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Research Article

Phytochemical Screening and Antimicrobial Activities of the Fractionated Leaf Extract of *Combretum Racemosum*

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Abstract: The phytochemical and anti-microbial properties of the leaves of *Combretum racemosum* were examined in this research work. The phytochemical screening of this plant leaves using ethanol, propanol and water reveal the presence of antraquinone, tannin, steroids, cardiac glycosides, saponin, reducing sugars, flavonoids, terpenoids and phlobatannin at various degrees of concentrations. The leaf extract also shows antibacterial and antifungal activities. Minimum inhibitory concentration for the ethanolic extract with *Staphylococcus aureus, Psudomonas aeruginosa, Proteus vulgaris, Escherichia coli and Salmonella typhi* range from 7.8125-31.25mg/ml; for the propanolic extract Minimum inhibitory concentration gave a range from 31.25-62.3mg/ml while for the aqueous extract the Minimum inhibitory concentration ranged from 15.625-125.0mg/ml respectively for the different bacteria. The ethanolic extract on *Candida albicans* gave a Minimum inhibitory concentration of 15.625mg/ml. Mycelial radial growth using the ethanolic and aqueous extract on *Aspergillus niger and Rhizopus spp* were examined. A percentage inhibition which ranged from 31.75-65.5% were obtained for *Aspergillus niger* for ethanolic extract and 7.7-9.6% for the aqueous extract while for *Rhizopus spp*, the percentage inhibition for ethanolic extract gave a range from 47.57-60.69% and 12.17-15.39% for the aqueous extract. The aqueous extract on *Aspergillus niger* and *Rhizopus spp* had no much significant activity on the plant extract. The hydrolysed alkaloid fraction obtained using the Stas Otto method gave good bacterial activity on all bacteria used and on *Candida albicans* and this was concentration dependent. Thus the presence of these secondary metabolites and the antimicrobial activities could be responsible for the potency of *Combretum racemosum* as a medicinal plant for the treatment of bacterial and fungal infections.
Keywords: *Combretum racemosum,* Antimicrobial activities, Traditional medicine, Extract, Mycelial radial growth.

INTRODUCTION

Medicinal plants are known to contain substances that are used for therapeutic purposes and are precursors for the synthesis of useful drugs. They are good sources of drugs that have found uses in the treatment of gastrointestinal diseases and other diseases in the underdeveloped world as these infectious diseases have become endemic and no accessibility modern to medical facilities[1]. Most of these plants contain antimicrobial properties that can inhibit the growth of bacteria by different mechanisms from modern methods of treatment with orthodox drugs.

Combretum is a tropical plant and is made of different species which include; *Combretum racemosum,Combretum molle, Combretum woodii, Combretum erythophyllum, Combretum apiculatum, Combretum mossanbicanse, etc.* [2, 3]. They could be shrubs, trees or climbers[3]. The leaves of *Combretum racemosum* are used mostly in Nigeria traditionally as a remedy for the treatment of some parasitic, bacterial and fungal infections. It is claimed to be effective against stomach pains, gastric ulcers, dysentery, abdominal disorder, fever and is believed to have good antibacterial activities against many organisms [2,3]. It is commonly called Ebi-odo among the Urhobos in Nigeria.

This is because it is used for the treatment of fever, jaundice and in some cases malaria. The plant belongs to the family *Combretaceae,* and in most parts of Africa it is used traditionally as a food spice. It has been proven to possess antihelmintic activities and could be used to treat haemorrhoids, convulsive coughing, tuberculoses, toothache and male sterility [4,5]. Some species are reported to have anti-inflammatory and anti-schistosomal activities and have antifeedant and insecticidal effects in plants [7].

Work done by Hutchings, reveals that some species of *Combretum* are used traditionally for the treatment of pneumonia,colds,syphilis and mumps [7]. Other ailments as claimed by traditionalists that could be treated with *Combretum species* are dysmenorrhoea, earache, fattening babies, hook worm, infertility in women, leprosy, scorpion bite, snake bite, heart disease, urinary tract infection, gallstone, sore throats, nosebleeds and general debility [5,6,8].

Most of the *Combretum species* are reported to be tanniferous and produce ellagic and gallic acids and sometimes proanthocyanins. They are also said to be cyanogenic and accumulate triterpenoids, especially saponins [6]. Some have also been known to contain acidic triterpenoids and glycosides, phenanthrenes, amino acids and stilbenes [9]. The presence of alkaloids have not been clearly reported [10]; but anti-inflammatory and mulluscidal compounds have been isolated from some species [11]. Saponins, Jessic acid of the alpha- L-arabinose have been isolated from the leaves of *Combretum eleagnoides* [9]. However, among the various species of *Combretum* no much study work has been done on *Combretum racemosum;* but phytochemical screening has been reported on it [12].

