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In vitro Antibacterial Activity and Toxicity Study of *Eucalyptus camaldulensis* Leaf extract against Clinical Isolates of *Salmonella spp*

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Abstract
The study was carried out to determine the antibacterial potential of Eucalyptus camaldulensis leaves against clinical isolates of salmonella species. The E. camaldulensis leaves were extracted separately and successively with ethanol, water and methanol using percolation method. The extracts were tested in vitro for activity against clinical isolates of Salmonella typhi and Salmonella paratyphi using agar well diffusion and broth dilution methods. The zones of inhibition, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined. The in vitro antimicrobial screening revealed that the extract exhibited varying activities against S. typhi and S. paratyphi A and S. paratyphi B with zones of inhibition ranging from 7mm-24mm, MIC ranging from 62.5µg/ml - 125µg/ml and MBC of 125µg/ml -500µg/ml. The highest activity observed with E. camaldulensis leaves extract was 24mm against S. paratyphi A. MIC of 62.5µg/ml against S.typhi, and MBC of 125µg/ml against, S. paratyphi A and S. paratyphi B. The activities observed were due to the presence of the secondary metabolites like, alkaloids, anthraquinones, sterols, glycosides, saponins, and terpenes detected in the plant.The toxicity study carried out revealed that the highest value for LD_{50} of 1308.872 which shows non toxic property was in E.camaldulensis leaves aqueous extractagainst hatched brine shrimps. All extracts shows activity against the test organism depending on the concentration of the extract.

Keywords: Antibacterial, Eucalyptus camaldulensis, Zone of inhibition, Toxicity.

INTRODUCTION
E.camaldulensis is a relatively large riparian tree, commonly growing to 20m in height, but rarely exceeding 50m. In open woodlands it usually has a short, thick bole and a large, spreading crown with heavy branching. In plantations it can have a clear bole up to 20m with a lightly-branched crown. *E.camaldulensisDehnh*Linn.is one of such medicinal plants belonging to the family *Myrtaceae* which is frequently seen occupying open waste spaces and grasslands, road sides, along river banks and wetlands. (Jacobs, 1955; Stone and Bacon, 1994; Brooker *et al.,* 2002).

The name originated from Greek word "eucalyptol" which means "well covered". The medicinal usefulness of the redgum tree has been the subject of numerous studies.The tree is widely used in traditional medicine to treat a variety of diseased conditions including colds, asthma, coughs, diarrhea and dysentery, hemorrhage, laryngalgia, laryngitis, sore throat, spasm, trachagia and vermifuge (Duke and Wain, 1981).

Commonly called "zaity" in Nigeria, the resinous exudates from the trunk is taken orally to cure bladder infections (Lassack and MacCarthy, 2006) and a decoction of the plant is used to

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treat enteric infections including diarrhea and dysentery, constipations and other stomach problems, asthma, oral thrush, boils, sores, skin and wound infections, asthma, bronchitis, eczema and athletes foot (Bala, 2006; Duke and Ayensu, 1985). There is still little evidence on the antimicrobial properties of the plant under investigation against majority of the economically significant bacteria that cause infections. Though, *E. camaldulensis* have shown high antibacterial activities against organisms like, *S. typhi, S. aureus , B. mirabilis* etc. Study like this tends to confirm the activity and also clarify issues of antibiotic resistance acquiring bacteria with prior history of susceptibility to antibiotics.

MATERIALS AND METHODS

Collection of Plant

The plants were collected then identified and authenticated at the Herbarium of the Department of Plant Biology, Bayero University, Kano where a voucher specimen numbered; BUKHAN 0116 was deposited at the herbarium of the Department. The whole plants were rinsed with clean water and air-dried for six days under shade, and then pulverized and homogenized using a mechanical grinder. The pulverized plant was kept in an air-tight cellophane bag until used.

