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ANTIMICROBIAL EVALUATION OF THE LEAF EXTRACT OF *EUPHORBIA MILII.VAR SPLENDENS*

OGAH CELINA ONOTSE[1,*], IGBOKWE NWAMAKA HENRIETTA[2], OWOLABI MBANG ADEYEMI[1], ALABI FAIZAH ABISOLA[1]

1. Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Lagos, Nigeria.
2. Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmacy, University of Lagos, Nigeria.

ABSTRACT

Plant-based medicines have become popular due to their low toxicity both to man and environment. In Nigeria, the leaves of *Euphorbia milii* are used in the treatment of variety of diseases, including microbial infections. This study screened ethanol and aqueous extracts of *E. milii* leaves for phytochemical constituents and evaluated their activity *in vitro*, against selected microorganisms. The leaves of *E. milii* were collected, washed, air dried and subsequently ground into coarse powder. The powder was extracted by cold maceration in ethanol and sterile distilled water to obtain the ethanol and aqueous extracts, respectively. Similarly, aqueous extract of fresh leaves of the plant was prepared by cold maceration in sterile distilled water. Phytochemical study was carried out and antimicrobial activity tests were done using agar well diffusion method. Levofloxacin and Bifonazole were used as reference antibacterial and antifungal drugs, respectively. Results showed that both ethanol and aqueous extracts contained an array of phytochemicals. The ethanol dry leaf extract was active against *Staphylococcus aureus* and *Bacillus subtilis* while the aqueous dry leaf extract was active only against *Bacillus subtilis*. Aqueous fresh leaf extract was not active against any of the bacterial test organisms. None of the three extracts inhibited the growth of the fungal organisms (*Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus, Trichophyton rubrum and Mycobacterium globose*) used in the study. The minimum inhibitory concentration (MIC) of the ethanol dry leaf extract was 50 and 75 mg/mL for *S. aureus* and *B. subtilis*, respectively.

KEYWORDS: **Antimicrobial, *Euphorbia milii,* Phytochemicals, Ethanol extract.**

INTRODUCTION

There is increased shift from synthetic to plant-based medicines because of their broad biological activities and low toxicity both to human and environment. It has been estimated that up to 80 % of African populations use some form of traditional herbal medicine, and plant extracts have been found to possess varieties of pharmacological activities [1, 2]. Thus, herbal medicine serves as a major and accessible means of treatment and continues to play an important role in healthcare management.

There has been a worldwide increase in incidence of microbial infections, partly attributable to increase in opportunistic infections in immunodeficient virus-positive patients and in the immunocompromised due to cancer chemotherapy. Urbanization and increased global travel are other factors that contribute to the spread of infectious diseases [3]. Some opportunistic infections such as cryptococcosis and histoplasmosis have been reported to cause death in 6-10 % of human immunodeficiency virus (HIV) infected patients [3]. Also, it is estimated that about 5.8 % of people living with AIDS have oral lesion caused by *Candida albicans* [4]. Furthermore, bacteria such as

**Corresponding author*: onotsec@yahoo.com; +234802 314 8053

Stapylococcus aureus and *Escherichia coli* are reported to be responsible for post-operative wound infections, toxic shock syndrome and urinary tract infections [4, 5]. In Nigeria, superficial mycoses of the glabrous skin caused by fungi belonging to the genera *Epidermophyton microsporum* and *Trichophyton rubrum* has been reported as one of the most prevalent human infections encountered in medical/clinical practice and has since been established as a public health problem in Africa [6]. These skin infections, though may not cause death, can result in serious discomfort and disfiguration.

Resistance by microorganisms to many synthetic antimicrobial agents is on the rise, and has been attributed to indiscriminate use of these agents, including unnecessary prescriptions by clinicians and patient self-medication, leading to frequent and inappropriate use [7, 8]. Antimicrobial resistance may cause therapeutic failure, leading to increase in cost of healthcare, morbidity and mortality. For instance, drug-resistant and multidrug-resistant tuberculosis (TB) in which strains of *Mycobacterium tuberculosis* develop resistance to one or more anti-TB drugs is a big challenge in the management of TB globally and has caused increase in the economic and social cost of the disease [9]. Furthermore, species of fungi such as *Candida* and *Aspergillus* have been associated with treatment failure and high death rates among patients in health facilities, especially intensive care units [10]. Although the use of multiple drug therapy can mitigate the effect of drug resistance, it also increases the chances of adverse drug reactions and interactions. There is a need to develop new effective antimicrobial agents, and screening of plants for antimicrobial activity is a step to finding safer and biodegradable alternatives to older drugs [11, 12].

