Effects of Crude Methanol Extracts of the Leaf, Root and Bark of *Combretum Molle* against Selected Test Organisms

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***Abstract*: - *Combretum molle* has been used in many traditional medicines for treatment of microbial infections (diarrhea, dysentery, fever) and several inflammatory conditions (abdominal pain, headache, and toothache). This work was carried out with the aim of determining the phytochemical compounds present in the methanol extracts of the leaves; stem-bark and roots of *C. molle* and their biological activities in some selected microorganisms. Phytochemical screening also revealed the presence of Tannins, Flavonoids, Glycosides (in leaves only), Terpenes and Saponins, whereas Alkaloids, Anthraquinones and Steroids were absent in both extracts. In a qualitative antimicrobial study, six microorganisms were tested (*Bacillus subtilis, Staphylococcus aureus, Shigella dysenteriae, Salmonella* Typhi*, Escherichia coli* and *Candida krusei*) using Ciprofloxacin and Fulcin as positive controls. *B. subtilis, S. dysenteriae* and *S. typhi* proved to be the most sensitive bacterial species with MIC values of as low as 1.5mg/ml, 3.125mg/ml and 6.25mg/ml respectively, whereas *S. aureus, E. coli* and *C. krusei* were resistant to the plant extract. The minimum bactericidal concentration also had low values such as 1.5mg/ml of the stem methanolic extract against *S. dysenteriae,* and 1.5mg/ml of the leaf methanolic extract against *B. subtilis.* There was no inhibition of the extract on *S. aureus, E. coli* and *C. krusei* for the MIC and MBC. The analysis of variance at $p < 0.05$ indicated that there was significant difference in the performance of the extracts (stem methanol, root methanol and leaf methanol) on the microorganisms (*B. subtilis, S. aureus, S. dysenteriae, S. typhi, E. coli* and *C. krusei*). Therefore the study above indicates that *C. molle* contains phytochemical compounds which makes it a good inhibitor of microbial growth and could be exploited through in-depth studies to determine the active compounds that could be utilized in the treatment of common ailments.**

***Key words:* *Combretum molle,* Traditional medicine, phytochemical compounds, Methanol extracts**

I. INTRODUCTION

It has been estimated that less than 1 – 10 % of the large diversity of 250,000 – 500,000 plant species on the Earth have been studied chemically and pharmacologically for their medicinal properties (Farnsworth, 1991; Verpoorte, 2000). This is especially true for the tropical flora, as at date only 1 % of the species in these habitats have been studied for their pharmaceutical potential (Gurib, 2006). Tropical forests and many other tropical ecosystems are rich sources of a diversity of plant derived chemical compounds, not only because of the high species diversity but also because of the "eternal summer" which forces the plant species to constantly produce chemical defense compounds against herbivores and pathogens as well as against other plant species (Wood *et al*., 1997). Plants in a tropical rainforest also have to compete for space and light and this forces species to develop more efficient means of utilizing energy and nutrients as well as to allocating more resources for the production of secondary metabolites (Wood *et al*., 1997). For these reasons a greater portion of the tropical plant species contains secondary metabolites, which are potentially useful as models for/as medicines (Wood *et al*., 1997). Plant derived compounds have been, and are still, important as models (lead compounds) for medicines: 50 % of the prescription products in various countries in Europe and the US are either natural products or natural product derivates (Cordell, 2002; Newman *et al.*, 2003). To date, about 500 drugs have came from tropical plants (Gurib, 2006). Plants continue to be a potent source of lead compounds.

Source and Preparation of Plant Materials

The plant materials were collected from neighboring communities near ABU dam, in Samaru, Zaria (latitude 11.07^o N, longitude 7.73^o E and altitude 613meters), Nigeria. These were brought and identified with voucher number 900191 at the Herbarium unit of the Department of Biological Sciences, Ahmadu Bello University Zaria. The plant parts were dried for two weeks at room temperature in the laboratory and then ground to powder.