MATERIALS AND METHOD

The leaves of *C. racemosum* were collected from the Delta State University, Abraka vicinity and authenticated in the Department of Pharmacognosy and Traditional Medicine of the Faculty of Pharmacy,Delta State University, Abraka.

The leaves were air dried for two weeks and made into powder by squeezing. The weight of the dry powder was 240g. This was divided into three parts of 80g each. Each of these parts was extracted using water maceration, and soxhlet using ethanol (50%) and n-propanol (50%) respectively. Water maceration gave 18g (22.5%) yield, while ethanol and n-propanol gave 25% yield respectively after concentration to dryness using hot air oven at a temperature of 40^0C. The concentrated extracts were kept in screw capped bottles and stored in the refrigerator at 4^0C prior to usage.

Percentage yield was calculated using the formula: $\frac{\text{weight of extract}}{\text{Weight of dry powder}} \times 100$

Preparation of Medium

The microorganisms used were human pathogens; *Salmonella typhi, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Proteus vulgaris* . These are both gram positive and gram negative organisms. The three fungi used were *Candida albicans, Aspergilus niger and Rhizopus species.* All were obtained from the Pharmaceutical Microbiology Laboratory stock of the Delta State University Abraka. The bacteria were grown in Nutrient Agar (Fluka) while the fungi were grown in Sabouraud Dextrose Agar (Himedia). The media were prepared according to the method of Wolfgang and Hilda [14].

Phytochemistry

Preliminary phytochemical screening for secondary metabolites- antraquinones, tannins, cardiac, terpenoids, glycosides, alkaloids, saponin glycosides, steroidal nucleus, etc; were carried out using Harborne method [13]. The ethanolic extract was hydrolysed in order to fractionate the alkaloid using the Stas Otto method [15]. 5g of the ethanolic extract was heated at 20^oc with 100ml of 3M H_2SO_4 solution under reflux for 24 hours. 30ml of this hydrolyzed extract was poured into a separation funnel and 20 ml of chloroform was added. This was agitated for differential separation to take place. 20ml of chloroform was further added in order to elute the chloroform phase. The aqueous phase was basified using 15ml of 0.5M sodium hydroxide solution. 20ml of chloroform was added to this basified extract and the chloroform phase eluted out. This fraction was labeled D while the sodium hydroxide phase was labeled E. 20 ml of 8% w/v sodium hydrogen carbonate was now added to the chloroform phase to extract the acidic compounds and the chloroform phase separated out into a beaker. The sodium hydrogen carbonate phase was labeled A. Further, 15ml of 0.5ml sodium hydroxide was then added to the chloroform phase, the separation funnel shaken to elute the chloroform phase labeled C and the sodium hydroxide phase labeled B. The whole procedure is represented in the flow chart (Fig-1).

Fraction D containing the alkaloid was screened and used for sensitivity and minimum Inhibitory concentration of the test organisms.

Anti-Microbial Assay

The agar well diffusion method was used for antibacterial sensitivity testing. An overnight broth culture of each bacterium was used after being standardized with 0.5 McFarland Standard. 500mg/ml of each extract was reconstituted in the solvents and concentrations ranging from 500-32.125mg/ml were used for the sensitivity test while concentrations ranging from 500-7.8125mg/ml were used for the minimum inhibitory concentration determination. Concentrations ranging from 200-3.125mg/ml were used to determine zones of inhibition and minimum inhibitory concentration of the alkaloid fraction of the leaf extract obtained using the Stas Otto method. The antimicrobial studies were done in duplicates and mean values obtained.

Antibacterial activity was carried out using Mueller –Hinton Agar (Biotech) and Sabouraud Dextrose Agar (Himedia) for the fungi. The pour plate method was used. The minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) were determined for the various

bacteria by sub-culturing all the MIC plates which had no growth on freshly prepared nutrient agar plates, and noting the plates that had no growth. The fractionated alkaloid was tested on the various bacteria- *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris and Salmonella typhi, and on Candida albicans* to determine the zone of inhibition and minimum inhibitory concentration if it has activity on these organisms.