Extraction of the Crude Extracts

The powdered samples of the plants were extracted following the method of Gupta *et al*. (2009). One hundred grams (100g) each of the dried powder of the leaves of the plant were weighed into 3 different glass containers and sequentially extracted with 500ml each of methanol, ethanol and distilled water by percolation method for three days during which the sealed bottles were undergoing vigorous shaking at regular intervals. The mixtures thus obtained were filtered through Whattman's filter paper No. 1. The filtrates were concentrated by complete evaporation of solvent using rotary evaporator at room temperature to yield the crude extracts with the exception of the aqueous extract, which was evaporated on the water bath at 45°C.

The percentage yield of each extract was calculated from the respective weights of the extracts using the formula below:

$$\text{Percentage yield} = \frac{\textit{weight of the extract}}{\textit{Total weight of sample extracted}} \times 100\%$$

Other physical parameters such as colour and texture of the extracts were also recorded.

Preparation of Extract Stock Concentration for Antimicrobial screening

A test stock concentration of 30mg/ml, 60mg/ml, 90mg/ml and 120mg/ml for aqueous, methanol and ethanol extracts were prepared by dissolving 0.3g, 0.6g, 0.9g and 1.2g respectively of each extract in 10mls of distilled water in separate test tubes. The same concentration was made for amoxicillin which serves as the control.

Phytochemical screening

The presence of some basic secondary metabolites in the pulverized plant material was determined using standard methods (Evans 2002; Sofowora 2008).

Antimicrobial Screening

The clinical isolates were obtained from the Department of Medical Microbiology Aminu Kano Teaching Hospital (AKTH).The test organisms were characterized using the methods of Cheesebrough, (2002)

A loopful of the test organism was taken from their respective agar slants and sub-cultured into test tubes containing nutrient broth andwere incubated for 24hrs at 37°C. The obtained microorganisms in the broth were standardized using normal saline to obtain a population density, equivalent to a 0.5 McFarland standard.

The media used (Blood agar and nutrient agar) were prepared according to manufacturer's instruction (AVONCHEM limited, Wellington House waterloo, west Macclesfield Cheshire, England).

A standard cork borer of 5mm in diameter was used to cut well. 10µl of the test solution (extract) was then introduced into the well. The plates were inoculated with test organism and were then incubated at 37°C for 24hrs. Equal concentration of amoxycilin was used as control.

Minimum Inhibitory Concentration - Broth Dilution Method

MIC of the extracts was also carried out using broth dilution method as described in Ibekwe*et al*, (2001). Two-fold serial dilution of the extract in the broth were made from the stock concentration of the extract to obtain 10, 5, 2.5, 1.25, 0.625mg/ml (1000µl, 500 µl, 250 µl, 125 µl, 62.5µl) 0.1ml of the standardized inoculum of the microbes were then inoculated into the different concentrations of the extracts in the broth then incubated at 37°C for 24hrs and observed for turbidity of growth. The lowest concentration which showed no turbidity in the test tube was recorded as the MIC.

Minimum Bactericidal Concentration

Blood agar was prepared, sterilized at 121°C for 15mins and was poured into sterile Petri-dishes and left to cool and solidify. The contents of the tubes without growth were then sub-cultured onto the blood agar plates and incubated at 37°C, and observed for colony growth. The MBC was the plate with the lowest concentration of extract and without colony growth.

Determination of activity index

The activity index of the crude plant extract was determined using the relation;

$$\text{Activity index (A.I.)} = \frac{\text{Mean of zone of inhibition of the extract}}{\text{Zone of inhibition obtained for standard antibiotic drug}}$$

Determination of proportion index

The proportion index was determined using;

$$\text{Proportion index (P.I.)} = \frac{\text{Number of positive results obtained for individual extract}}{\text{Total number of tests carried out for each extract}}$$

Brine shrimps lethality assay

Test sample preparation for Brine shrimp bioassay

Test samples were dissolved in DMSO (Dimethyl sulfoxide) to obtain stock solution from which various concentrations of 10, 100, and 1000 µg/ml were made by serial dilution after dissolving 1g of the extract in 100ml of the DMSO. Pure DMSO and artificial seawater were used as negative control.

Hatching of Brine shrimp eggs

Brine shrimps eggs were obtained from Chemistry Department Bayero University Kano. The cysts were hatched in a tank containing artificial seawater made through dissolving a commercial marine salt 38g/L in distilled water (mineral water). The tank was well aerated and the proper light source was also provided. The nauplii were hatched within 24-36 h.