Extraction Procedures

The ground plant parts were extracted at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Ahmadu Bello University Zaria, following the methods of Sofowora (2006). Separation funnels were taken and 200g of each ground plant parts (stem, leaf and roots) were placed in three separation funnels. One litre of 80% methanol extraction fluid was added to the plant parts in the funnel. The mixture was allowed to stand for an hour and a conical flask was placed below the funnel which was then opened for the methanol extract to flow in. The extract was heated at 100° C in the water bath so as to obtain the dry extract.

Phytochemical Analysis

The method of Sofowora, (2006) was employed for the test of the presence of the phytocemical properties.

Source and Preparation of Test Microorganisms

The test microorganisms stock cultures were collected at the Department of Microbiology, Ahmadu Bello University, Zaria. Their validity was determined by sub culturing onto nutrient agar and confirmed by standard cultural, morphological and biochemical techniques as described by Cowan and Steel (1974). The inocula of the test organisms were standardized by the method of Barry and Thormsberry, (1991). This was done by suspending each test organism in 5ml of nutrient broth and the turbidity was compared with that of 0.5 McFarland standard. McFarland standard was prepared by adding 0.5ml of 1% barium chloride ($Bacl_2$) to 99.5ml of 1% sulphuric acid (H_2S0_4) solution. The turbidity of the 0.5 McFarland standards was used for estimation of the amount of bacteria in broth culture (culture for 24 hours at 37^0C) to pour into 5ml of distilled water in order to obtain a standard bacterial suspension of 1 x 10^5 cfu/ml (Bauer *et al.,* 2003).

Preparation of Concentration of Extracts

Approximately 1g of each extract was dissolved in 5mls of distilled water to yield 200mg/ml. 1ml of the 200mg/ml was taken and added to 1ml of distilled water to give a concentration of 100mg/ml. 1ml of the 100mg/ml extract concentration was also taken and added to 1ml of distilled water to get a concentration of 50mg/ml. The procedure was repeated twice to give concentrations of 25mg/ml and 12.5mg/ml

Antimocrobial Supsceptibility Testing

The methods of Cowan and Steel, (1974) were used. Standard aseptic microbiological methods were followed throughout this antibacterial study. McFarland standard was prepared by adding 0.5ml of 1% barium chloride ($Bacl_2$) to 99.5ml of 1% sulphuric acid (H_2S0_4) solution. The turbidity (density) of the 0.5 McFarland standards was used for estimation of the amount of bacteria in broth culture (culture for 24 hours at 37^0C) to pour into 5ml of distilled water in order to obtain a standard bacterial suspension of 1 x 10^5 cfu/ml (Bauer *et al.,* 2003).

II. PREPARATION OF THE MEDIA

Potato Dextrose Agar (PDA)

38 grams of potato dextrose agar powder (Oxoid Manufacturers, UK) was weighed and dissolved in 1 litre of distilled water. It was then boiled to dissolve the media completely. The media was then sterilized by autoclaving at 121^0 C for 15 minutes. After sterilization, the media was allowed to cool to about 40^0 C and then poured onto sterile petri-dishes.

Nutrient Agar

28 grams of the nutrient agar powder (Oxoid Manufacturers, UK) was weighed and dissolved in 1 litre of distilled water. It was then boiled to dissolve the media completely. The media was then sterilized by autoclaving at 121^0 C for 15 minutes. After sterilization, the media was allowed to cool to about 40^0 C and then poured onto sterile petri-dishes.

Well Method for Antibacterial Activity

The well method was employed to assay the plant extracts for antimicrobial activity. Petri dishes were poured with nutrient agar (for bacteria) and potato dextrose agar (for fungi) and allowed for 30 minutes to solidify (This was done in duplicate for each extract and test organism). The test organisms were then spread on the surface of the media using a sterile swab stick. A sterile Cork borer (size 3) was used to bore 6 wells in the medium. The different concentration of the plant extracts was injected into the wells using a sterile syringe and needle (different for each sample and test organism). These were then allowed a diffusion time of 1 hour after which the plates were incubated at 37 ^{0}C for 24 hours. The positive controls were ciprofloxacin for the bacteria and greseofulvin for the fungi. The potency of the crude extracts was determined by the clear zones of inhibition around the wells and was respectively measured as the diameter zones of inhibition.