Euphorbia milii (family *Euphorbiacea*) is a popular evergreen garden shrub, native to Madagascar but commonly found in Nigeria. It grows up to a height of 10 - 50 cm and bears small, green, oval leaves which are alternately arranged round its shoots, surrounded by spiky thorns; the reason the plant is commonly referred to as 'Crowns of thorns'. It is a flowering plant, bearing red, pink, white or yellow flowers at the shoot tips, depending on the variety. Different parts of the plant have been reported to contain an array of secondary metabolites and possess anti-inflammatory, antioxidant and antinociceptive properties for which they are used in several parts of the world to treat ailments such as cancer, genital wart and hepatitis [13-16]. In Nigeria, juice from the leaves is used by local herbal medicine practitioners for the treatment of various ailments including skin infections, boils, sores and promotion of wound healing.

This study screened the ethanol and aqueous leaf extracts of *E. milii* for phytoconstituents and evaluated their antimicrobial activity with a view to justifying its local use in the treatment of skin and other infections.

MATERIALS AND METHODS

Collection and identification of plant material

The fresh aerial parts of *E. milli* were collected from the flower beds around the Faculty of Pharmacy, University of Lagos, in July, 2018. The plant was identified and authenticated at Department of Botany, University of Lagos, Nigeria with voucher number LUH: 7815 and the plant specimen was deposited in the herbarium for future reference.

Collection of microorganisms

Clinical isolates of bacteria (*Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae*) and fungi (*Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus, Trichophyton rubrum, Mycobacterium globosa*) collected from Lagos University Hospital (LUTH) and Department of Pharmaceutical Microbiology Laboratory, Faculty of Pharmacy of the University of Lagos, Nigeria were used for the study.

Preparation of leaf extracts

The leaf extracts were prepared using the method of Igbokwe et al., 2018 [17]. Fresh and dry (shade dried at room temperature) leaves of *E. milii* were pulverized to coarse powder with laboratory mill (Christy and Norris Ltd, Chelmsford, England). Each of 100 g sample of the powdered dry leaf was extracted with 1 L of 80 % ethanol and 1 L of distilled water, respectively using cold maceration for three days. The same maceration process was used in extracting 100 g ground fresh leaves using distilled water. Each extract was filtered using a fine pored muslin cloth, concentrated in a rotary evaporator (Buchi V-801) and dried in an oven at 37^0C. Each dry extract was weighed and the % w/w yield calculated.

Phytochemical screening

The phytochemical screening of ethanol and aqueous extracts of dry and fresh leaves was carried out using standard procedures [18].

Evaluation of antimicrobial activity of the extracts

The antimicrobial activities of the ethanol and aqueous extracts on clinical isolates of *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus, Trichophyton rubrum* and *Mycobacterium globosa* were determined using the agar well diffusion method [19, 20].

A 1 ml suspension of each of the test organisms was seeded in sterile 25mL Mueller Hinton Agar (MHA) and allowed to set. Wells were bored on the seeded agar using a sterile cork-borer (12mm in diameter) and filled with 0.5mL each of the graded concentrations (75, 150 and 300 mg/mL) of each of the extracts. A blank well in each of the culture plates was filled with 0.5 mL sterile water which served as the negative control. Levofloxacin and Bifonazole at the concentrations of 6.25, 12.5, 25 and 50 µg/mL were used as positive control for bacteria and fungi, respectively. The cultures were allowed to diffuse into the agar at room temperature for one hour before incubation at 37 ^{0}C for 24 hours and the zones of inhibition recorded. The experiment was carried out in triplicates and the mean calculated.

Evaluation of minimum inhibitory concentration (MIC)

The MIC of the ethanol extract was determined using agar dilution method [20, 21]. Different concentrations of the leaf extract ranging from 40mg/mL – 100 mg/mL were prepared in 5% ethanol by serial dilution. Each concentration was seeded in 25mL MHA and allowed to solidify. With a sterile micropipette, susceptible bacteria were introduced to the surface of the agar plates which were left to stand for 1 hour on the bench to allow pre-diffusion of the plant extract before incubating at 37^{0}C for 24 hours and the plates observed for growth. The least concentration at which there was no growth was recorded as the MIC for each microorganism.

