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Evaluation of phytochemical analysis and antibacterial potential of different solvent extracts of leaves and stem bark of *Eucalyptus camaldulensis* against selected pathogenic bacteria

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ABSTRACT: Research into the phytotherapeutic potentials of plants in the treatment of ailments has significantly received attention in recent years. Here, using two solvent extracts, we aimed to evaluate the antibacterial efficacy of phytochemicals extracted from leaf and stem bark of *Eucalyptus camaldulensis* against selected pathogenic bacteria. Solvent extracts of the leaf and stem bark of *E. camaldulensis* were prepared with methanol and chloroform. Phytochemical screening of crude extract was performed using standard methods. The leaf and stem bark extracts were tested for their antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Bacillus subtilis* and *Escherichia coli* using the agar well diffusion method. Aqueous extract of plant (50 mg/mL) was used to prepare 50 mg/mL, 25 mg/mL, 12.5 mg/mL and 6.25 mg/mL concentrations of extract. Qualitative phytochemical analysis showed that in the methanolic leaf extract; cardiac glycosides, saponins, flavonoids and terpenoids were present while alkaloids and steroids were found in the chloroform leaf extract. Tannins were present in both extracts. Also, methanolic and chloroform stem bark extracts showed the presence of alkaloids, flavonoids, cardiac glycosides, steroids and tannins. Furthermore, both methanolic and chloroform extracts showed inhibitory effects at varying concentrations. Methanolic extract was effective on *E. coli*, *B. substilis* and *P. aeruginosa* at 25 mg/mL while chloroform extract exhibited inhibitory effect on *E. coli* at concentrations of 12.5 mg/mL and 6.25 mg/mL. Phytochemical screening of leaf and stem bark extract of *E. camaldulensis* revealed the presence of some active secondary metabolites; with the methanolic extracts showing more considerable antimicrobial effects.

Keywords: *Eucalyptus camaldulensis*, phytochemical screening, antibacterial activity, stem bark and leaves.

Introduction

For a long time, synthetic drugs have been a standard in the treatment of different microbial infections. In recent years, the surge of antibiotics resistance cases as reported by several studies and the detrimental effect of synthetic drugs have prompted the search for safer alternatives from plant origin as potential sources of new antimicrobials (Mégraud *et al.*, 2013, Tacconell *et al.*, 2018, Musa *et al.*, 2020).

Consequently, many researchers have therefore searched for alternatives with the use of medicinal plants as therapeutic remedies.

Several studies have reported the medicinal properties of different plant species (Musa *et al.,* 2011; Abd EL-Tawab *et al.,* 2017, Omer *et al.,* 2020). Many of these plants have also been cataloged by the World Health Organization (WHO) for their considerable therapeutic potentials against several diseases. According to Krishnananda *et al.* (2017), in modern medicines, medicinal plants are regarded as an important source of drugs for diseases treatment as well as for nutraceuticals, food supplements, among others. The therapeutic tendencies in medicinal plants are attributed to their bioactive secondary metabolites (Omer *et al.,* 2020). In addition, traditional medicinal plants have been considered to be relatively safer than chemical drugs (Abd EL-Tawab *et al.,* 2017). This has prompted their extensive usage in health care settings (WHO, 2005). Nevertheless, there is a continued search for effective antibiotics with an emphasis on active substances from plant origins.

Eucalyptus camaldulensis is a plant belonging to the *Myrtaceae* family mostly found in tropical, subtropical and temperate environments (Behbahani *et al.,* 2013). The plant is commonly referred to as "zaity" in the northern part of Nigeria and has been regarded as one of the most planted *Eucalypts* (Musa *et al.,* 2011). For several years, attention has been given to *Eucalyptus* species due to their medical characteristics. The ethnomedicinal benefits of the plants have also been ascribed to several valuable compounds such as flavonoids, phenols, sterols, terpenes, resins, saponin, cardiac glycosides, tannin among others (Musa *et al.,* 2011).

Several studies have also reported the antihyperglycemic, antibacterial activity, antioxidants, and anti-inflammatory tendencies of leaves extracts of *Eucalyptus* species (Bachir *et al.,* 2012; Islam *et al.,* 2015, Al- Snafi *et al.,* 2017) as well as antimicrobials activity (Pandey *et al.,* 2014). Here, we investigated the antimicrobial efficacies of leaves and stem bark of *E. camaldulensis* from the north-central origin of Nigeria to some pathogenic bacteria.