Minimum Inhibitory Concentration (MIC)

Nutrient broth was used on the test organisms. Seven test tubes were selected. Each test tube contained 2ml of double strength broth. 2ml of the extract in the various concentrations was introduced into tube 1 and mixed thoroughly. 2ml of the content was then transferred into tube 2. The procedure was repeated for the remaining tubes up to tube 8. 2ml was then discarded from tube 7 with tube 8 containing no extract (control). To all test tubes a drop (0.02ml) of the inocula (18 hr broth culture of the microorganism) was added. The tubes were then incubated overnight at 37 ^{0}C after which they were examined for turbidity (microbial growth). The growth of the organisms was observed as a change in turbidity. The minimum inhibitory concentration of the extracts is the smallest concentration that is capable of inhibiting the growth of a specific inoculum of the microorganism. The test tube that shows no growth and had a clear solution represents the MIC. This procedure was carried out for all the organisms.

Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration (MBC) was determined by the method of Rotimi *et al.* (1988). All the tubes from the MIC showing no growth were subsequently sub cultured on freshly prepared nutrient agar and incubated at 37^0C for 24hrs. The MBC was regarded as the lowest concentration of the extract that inhibited bacterial growth on the nutrient agar after incubation at 37^0C for 24hrs.

Statistical Analysis

Analysis of Variance (ANOVA) of was used to assess the efficacy of the plant parts in terms of the activity as was shown by the zones of inhibition. Duncan's multiple range test (DMRT) was used to separate and rank the means where significant. Student t-test was carried out to determine if there was significant difference between the activity of the aqueous and methanolic extracts on the microorganisms.

III. RESULTS AND DISCUSSION

Table 1. Qualitative Phytochemical Screening of crude methanol *Combretum molle* Extracts

	Leaves	Stem	Root
	Methanol	Methanol	Methanol
Tannins	+	+	+
Flavonoids	+	+	+
Glycoside	+	-	-
Alkaloids	-	-	-
Anthraquinones	-	-	-
Steroids	-	-	-
Triterpenes	+	+	+
Saponins	+	+	+

Key: + = Present, - = Absent.

Table 2 Analysis of inhibition zones by Extracts

Source	Df	Leaf methanol	Stem methanol	Root methanol
Bacteria	5	270.68*	438.24	322.08
Concentration	4	843.90	1446.77	764.10
Interaction	20	51.48	30.41	48.55
Error	30	2.70	0.80	1.20

* =Significantly different at 95% ($p \geq 0.05$)

Table 3 The Mean of the Sensitivity Test of the Microorganisms to the Methanol stem Extract of *Combretum molle*

Microorganism	Zone of inhibition (mm) (Mean±SE)				
	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	control
B. subtilis	22±1.0	20±1.0	18±1.0	16±1.0	42±1.0
S. Typhi	22±0.5	19±1.0	18±1.0	13±1.0	43±1.0
S. dysenteriae	17±1.0	16±1.0	13±1.0	11±0.5	28±1.0
S. aureus	-	-	-	-	29±1.0
E.coli	-	-	-	-	26±1.0
C. krusei	-	-	-	-	-

- = No activity, Control: Ciprofloxacin for bacteria, Greseofulvin for fungi

Table 4 The Mean of the Sensitivity Test of the Microorganisms to the Methanol root Extract of *C. molle*