The antifungal activity was carried out by determining the effect of the various extracts on the mycelial radial growth of the different fungi using the methods of Oloke et a. [16]. A sterilized 5mm flamed cork borer was used to cut mycelia discs from 7 day old cultures of the fungi and transferred aseptically to the centre of petri dishes containing Sabouraud dextrose agar containing the various concentrations of the different extracts. The bottom of each plate was marked with two perpendicular lines intersecting at the centre. The plates were then incubated in duplicates at 30°c and radial growth measured in two directions along the perpendicular lines daily for fourteen (14) days and their mean calculated for each colony, to ascertain the antifungal property of the extracts at the different concentrations. Control plates were also cultured and their radial growth measured. Percentage of growth inhibition was calculated using the formula

$$(A\ \%) = \frac{W-X \times 100}{W}$$

Where W= Diameter of radial growth on Sabouraud Dextrose Agar (SDA) without the extract

X= Diameter of radial growth on SDA with the extract.

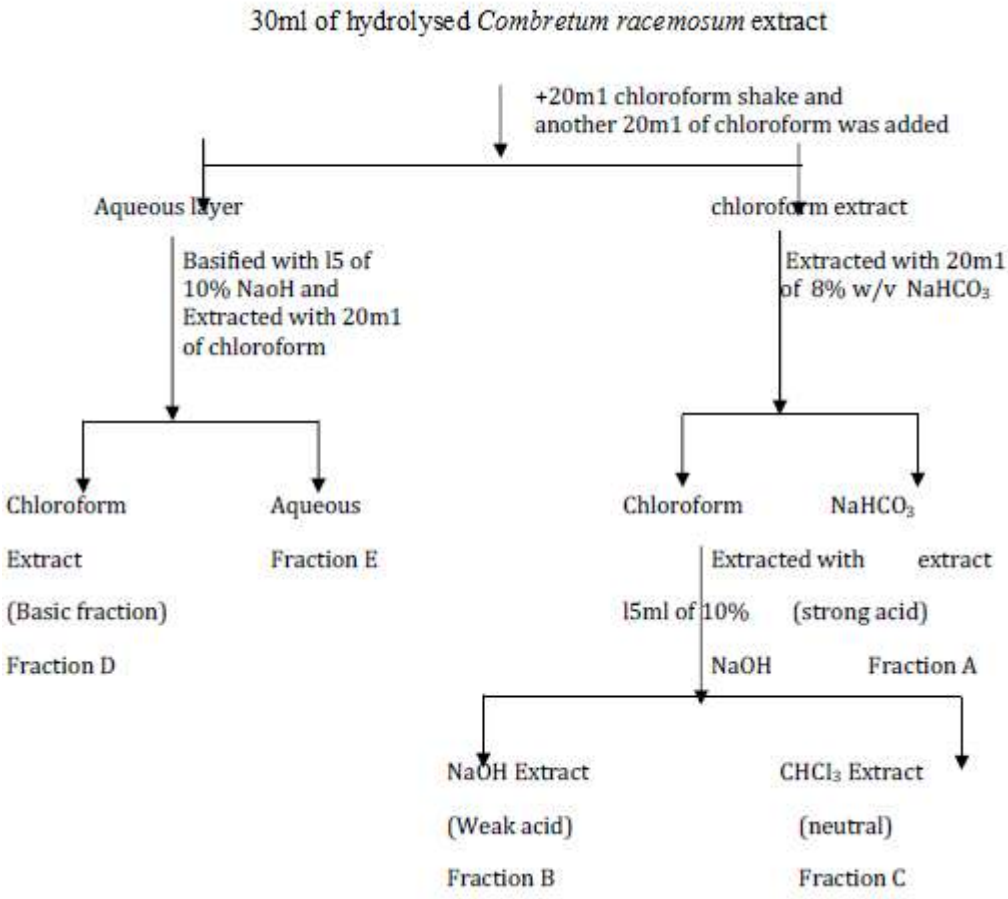

Fig-1: Schematic flow chart of the ethanolic extract of *Combretum racemosum* leaves.

RESULTS

Phytochemical screening of the various extracts (ethanolic, propanolic and aqueous) reveals the presence of alkaloids, antraquinone, tannin, steroids, cardiac glycosides, saponin, reducing sugars, flavonoids, terpenoids and phlobatannin at various degrees of concentrations from low to high concentration as shown in Table 1. The anti-bacterial and anti-fungal activities of the leaf extract s also reveal that *Combretum racemosum* has inhibitory effect on *Staphylococcus aureus, Pseudomonas aeruginosa, Proteus vulgaris, Escherichia coli and Salmonella typhi* as shown in Table 2 ; and fungal activities was shown in *Aspergilus niger and Rhizopus spp* with ethanolic extract but no significant activity was shown with aqueous extract as can be seen in Tables 3, 4, 5 and 6.