Materials and Methods

Sample collection and identification

Eucalyptus camaldulensis leaves and stem bark were collected from Minna, north-central Nigeria, and was duly authenticated by a Botanist in the Department of Biological Science Ibrahim Badamasi Babangida University Lapai, Niger State.

Preparation of plant sample

Fresh leaves and stem bark of *E. camaldulensis* were rinsed thoroughly with distilled water to remove any debris and air-dried for 2 weeks at room temperature. Dried plant samples were then pulverized using an electrical blender (Qasa) and filtered with 8 mm mesh sieve. The fine powdered sample obtained were stored in clean air-tight container and kept in clean, dry place until required for use.

Preparation of the crude extract

The method of Chukwu *et al.,* (2016), was adopted for the extraction of plant samples with modification. Two hundred grams (200 g) of stem bark and leaves were macerated separately in 800 mL of methanol and chloroform. The mixtures were placed on an orbital shaker for 48 hrs to attain a homogenous mixture (shaken at an interval of 3 hrs) and filtered with Whatmann no.1 paper. Using rotary evaporator, each filtrate was concentrated at 40 ^{o}C and dried in water-bath to obtain a crude extract. A concentration of 50 mg/mL of crude extract was prepared by dissolving 4 g of crude extract in 10 mL sterile distilled water. Then, through serial dilution, 6.25, 12.5 and 25 mg/mL concentrations were prepared and kept at 4 °C before use.

Test Organisms

Clinical isolates, pure cultures of pathogenic bacteria: *Staphylococcus aureus, Bacillus substillis, Klebsiella pneumonia, Escherichia coli,* and *Pseudomonas aeruginosa* were obtained from the National Institute of Pharmaceutical Research and Development (NIPRID), Abuja, Nigeria. These bacterial isolates were cultured on nutrient agar and incubated for 24 hrs at 37^{o}C. Isolates were further sub-cultured on Mueller-Hilton Broth and incubated at 37^{o}C for 24 hrs.

Phytochemical Analysis of crude extract

The chloroform and methanol extracts from stem bark and leaves were subjected to qualitative phytochemical analysis for the test of alkaloids, saponins, steroids, flavonoids, tannins, terpenoids and cardiac glycosides. The standard procedures used were as established by Harborne, (1978) and Sofowora (1993).

Test for alkaloids

In a steam bath, 2 mL of extract and 5 mL of 1% aqueous HCl was stirred and filtered while hot. Distilled water was added to the residue, as well as 2-3 drops of potassium mercuric iodide (Mayer's reagent). The appearance of white turbid residue was indicative of the presence of alkaloids.

Test for saponins

In a controlled water bath, 5 mL of extract was boiled in distilled water and filtered appropriately. Distilled water was added to the filtrate and properly shaken until froth appears. About 2-3 drops of olive oil were added to the froth until a formation of the emulsion was observed which was positive for saponins.

Test for steroids

About 1 mL of conc. H_2SO_4 was carefully added in layer to the test tube containing 2 mL of each extract. The presence of steroids was confirmed with the development of reddish-green coloration.

Test for cardiac glycosides

Conc. H_2SO_4 was carefully added in layer into a test tube containing 2 mL of extract and 1 mL of chloroform. The appearance of reddish-brown coloration confirms the presence of a steroidal ring of glycosides.

Test for tannins

In a test tube, 1.5 mL of extract was boiled in water and filtered. Two drops of $FeCl_3$ were added and color changes were observed. A blue-black or green color change confirms the presence of tannin.

Test for terpenoids

Conc. H_2SO_4 (3 mL) was carefully added in layer to test-tube containing 3 mL of plant extract and 2 mL of chloroform. The development of reddish-brown coloration at the interface shows the presence of terpenoids.

Test for flavonoids

About 5 mL of each extract is added to 3 mL of 1% Al_2Cl_3. The presence of flavonoids is observed with yellow color change. Dilute ammonia solution of 5 mL was added to the mixture and conc. H_2SO_4. The disappearance of yellow coloration is further indicative of the presence of flavonoids.