Microorganism	Zone of inhibition (mm) (Mean±SE)				
	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	control
B. subtilis	18±1.0	15±1.0	13±1.0	11±1.0	43±1.0
S. Typhi	11.5±0.5	9±1.0	7±1.0	5±0.5	41±1.0
S. dysenteriae	12.5±0.5	11±1.0	9±1.0	7±1.0	30±2.0
S. aureus	-	-	-	-	33±1.0
E.coli	-	-	-	-	23±1.0
C. krusei	-	-	-	-	-

- = No activity, Control: Ciprofloxacin for bacteria, Greseofulvin for fungi

Table 5 The Mean of the Sensitivity Test of the Microorganisms to the Methanol leaf Extract of *C. molle*

Microorganism	Zone of inhibition (mm) (Mean±SE)				
	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	control
B. subtilis	14±1.0	12±1.5	10.5±0.5	8±1.0	40±1.0
S. Typhi	12±2.0	11±1.0	9±1.0	5±1.0	40±0
S. dysenteriae	12±1.0	10±1.0	8.5±0.5	6±1.0	30.5±0.5
S. aureus	-	-	-	-	27±1.0
E.coli	-	-	-	-	15±1.0
C. krusei	-	-	-	-	-

- = No activity, Control: Ciprofloxacin for bacteria, Greseofulvin for fungi

Table 6 Minimum inhibitory concentration **(**MIC) for microorganism of different extracts of *C. molle* in mg/ml

	Leaf methanol	Stem methanol	Root methanol
S. aureus	-	-	-
E.coli	-	-	-
B. subtilis	3.125	25.00	6.25
S. Typhi	50.00	25.00	25.00
S. dysenteriae	12.50	1.50	12.50
C. krusei	-	-	-

- = no activity

Table 10 Minimum bactericidal concentration (MBC) for microorganism at different extracts of *C. molle in* mg/ml

	Leaf methanol	Stem methanol	Root methanol
S. aureus	-	-	-
E.coli	-	-	-
B. subtilis	1.50	25.00	6.25
S. typhi	25.00	12.50	25.00
S .dysenteriae	12.50	1.50	12.50
C. krusei	-	-	-

- = no activity

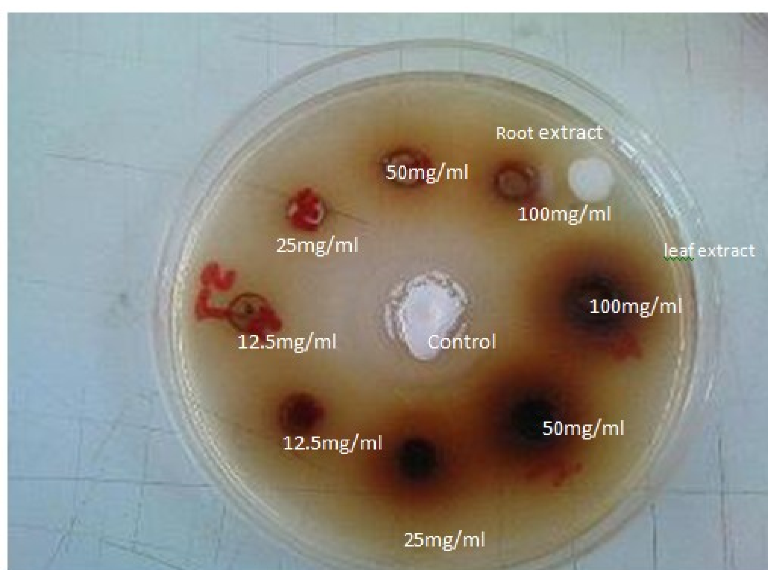

Plate 1 No zone of inhibition of the extracts at different concentrations against *Staphylococcus aureus.*