Fungal activity was also shown on *Candida albicans* using ethanolic extract but not with aqueous extract (Table 9.) The propanolic extract was not used to investigate the fungal activity of the fungi. The alkaloid fraction obtained showed significant anti-bacterial activity on all the bacteria and on *Candida albicans* which were concentration dependent. The minimum inhibitory concentration (MIC) for the ethanolic extract of the bacteria (*Staphylococcus aureus, Pseudomonas aeruginosa, Proteus vulgaris, Escherichia coli and Salmonella typhi*) range from 7.8125-31.25mg/ml, while for the propanolic extract the MIC range from 31.25-62.5mg/ml and the MIC values for the aqueous extract range from 15.625-125mg/ml. Results obtained from the zones of inhibition of the various extracts shows that the values are not concentration dependent but the inhibition zones obtained from the alkaloid fraction are concentration dependent. Fungal activities were determined using radial growth of the different fungi on the extracts for fourteen (14) days; and the percentage inhibition determined. The percentage inhibition for *Aspergilus niger* on the ethanolic extract range from 31.78%-65.3% while for the aqueous extract the percentage inhibition range from 7.7%-9.6% which shows that the aqueous extract has no good fungal activity compared to the ethanolic extract. For *Rhizopus spp* ; percentage inhibition gave a range of 47.57%-60.69% for ethanolic extract and 12.17%-15.39% for aqueous extract (Table 8) which shows that the aqueous extract has no significant fungal activity compared to the ethanolic extract.

DISCUSSION

The presence of alkaloid in the leaf extract of *Combretum racemosum* has not been fully reported [10], but this work shows that alkaloid fraction had inhibitory effect on the bacteria used and on *Candida albicans* with MIC values of 3.125mg/ml for *Staphylococcus aureus and Candida albicans* and 100mg/ml for *Pseudomonas aeruginosa, Proteus vulgaris, Escherichia coli* and *Salmonella typhi* respectively.

Alkaloids are known to be the largest groups of secondary metabolites in plants. They are claimed to have powerful effects on humans and hence could be used as pain killer medications[17]. *Combretum racemosum* could be a potential source of anti-microbial agent and a detailed assessment of its *in vivo* potencies and toxicological assay could be further worked upon. The presence of tannin in the various extracts (ethanolic, propanolic and aqueous) of *Combretum racemosum* is also of importance. Tannins have been found to form irreversible complexes with prolin-rich protein[18] and these results in the inhibition of cell protein synthesis. It has also been revealed that tannins react with proteins to provide tanning effect which helps in the treatment of inflamed/ ulcerated tissues[19]. Most herbs that contain tannin as a major constituent are claimed to be astringent in nature and find use in the treatment of intestinal disorders like diarrhea and dysentery[20]. These observations could be responsible for the use of *Combretum racemosum* in herbal cure remedies. It is also suggestive that tannin has anticancer activities as shown by Li and Wang [21] and hence could be used for cancer prevention. Thus it can be suggested from the above that *Combretum racemosum* is a source of bioactive compound that could have effect on the treatment and prevention of cancer.

The presence of phenolic compounds like flavonoids, glycosides, etc, in the extracts reveals its anti-microbial activity. Phenolic compounds are synthesized in plants as secondary metabolites. They have several biological activities which include anti-oxidant, anti-inflammatory, anti-aging and inhibitory properties. It is believed[22] that these biological activities are due to the intrinsic reducing capability towards pro-oxidants. It has also been proved that total phenolic content is due to the anti-oxidant properties[23, 24, 25]. Therefore it could be inferred that any anti-oxidant/anti-microbial activity of this plant, *Combretum racemosum* may be connected with the presence of phenolic compounds present in the extract and their isolation will enhance its clinical usage.