Antibacterial activity of crude extract

Antibacterial testing of various solvent extracts of the stem bark and leaves of *E. camaldulensis* was estimated using the method of Chukwu *et al.,* (2016). The sensitivity of the test organisms to the stem bark and leaves extracts was determined using the agar well diffusion method as described by the National Committee for Clinical Laboratory Standards (NCCLS, 1999). Mueller-Hilton agar plates were prepared in

triplicates for each of the test organisms and labeled appropriately. A volume of 0.1 mL of each test organism was made to the equivalent of 0.5 McFarland standards and inoculated into separate plates using the spread plate method. Sterile cork borer was used to bore 5 equidistant holes on the surface of the plate with one at the center. One-tenth of a milliliter (0.1 mL) of each extract concentration was introduced into the four peripheral holes while the hole in the middle contained a control (ciprofloxacin). Diffusion of extracts through the medium was allowed for 1 hr and the plates were kept in incubator at 37 °C for 24 hrs. The inhibition zones around each well were measured and the mean value was obtained in millimeters (mm).

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The Minimum Inhibitory Concentration was determined according to the National Committee for Clinical Laboratory Standards (NCCLS, 1999). One millimeter of the extract was inoculated into test tube containing 9 mL of sterile Mueller-Hilton Broth and properly shaken before being serially dispersed into the other 3 test tubes containing 9 mL of sterile Mueller-Hilton Broth to obtain 6.25, 12.5, 25, and 50 (mg/mL) concentrations. Standardized inoculums of 0.1 mL were then inoculated into each tube containing the various concentrations. The tubes were incubated at 37 ^{o}C for 24 hrs. This procedure was repeated for all test isolates. From the MIC results obtained, MBC was estimated. Plant extract concentrations with no visible growth through turbidity observation were further sub-cultured onto freshly prepared sterile Mueller-Hilton Agar plates and incubated at 37 °C for 24 hrs. After incubation, the least concentration at which the organism did not recover or seen to grow was considered and recorded as the MBC.

Results

Plant yield: the result showed plant yield was higher in leaves with values 27.10 g and 7.91 g for methanol and chloroform respectively while stem bark yield was 4.78 g and 3.68 g for methanol and chloroform respectively (Table 1).

Phytochemical analysis

Secondary metabolites such as alkaloids, cardiac glycosides, steroids, tannins, flavonoids were present in leaves and stem bark extracts of *E. camaldulensis* while saponins and terpenoids were also present in leaf extract (Table 2).

Screening for antibacterial activities

Table 3 shows the antibacterial activities of methanolic and chloroform of *E. camaldulensis* leaf and stems bark extracts. In the methanolic fraction of leaf extracts, the antibacterial activity against test isolates showed an inhibitory effect at 25 and 50 mg/mL. No inhibitory effect was observed on *S. aureus* and *K. pneumoniae* at 25 mg/mL. In stem bark extract, an inhibitory effect was observed at 12.5, 25 and 50 mg/mL. As shown, a greater inhibitory effect was observed at 25 and 50 mg/mL against *S. aureus* and *P. aeruginosa*. In the chloroform fraction of leaf extracts, an inhibitory effect was observed against *P. aeruginosa, E. coli,* and *S. aureus*. The inhibitory concentrations observed for these isolates range between 6.25 – 50 mg/mL. In stem bark extract, at 12.5 mg/mL and 6.25 mg/mL, an inhibitory effect against *P. aeruginosa* was observed.

Determination of minimum inhibitory concentration (MIC)

As observed in Table 4, extracts of *E. camaldulensis* leaf and stem bark showed MIC values within the range of 12 - 50 mg/mL. In the stem bark extracts, the MIC value is 50 mg/mL, while the leave extracts values are between the ranges of 12.5 – 50 mg/mL. In addition, the lowest MIC value of methanol and chloroform extracts is 12.5 mg/mL. Also, MBC values of chloroform leaf extract range between 25 – 50 mg/mL while methanol leaf extract is 50 mg/mL.