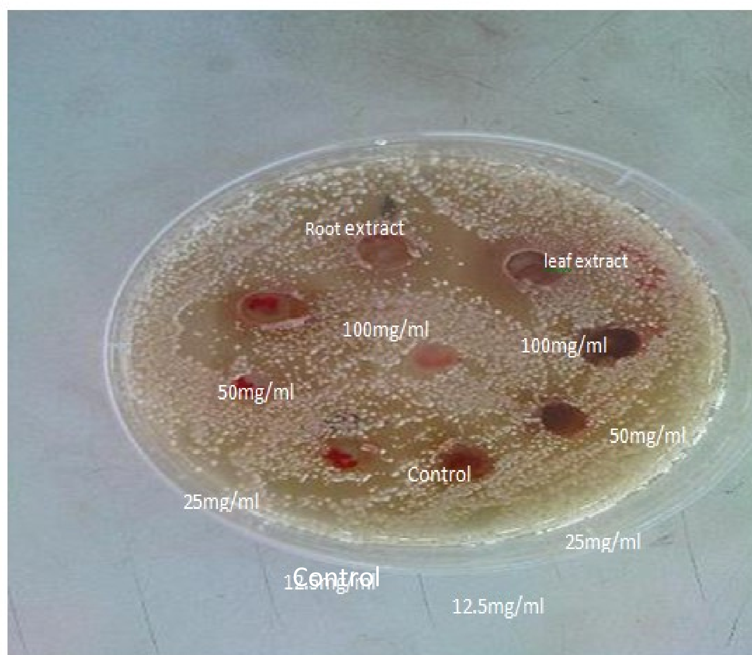

Plate 2 The zone of inhibition of the methanol stem extract at different concentrations against *Candida krusei.*

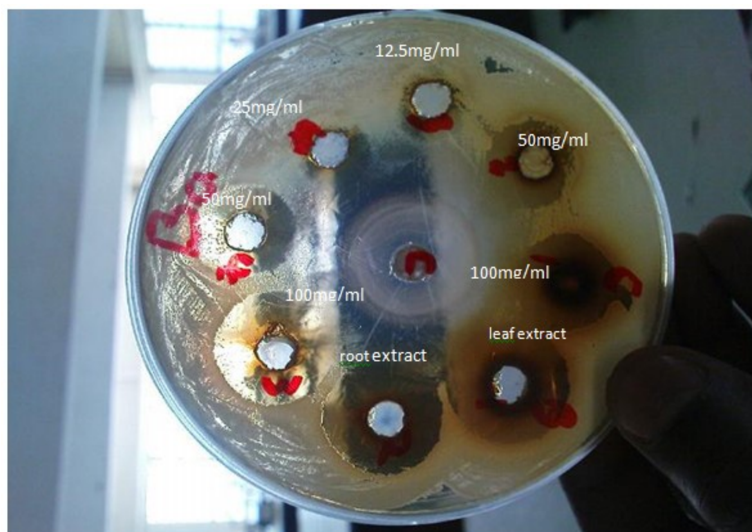

Plate 3 The zone of inhibition as a result of the aqueous and methanolic extracts at different concentrations against *Bacillus subtilis.*

The phytochemical screening of methanol extracts of the leaves, stem and root *Combretum molle* are presented in Table 1. The methanol extract revealed the presence of; Tannins, Flavonoids, Saponins and Triterpenes, although Triterpenes were absent in aqueous leaf extract. Alkaloids, Steroids and Anthraquinones were absent in the methanol extracts of leaves, stem and root of *C. molle*. Similarly tannins, flavonoids and saponins were found in the methanol extracts of the leaves, stem and roots of *C. molle*. This confirms the report by many researchers that *C. molle* contains phytochemical compounds (this also agrees with the work of Fyhrquist *et al*. (2002) who reported the presence of tannins, flavonoids and saponins in the roots and leaves of extracts of *C. molle*). Harborne (1999) found tannins and anthraquinones (the largest group of quinones) to posses antibacterial effects by inhibiting nucleic acid synthesis. Bajaj (1988) reported that tannins have been used for immediate relief of sore throats, diarrhea, dysentery, hemorrhage, fatigue, skin ulcers and as a cicatrizant on gangrenous wounds. The of the analysis of variance carried out to determine the significant effects in the performance of the extracts for the three different plant parts a indicated that for the bacteria, there was significant difference for the methanol leaf, but there was no significant difference for the methanol root and methanol stem extracts. Similarly, there was no significant difference for interactions for all the extracts used in the study (Table 2).