Brine shrimp lethality test

The toxicity of extracts was tested at various concentrations viz. 10, 100, and 1000 µg/ml in seawater. About 0.5 ml of diluted test solution was added to the pre marked test tubes containing 4.5ml of artificial sea water. Finally 10 active shrimps were added into each test tube. A vial containing 50µl DMSO diluted to 5 ml was used as control After 24 hours, survivors were counted using a dissection microscope (hand lens) and the percentage of the mortality (%M) of each dose calculated.

Statistical analysis

Using probit analysis, the lethality concentration (LC_{50}) was assessed at 95% confidence intervals. LC_{50} of less than 100 ppm was considered as potent (active) Gupta *et al.*, 1996). As mentioned by (Meyer *et al.*, 1982), LC_{50} value of less than 100µg/mL is toxic while LC_{50} value of greater than 1000 µg/mL is non-toxic. The percentage mortality (%M) was also calculated by dividing the number of dead nauplii by the total number, and then multiplied by 100%. This is to ensure that the death (mortality) of the nauplii is attributed to the bioactive compounds present in the plant extracts.

RESULTS

Physical Characteristics and the percentage yield of the extracts

The physical characteristics and the percentage yield of the aqueous extract, ethanolic extract and methanolic extracts are shown in (Table 1). During the extraction, the filtrates appeared brownish in colour, whereas the dried extracts appeared as deep brown, in colour with crystalline textures. The highest percentage yield of the extract was observed in aqueous extract of *E. camaldulensisi* which was 25.1% of the total sample extracted, followed by the methanolic extract which has a percentage yield of 23.1% and least in terms of percentage yield was ethanolic extract which was 19.1% as shown in (table 1).

Preliminary Qualitative Phytochemical Screening Tests of the various extracts

Phytochemical screening for the bioactive components present in the aqueous extract, ethanolic extract and methanolic extract of *Eucalyptus camaldulensis* revealed that the extracts were very rich in secondary metabolites including of alkaloids, saponin, terpenoid, anthraquinone,

tannins, glycosides and steroids as shown in (Table 2). The methanolic extract has the highest number of phytochemical in the plants extract followed by the ethanolic extract the least is the aqueous extract. Tannin, saponin and steriod were detected in all the plants extracts, while flavonoid was not detected in all the extract. Table 2 shows the distributions of the bioactive phytochemicals in each of the plants extracts.

Table 1 Physical characteristic of extracts of *Eucalyptus camaldulensis* leaves.

Extract	**Colour**	**Texture**	**%yield**
Methanol	Deep brown	Crystalline	23.1
Ethanol	Deep brown	Crystalline	19.6
Aqueous	Brown	Crystalline	25.1

Table 2: Phytochemical constituents' of *Eucalyptus camaldulensis* leaves extracts.

EXTRACT	Alkaloids	Saponins	Tannins	Flavonoids	Steroids	Glycosides	Terpenoids	Anthraquinones
Aqueous	-	+	+	-	+	+	-	+
Ethanolic	+	+	+	-	+	-	-	-
Methanolic	-	+	+	-	+	-	+	-

Antimicrobial Activity of the plants extracts against clinical isolates

The antimicrobial activities of the methanolic, ethanolic, and aqueous extract and that of Amoxycillin antibiotic at four different concentrations (120mg/ml, 90mg/ml, 60mg/ml, and 30mg/ml for each extract) against the test organisms are indicated in (Table 3). Methanolic extract shows the highest activity with a zone diametre of 24mm at a concentration of 120mg/ml against *Salmonella paratyphi A,* no resistance was observed against the extracts at higher concentration. The susceptibility pattern of all the test organisms against the various extract is generally very high as resistance was only observed at lower concentration 30mg/ml of aqueous extract. *Salmonella typhi*appeared the most susceptible organism which showed no resistance to the extracts.

MIC and MBC of the plants Extracts.

The minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of the extracts of *E. camaldulensis* leaf against the test organisms are presented in (Table 4). *Eucalyptus camaldulensis* leaf extract had MIC range of (62.5 – 125µg/ml) against all the test organisms. Similarly, the minimal bactericidal concentration (MBC), generally do not exceed the minimal inhibitory concentration (MIC) by more than a factor of 2 (Table 4)

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Table 3: Antimicrobial activity of the *Eucalyptus camaldulensis* leaves extract against the test organisms by agar well diffusion method.

S/N	Test organism	Mean zone of inhibition in (mm) produced by concentration of each extract against the test organism																ACTIVITY INDEX		
		Methanolic extract concentration (mg/ml)				Ethanolic extract concentration (mg/ml)				Aqueous extract concentration (mg/ml)				Amoxicillin concentration (mg/ml)						
		120	90	60	30	120	90	60	30	120	90	60	30	120	90	60	30	Met	Eth	Aqu
1	*Salmonella typhi*	21	18	13	10	19	15	12	8	17	15	09	0	20	17	15	10	1.00	0.87	0.66
2	*Salmonella paratyphi A*	24	19	15	8	20	17	13	9	18	15	10	0	21	18	15	13	0.98	0.74	0.64
3	*Salmonella paratyphi B*	22	19	13	8	20	18	15	9	19	15	10	0	21	17	15	15	0.91	0.77	0.64

Proportion **I**ndex **:** MET=1, ETH=1, AQU= 0.75.

Table 4: Minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of *Eucalyptus camaldulensis* leaves extracts.

S/N	Test organisms	Methanolic extract		Ethanolic extract		Aqueous extract		Amoxycillin	
		MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
1	*Salmonella typhi*	62.5	250	125	500	125	500	62.5	250
2	*Salmonella paratyphi A*	125	500	62.5	250	125	500	125	500
3	*Salmonella paratyphi B*	125	500	125	500	125	500	125	500

Table 5: Brine Shrimp cytotoxicity Assay of *E. camaldulensis* extracts

Plant	Extract	Concentration (ppm or µg/ml)	No. of Shrimps	No. of Survivors	% Mortality	LC_{50} (µg/mL) Brine Shrimp Lethality
*E. camaldulensis*leaves	Aqueous	1000	10	6	40	
		100	10	7	30	1308.872
		10	10	10	00	
	Methanol	1000	10	4	60	
		100	10	7	30	469.630
		10	10	8	20	
	Ethanol	1000	10	4	60	
		100	10	7	30	472.221
		10	10	9	10	

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DISCUSSION

Water as a solvent of extraction gives a yield of the extract, the extracts appears crystalline after drying with brown to deep brown colour. Methanol gives more yields of the phytochemical compounds. Tannin was found in the extract. Babayi *et al.,* (2004) in his work also report the presence of the phytochemicals identified in this study. The antibacterial activity of *Eucalyptus camaldulensis* agrees with earlier studies conducted by Ayopola *et al.,* 2008. However, the antibacterial potential of *E. camaldulensis* recorded contradicts earlier findings by Babayi *et al.,*(2004).In brine shrimp lethality bioassay, % mortality increased gradually with increase in concentration of the test samples. An LC_{50} (concentration killing fifty per cent of the brine shrimp larvae), value greater than 100µg/ml is considered to present a non-toxic compound or extract (Moshi *et al.,* 2010). The brine shrimp toxicity assay showed 1 extract had LC_{50} value greater than 1000µg/ml, while the remaining had LC_{50} value between 100 and 700µg/ml and therefore classified as moderately toxic.

CONCLUSION

Methanol, ethanol and water have the ability of extracting phytochemical from *E. camaldulensis.* All extract showed activity against the test organisms in accordance with the extract concentration. The results from MIC and MBC indicated the bacteriostatic property against the test organism, in accordance with the extract concentration. *Eucalyptus camaldulensis* shows higher activity with P.I value of 1.00 similar to control antibiotic. Toxicity study carried out on the plants extract revealed that aqueous extracts *E. camaldulensis* leaf was non-toxic.

From the results obtained, brine shrimp lethality assay conducted shows greater percentage of the extract tested are toxic, contrary to the claims by traditional medicine practitioners. Prolong administration of such plants should be avoided especially by communities that patronize such plants as herbal cure.

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