Statistical analysis

Data were expressed as mean ± standard error of mean (SEM). One-way ANOVA and Student's t-test were used to compare means of samples and between samples and standards on SPSS statistical software. Differences were significant at $p< 0.05$.

RESULTS

Percent yield and phytochemical screening of the extracts

Ethanol was more efficient as a solvent for extraction of *Euphorbia milii* leaves, as it produced a higher yield of extract than water. Extraction with water produced a higher yield from dry leaves than from fresh leaves (Table 1).

The results of phytochemical screening showed high presence of flavonoids in ethanol and aqueous extracts of dry leaves. Condensed tannins, cardiac glycosides, terpenoids, saponins and free anthraquinones were also moderately present in both extracts (ethanol and aqueous) of dry leaves, with hydrolysable tannins slightly present in both. Steroids were moderately detected in ethanol dry leaf extract only. Reducing sugars and carbohydrates were the only phytoconstituents slightly present in aqueous fresh leaf extract of *E. milii*. None of the extracts showed any presence of alkaloids (Table 2).

Antimicrobial activity of the extracts

Levofloxacin inhibited the growth of all test bacteria except *Klebsiella pneumoniae* (Table 3). Ethanol dry leaf extract was active against *Staphylococcus aureus* and *Bacillus subtilis* (Table 4). The aqueous dry leaf extract was active against *Bacillus subtilis* only (Table 5). The antimicrobial activities of the extracts and levofloxacin were concentration dependent. The aqueous fresh leaf extract was not active against any of the test bacteria.

Antifungal susceptibility test results showed that the reference drug, bifonazole was active against *Aspergillus niger* and *Trichophyton rubrum* (Table 6). All the extracts showed no activity against any of the test fungi.

The minimum inhibitory concentration (MIC) for ethanol dry leaf extract was determined and found to be 50 mg/mL and 75 mg/mL for *S. aureus* and *B. subtilis,* respectively (Table 7).

DISCUSSION

Phytochemical constituents and antimicrobial activity of ethanol and aqueous extracts of dry and fresh leaves of *Euphobia milii* were investigated in this study. The extract obtained using ethanol as solvent was more than that obtained from water as solvent. Solvent of similar polarity to the solute will more effectively extract the solute, hence the higher yield of ethanol extract could be as a result of more permeation of ethanol, an organic solvent, into the leaf polymer leading to enhanced solubility of compounds of similar polarity.

(A)

(B)

Figure 1: Pictures of *Euphorbia milii* showing: [A] aerial parts and [B] leaves and flowers.

Table 1: Percentage yield of extracts

Solvent	Weight of powdered dry leaves (g)	Weight of fresh leaves (g)	Weight of dry extract (g)	Percentage yield (%)
Ethanol	100	-	12.13	12.13
Water	100	-	9.85	9.85
Water	-	100	6.21	6.21

Table 2: Results of phytochemical screening of the extracts

Phytoconstituents	Ethanol dry Leaf extract	Aqueous dry Leaf extract	Aqueous fresh leaf extract
Alkaloids	-	-	-
	-	-	-
	-	-	-
Tannins	+	+	-
	+ +	+ +	-
Flavonoids	+ + +	+ + +	-
Cardiac Glycosides	+ +	+ +	-
Steroids	+ +	-	-
Terpenoids	+ +	+ +	-
Reducing Sugars	+ +	+ +	+
Carbohydrates	+ +	+ +	+
Saponins	+ +	+ +	-
Anthraquinones	+ +	+ +	-

Highly Present (+++); Moderately present (++); Slightly present (+); Absent (-).

Table 3: Antibacterial activity of standard drug (Levofloxacin) against test bacteria

Organism	50 µg/mL	25 µg/mL	12.5 µg/mL	6.25 µg/mL
Zone of inhibition(mm) ± SEM				
Staphylococcus aureus	29.75±0.5	27.95±0.42	21.95±0.33	17.55±0.64
Bacillus subtilis	37.825±0.42	32.95±0.33	31.18±0.24	26.95±0.10
Escherichia coli	32.63±0.48	30.88±0.63	27.53±0.61	16.43±0.81
Pseudomonas aeruginosa	22.0±00	22.0± 00	18.5±0.90	–
Klebsiella pneumoniae	–	–	–	–

(-) No zone of inhibition; (n=3).

