, and stigmasterol can be responsible for the antioxidant activities which are present in the ethanolic extract of *C. albidum* leaves [39-41]. The results of this study indicated that the constituents from the ethanolic extract of *C. albidum* act as good antibacterial agent as well as the potent antioxidant source (Table 4). The presence of bioactive compounds confirmed the antibacterial and antioxidant activity of the ethanol extract from leaves of *C. albidum*.

CONCLUSION

The present investigation aimed the antioxidant, the antimicrobial potential of phytoconstituents present in the ethanolic extract of *C. albium* and identification of chemical compounds by GC-MS analysis. The presence of phytoconstituents such as long-chain fatty acids, steroids, terpenoids, aliphatic, and aromatic hydrocarbons is responsible for the antimicrobial activity and antioxidant potential. Screening of *C. albidum* by GC-MS confirmed the plant to be a potential source for bioactive substances that supports several pharmaceutical uses and therapeutic value.

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