Table 1: Plant yield of methanol and chloroform extracts of stem bark and leaf of *E. camaldulensis*

Solvent	Plant	Plant weight (g)	Plant extract weight (g)	Percentage Plant yield (%)
Methanol	Leaves	200	27.10	13.55
	Stem bark		7.91	3.96
Chloroform	Leaves		4.78	2.39
	Stem bark		3.68	1.84

Table 2: Phytochemical analysis of methanolic and chloroform leaf and stem bark extracts of *E. camaldulensis*

Solvent	Flavonoid	Alkaloids	Saponins	Steroids	Cardiac glycosides	Tannins	Terpenoids
Ethanol							
Leaves	+	-	+	+	+	+	+
Stem bark	+	+	-	+	+	+	-
Chloroform							
Leaves	-	+	-	+	-	+	-
Stem bark	-	+	-	+	+	+	-

+: present, -: not present

Table 3: Antibacterial activity of methanolic and chloroform leaf and stem bark extracts of *E. camaldulensis*

Isolate	Solvent	Plant	Zone of Inhibition(mm) Concentration (mg/mL)			
			50	**25**	**12.5**	**6.25**
E. coli	Methanol	Leaves	9.2	5	-	-
		Stem bark	-	-	-	-
	Chloroform	Leaves	-	-	4.5	2.1
		Stem bark	-	-	-	-
S. aureus	Methanol	Leaves	1.5	-	-	-
		Stem bark	7	1	-	-
	Chloroform	Leaves	4	2	1	-
		Stem bark	-	-	-	-
K. pnuemoniae	Methanol	Leaves	6.5	-	-	-
		Stem bark	3	-	-	-
	Chloroform	Leaves	-	-	-	-
		Stem bark	-	-	-	-
P. aeruginosa	Methanol	Leaves	4	6.5	-	-
		Stem bark	9	7.2	4	-
	Chloroform	Leaves	1	-	-	-
		Stem bark	-	-	5	4.5
B. substilis	Methanol	Leaves	3.5	1.5	-	-
		Stem bark	-	-	-	-
	Chloroform	Leaves	-	-	-	-
		Stem bark	-	-	-	-

keys: mm = Millimeter; mg/mL = milligram per milliliter; - = no zones of inhibition

Table 4: Minimum Inhibitory concentration (MIC) of leaf and stem bark extract of *E. camaldulensis* (mg/mL)

Solvent	**Plant**	**Minimum Inhibitory Concentration (mg/mL)**				
		Test Organism				
		E. coli	*S. aureus*	*K. pnuemoniae*	*P. aeruginosa*	*B. substilis*
Methanol	Leaves	50	12.5	50	25	25
	Stem bark	50	50	50	50	50
Chloroform	Leaves	12.5	50	50	50	50
	Stem bark	50	50	50	50	50

Table 5: Minimum Bacterial Concentration (MBC) of leaf and stem bark extract of *E. camaldulensis* (mg/mL)

Solvent	**Plant**	**Minimum Bacterial Concentration (mg/mL)**				
		Test Organism				
		E. coli	*S. aureus*	*K. pnuemoniae*	*P. aeruginosa*	*B. substilis*
Methanol	Leaves	-	50	-	-	-
	Stem bark	-	-	-	-	-
Chloroform	Leaves	-	50	-	25	-
	Stem bark	-	-	-	-	-

Discussion

The phytochemical screening and antimicrobial activity of *E. camaldulensis* leaf and stem bark using methanol and chloroform solvents were investigated. Previous studies have highlighted the rich quantity of bioactive phytochemicals in various parts of the plant. These metabolites are highly essential and; have properties that are employed for medicinal purposes against several diseases. Importantly, the type of solvent used during extraction is important to obtain maximum active phytochemical. Here, the results of phytochemical extraction from all extracts in leaves and stem bark indicated the presence of flavonoids, alkaloids, saponins, cardiac glycoside, tannins, steroids and terpenoids. Analysis of phytochemical constituents of methanolic leaf extract of *E. camaldulensis* showed the presence of flavonoids, cardiac glycosides, saponnin, steroids, tannin and terpenoids while alkaloids was absent. This is consistent with the findings of Musa *et al.* (2011), Pandey *et al.* (2014) and Chukwu *et al.* (2016) who reported flavonoids, tannins, saponnins and terpenes in the leaf of *E. camaldulensis*.