Table 1: Phytochemical screening of *Combretum racemosum*

Secondary metabolites	Ethanolic extract	Propanolic extract	Aqueous extract	Degree of presence
Alkaloids	++	+	++	Low/medium conc
Antraquinone	+	+	+	Low conc
Tannin	++	++	++	Medium conc
Steroids	+	++	++	Low/medium conc
Cardiac glycosides	++	+++	+	Low, mediu/high conc
Saponin	+++	+++	+++	High conc
Reducing sugars	++	++	++	Medium conc
Flavonoids	++	++	+++	Medium/high conc
Terpenoids	+	+	++	Low/medium conc
Phlobatannin	+++	++	++	Medium/high conc
+ Low conc, ++ Medium conc, +++ High conc				

Table 2: Inhibition zones of extracts/ minimum inhibitory concentration (MIC) / minimum bactericidal concentration (MBC)

Organism/extract conc (mg/ml)	**Ethanolic extract zone of inhibition (mm)**	**Propanolic extract zone of inhibition(mm)**	**Aqueous extract zone of inhibition(mm)**	**Organism/extract conc(mg/ml)**	**Ethanolic extract MIC (mg/ml)and MBC(mg/ml)**	**Propanolic extract MIC(mg/ml)and MBC(mg/ml)**	**Aqueous extract MIC (mg/ml)and MBC(mg/ml)**
Staphylococcus aureus 500 250 125 62.5 31.25	 19 20 22 22 25	 14 12 12 10 8	 19 14 15 15 13	*Staphylococcus aureus* 500 250 125 62.5 31.25 15.625 7.8125	 7.8125 MBC=15.625	 31.25 MBC=15.625	 15.625 MBC=62.5
Pseudomonas aeruginosa 500 250 125 62.5 31.25	 11 9 10 12 12	 7 7 6 4 6	 - - - - -	*Pseudomonas aeruginosa* 500 250 125 62.5 31.25 15.625 7.8125	 15.625 MBC=31.25	 31.25 MBC=31.25	 125 MBC= -
Proteus vulgaris 500 250 125 62.5 31.25	 12 15 8 9 9	 9 10 7 8 5	 17 19 20 10 12	*Proteus vulgaris* 500 250 125 62.5 31.25 15.625 7.8125	 31.25 MBC=62.5	 31.25 MBC=125	 15.625 MBC=125
Escherichia coli 500 250 125 62.5 31.25	 4 4 3 2 2	 8 7 10 6 6	 15 15 17 20 21	*Escherichia coli* 500 250 125 62.5 31.25 15.625 7.8125	 - MBC=-	 31.25 MBC=-	 31.25 MBC=62.5
Salmonella typhi 500 250 125 62.5 31.25	 3 5 4 4 6	 5 3 5 6 4	 - - - - -	*Salmonella typhi* 500 250 125 62.5 31.25 15.625 7.8125	 31.25 MBC=-	 62.5 MBC= -	 - MBC=-
Positive control(ciproflox acin) (mm)	S.A 30 P.A 30 P.V 40 E.C 15 S.T 25	S.A 35 P.A 35 P.V 30 E.C 25 S.T 25	S.A 40 P.A 17 P.V 40 E.C 40 S.A 25				
S.A-*Staphylococcus aureus*	P.A-*Pseudomonas aeruginosa*	P.V- *Proteus vulgaris*	E.C-*Escherichia coli*	S.T- *Salmonella typhi*			

Table 3: Radial growth (in mm) of *Aspergilus niger on Combretum racemosum* (Ethanolic extract) for fourteen (14) days.

Conc mg/ml	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Average
500	5	12	18	22	24	24	24	32	36	36	36	39	42	48	28
250	6	12	18	24	28	32	35	48	53	53	58	62	66	70	40.35
125	5	15	22.5	23.5	26	29	33	45	51	51	65	68	72	78	41.71
62.5	6	16	25	39	39	42	46	62	67	67	72	85	85	85	52.57
31.25	5	20	28	35	42.5	48	50	68	70	70	85	85	85	85	55.46

Table 4: Radial growth (in mm) of *Aspergilus niger on Combretum racemosum* (Aqueous extract) for fourteen (14) days.

Conc mg/ml	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Average
500	5	27	62	85	85	85	85	85	85	85	85	85	85	85	73.5
250	10	32	65	85	85	85	85	85	85	85	85	85	85	85	74.43
125	12	30	67.5	85	85	85	85	85	85	85	85	85	85	85	74.61
62.5	12	32	67	85	85	85	85	85	85	85	85	85	85	85	74.71
31.25	11	35	69	85	85	85	85	85	85	85	85	85	85	85	75

Table5: Radial growth of *Rhizopus spp on* Ethanolic extract of *Combretum racemosum* for fourteen (14) days;

Conc mg/ml	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Average
500	5	18	20	20	24	27	28	28	28	32	45	52	60	65	32.29
250	5	10	12	13	17	20	24	26	26	29	32	45	54	58	26.5
125	6	15	18	18	18	22	26	32	36	49	54	62	66	78	35.71
62.5	5	12	15	17	19	24	27	46	55	63	75	75	85	85	43.07
31.25	6	16	18	19	21	26	28.5	49	57	65	80	85	85	85	45.75