Results obtained from the methanol extract of the stem in table 3 showed activity against *Bacillus subtilis, Salmonella typhi,* and *Shigella dysenteriae* at all concentrations of 100mg/ml, 50mg/ml, 25mg/ml and12.5mg/ml, whereas *Candida krusei* had inhibition at 100mg/ml only. The positive control performed better than the extracts in terms of activities. *Candida krusei* was not susceptible to the control. Results obtained from the methanol extract of the root in table 4 showed activity against *Bacillus subtilis, Salmonella typhi* and *Shigella dysenteriae* at all concentrations of 100mg/ml, 50mg/ml, 25mg/ml and 12.5mg/ml. The extract, however did not inhibit *Staphylococcus aureus, Escherichia coli* and *Candida krusei* at all concentration levels. The positive control performed better than the extracts with the highest zone of inhibition of 43 mg/ml against *B. subtilis*. Results obtained from the methanol extract of the leaf in table 5 showed significant zones of inhibition against *Bacillus subtilis, Salmonella typhi* and *Shigella dysenteriae* at all concentrations of 100mg/ml, 50mg/ml, 25mg/ml and12.5mg/ml. The extract, however did not inhibit *Staphylococcus aureus, Escherichia coli* and *Candida krusei* at all concentration levels. The positive control performed better than the extracts with the highest zone of inhibition of 40 ml/ml against *B. subtilis*. The results of the minimum inhibitory concentration (MIC) (Table 6) indicated that the stem methanol extract had the greatest activity against *S. dysenteriae* with an MIC value of 1.5mg/ml on *S. typhi* had its greatest activity against the leaf extract with a value of 6.25mg/ml. The root methanol extract had its greatest activity against *B. subtilis* with an MIC value of 6.25mg/ml. There was no inhibition on *E. coli, S. aureus* and *C. krusei*. . This is in line with findings of Haerdi, (2001) that the juice of the leaves and roots of *C. molle* are used as antidiarrhoic remedy and with Kokwaro, (2000) and Chhabra *et al*, (1999) on its use to treat typhoid fever. This also confirms Fyhrquist *et al*, (2002) that the extracts of *C. molle* gave good antibacterial effects. There was evidence of activity of the extract against *C. krusei* at 100mg/ml. Fyhrquist *et al*, (2002) reported that the leaf extracts of *C. molle* showed no activity against *Candida* species. Arses *et al*, (2001) reported that the extract inhibited completely the growth of *Candida* at a concentration of 400mg/ml. This may not be fully explained as the highest concentration used during the test was100mg/ml. The least activity was obtained from the leaf methanol extract on *S. typhi*, with an MIC of 50mg/ml. The results from the minimum bactericidal concentration (MBC) (Table 7) showed that the stem methanol extract had the greatest activity against *S. dysenteriae* with an MBC value of 1.5mg/ml whereas the leaf methanol extract performed best on *B. subtilis* with an MBC value of 1.5mg/ml The least activity was given by the leaf methanol extracts on *S. typhi* with an MBC value of 25mg/ml.

IV. CONCLUSION

The use of *C. molle* by many traditional cultures in folk medicine prompted the investigation the antibacterial and antifungal properties of the plant. The study shows that *C. molle* contains secondary metabolites such as tannins, flavonoids, triterpenes and saponins, where as compounds such as anthraquinones, alkaloids and steroids where absent. The leaf, stem and roots of *C. molle* contain several phytochemical compounds. These compounds possess antibacterial effects against both gram-positive and gram-negative bacteria as they were active against *S. typhi* (gram-negative), *B. subtilis* (gram-positive) *and S. dysentriae* (gram-negative). These results validate the ethno botanical use of *C. molle* for ailments that may be of bacterial aetiology.

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