Notably, qualitative estimation in methanolic extract of leaves and stem bark showed the presence of more phytochemicals compared to chloroform extracts which lacked flavonoids, saponins and terpenoids in leaves and stem bark. This indicates the inefficiency of chloroform as an extraction solvent for

phytochemicals in *E. camaldulensis* leaves and stem bark. This is consistent with other studies that have observed the poor solubility of chloroform for phytochemicals (Javaid, and Saddique, 2012; Dixon and Jeena, 2017). Thus, the plant yield of methanol showed better extraction potential than chloroform. This agrees with the studies of Do *et al.* (2014) and Azzah and Ibtisam, (2019) that have found methanol as one of the preferred solvents in the extraction of secondary metabolic compounds from *E. camaldulensis*. Moreover, based on the phytochemical studies of Ayepola and Adeniyi, (2008) and Babayi *et al.* (2004) on stem bark and leaves, *E. camaldulensis* have been observed to possess high levels of isoprenoids, tannins, phenolic compounds, saponins, cardiac glycosides, flavonoids, terpenes and essential oils.

In the current study, *E. camaldulensis* showed a broad spectrum against gram-negative and gram-positive bacterial isolates similar to the previous study of Azzah and Ibtisam, (2019). According to different studies, the antibacterial efficacy of plant extract is dependent on the solvent used for extraction (Asfere *et al.,* 2020). Here, the antibacterial activity of methanolic leaf extract showed more effective growth inhibition of bacterial isolates. At the highest concentration, methanolic leaf extract showed better inhibition against all bacterial isolates. As suggested by several studies, the better inhibitory activity observed in methanolic leaves extracts might be attributed to the presence of other antimicrobial agents such as phytol, pyranone derivative and 3,5-dihydroxy-2-methyl (Jananie *et al.*, 2011, Tyagi *et al.*, 2017). This is consistent with the previous report that has shown that methanolic extract of *E. camaldulensis* is effective in the treatment of a wide range of *E. coli, S. aureus, P. aeruginosa* infections.

For fraction of chloroform leaf extract, optimal inhibition was observed at 12.5, 50 and 50 (mg/mL) against *E. coli, S. aureus, K. pnuemoniae* respectively. While stem bark extract showed maximum inhibitory activity at 12.5 mg/mL. The outcome of antibacterial activity of *E. camaldulensis* in this present study is consistent with other studies that have proved the growth inhibitory effect of extracts of *E. camaldulensis* to gram-positive and gram-negative bacteria (Pandey *et al.,* 2014; Traore *et al.,* 2014; Ibrahim *et al.,* 2016).

The MIC of *E. camaldulensis* leaf extract indicates an inhibitory effect within the range of 6.25 -50 mg/mL against all test isolates. The growth inhibitory effect of methanolic extract was optimal at 50 mg/mL against *E. coli* and *K. pneumonia*; 25 mg/mL for *P. aeruginosa* and *B. substilis* and 12.5 mg/mL for *S. aureus*. This is in agreement with Behbahani *et al*. (2013) which observed a similar range of inhibition. On the other hand, at 50 mg/mL chloroform extract showed inhibition against test isolates except for *E. coli* which had an inhibitory effect at 12.5 mg/mL. The stem bark extract indicated an inhibitory effect at 50 mg/mL against all test isolates for both methanolic and chloroform extracts.

The minimum bactericidal concentration of the leaf extract of *E. camaldulensis* against all test isolates in the study ranged from 6.25 – 50 mg/mL. The methanolic extract had bactericidal effect at 50 mg/mL against *S. aureus* only, while other test isolates were resistant. The chloroform extract had bactericidal effect at 50 mg/mL and 25 mg/mL for *S. aureus* and *P. aeruginosa* respectively. The stem-bark extract of both methanol and chloroform had no bactericidal effect against test isolates. Thus, the little or no bactericidal effect of extract may be attributed to the stage of development of the plant or organic solvent used for extraction. Other studies have observed that concentration of plant extracts has an effect on the bactericidal properties (Asfere *et al.,* 2020). Therefore, it can be suggested that a higher concentration is used against these organisms. In this study, the result revealed that the methanolic extract of *E. camaldulensis* is more compelling. It showed a greater inhibitory effect as compared to other extracts of *E. camaldulensis* used in this study.

In conclusion, the inhibitory potential of *E. camaldulensis* extracts depends on the type of solvent under study. Among the solvents, methanol extracted fractions were more effective to inhibit the activity of the tested pathogenic bacteria with extracts from leaves showing better susceptibility. Phytochemical analysis of methanolic extract of leaves and stem bark showed the presence of more phytochemicals compared to chloroform extracts.

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