Table 6: Radial growth of *Rhizopus spp on aqueous extract of Combretum racemosum* for fourteen (14) days

Conc mg/ml	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Average
500	5	25	30	85	85	85	85	85	85	85	85	85	85	85	71.07
250	10	22	35	85	85	85	85	85	85	85	85	85	85	85	71.57
125	6	15	17	85	85	85	85	85	85	85	85	85	85	85	69.5
62.5	12	17	21	85	85	85	85	85	85	85	85	85	85	85	70.36
31.25	12	28	35	85	85	85	85	85	85	85	85	85	85	85	72.14

Table 7: Radial growth of *Aspergilus niger and Rhizopus spp* without the extract and with Fluconazole drug for fourteen (14) days.

Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Average
Aspergilus niger Without extract	60	65	78	85	85	85	85	85	85	85	85	85	85	85	81.3
Aspergilus niger with Fluconazole	5	5	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	5
Rhizopus spp without extract	45	85	85	85	85	85	85	85	85	85	85	85	85	85	82.14
Rhizopus spp with Fluconazole	5	12	12	22.5	26	32	32	34	37	37	42.5	45	46	49	28.43
NG- No Growth															

Table 8:Percentage Inhibition Growth from radial growth of *Aspergilus niger and Rhizopus spp.*

Fungal extract type	Conc (mg/ml)	Average radial growth(mm)	Control without extract(mm)	% growth inhibition	Control with Fluconazole(mm)
Aspergilus niger ethanolic extract	500	28	81.3	65.3%	5
	250	40.35		50.37%	
	125	41.71		48.7%	
	62.5	52.57		35.34%	
	31.25	55.46		31.78%	
Aspergilus niger aqueous extract	500	73.5	81.3	9.6%	5
	250	74.43		8.5%	
	125	74.61		8.2%	
	62.5	74.75		8.1%	
	31.25	75.0		7.7%	
Rhizopus spp ethanolic extract	500	32.29	82.14	60.69%	28.43
	250	26.5		67.74%	
	125	35.71		56.53%	
	62.5	43.07		47.57%	
	31.25	45.75		44.30%	
Rhizopus spp aqueous extract	500	71.07	82.14	13.48%	28.43
	250	71.57		12.87%	
	125	69.5		15.39%	
	62.5	70.36		14.34%	
	31.25	72.14		12.17%	

Table 9: Inhibition zones and Minimum inhibitory concentration of Ethanolic extract and Aqueous extract of *Combretum racemosum on Candida albicans.*

conc (mg/ml)	Ethanolic extract	Aqueous extract	MIC (mg/ml)
500	10	No inhibition	15.625
250	8	No inhibition	
125	5	No inhibition	
62.5	5	No inhibition	
31.25	6	No inhibition	

Table 10: Zones of inhibition (in mm) on organisms using the alkaloid fraction of *Combretum racemosum*

Conc mg/ml	*S. aureus*	*E. coli*	*P. aeruginosa*	*P. vulgaris*	*S. typhi*	*C. albicans*
200	18	15	15	17	18	16
100	16	10	12	12	12	13
50	13	6	7	10	7	8
25	7	4	6	6	5	5
12.5	7	3	5	5	2	5
6.25	5	3	2	5	No inhibition	4
3.125	4	3	No inhibition	5	No inhibition	2

Table 11: Minimum inhibitory concentration of the alkaloid fraction of *Combretum racemosum* on organisms (- No Growth, + Growth)

Conc mg/ml	*S. aureus*	*E. coli*	*P. aeruginosa*	*P.vulgaris*	*S. typhi*	*C. albicans*
200	-	-	-	-	-	-
100	-	-	-	-	-	-
50	+	+	+	+	+	-
25	-	+	+	+	+	+
12.5	-	+	+	+	+	-
6.25	-	+	+	+	+	-
3.125	-	+	+	+	+	-
MIC (mg/ml)	3.125	100	100	100	100	3.125

CONCLUSION

The phytochemistry and anti-microbial activity of *Combretum racemosum* leaf extract as studied in this work shows that it contains secondary metabolites. Its anti-microbial activity against bacteria and fungi may be attributed to the presence of these secondary metabolites. Thus *Combretum racemosum* is found to be a good source of medicinal remedy for the treatment of bacterial and fungal infections and further studies on it should be carried out for its clinical usage.

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