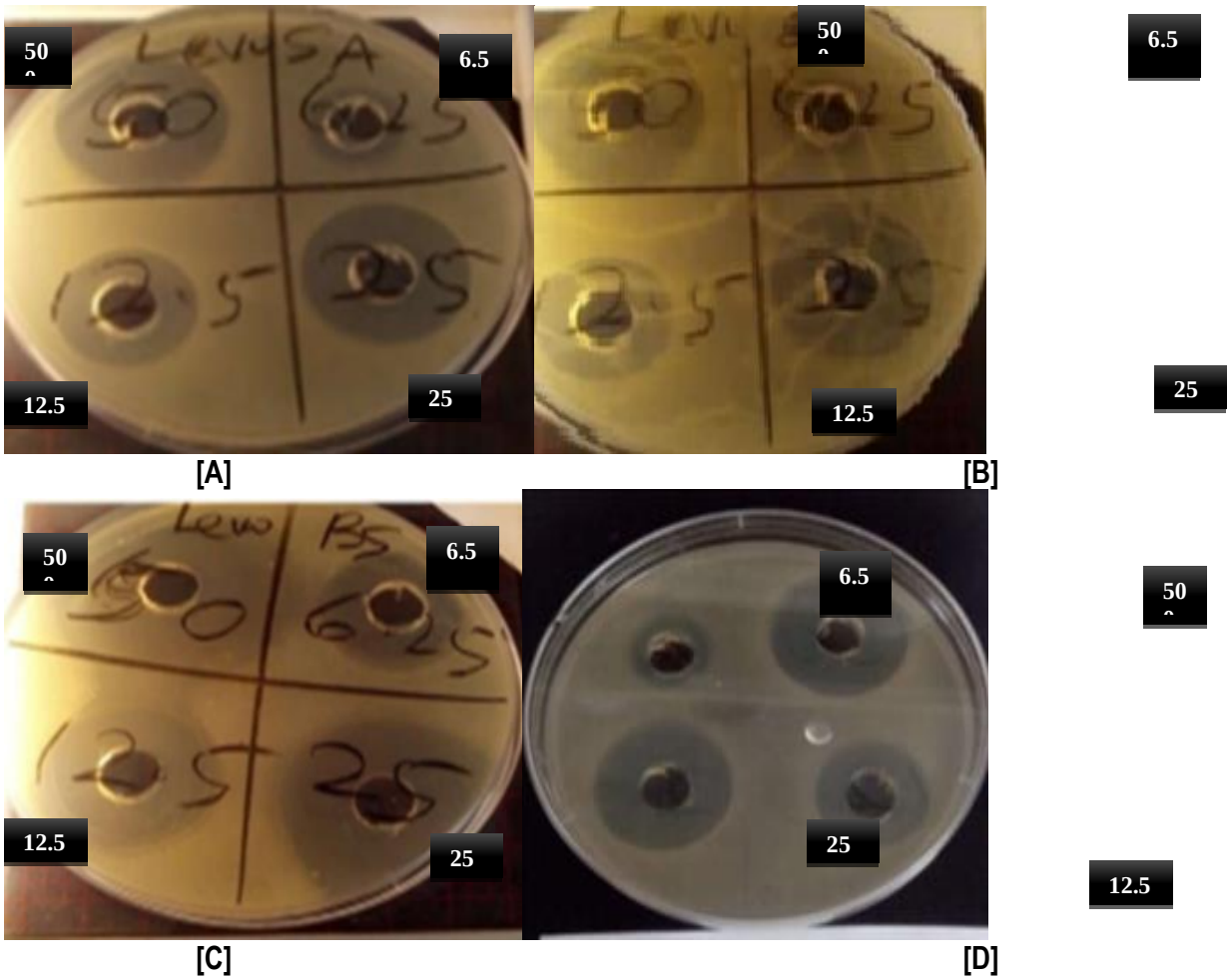

Figure 2: Zones of Inhibition of Levofloxacin on [A] *Staphylococcus aureus,* [B] *Escherichia coli,* [C] *Bacillus subtilis* and [D] *Pseudomonas aeruginosa* in concentrations of 6.5 to 50 µg/mL.

Table 4: Antibacterial activity of ethanol dry leaf extract against test bacteria

Organism	300 mg/mL	150 mg/mL	75 mg/mL	Control (Solvent)
		Zone of inhibition (mm) ±SEM		
Staphylococcus aureus	17.15±1.50*	12.75±0.50	8.33±0.80	–
Bacillus subtilis	15.30±0.40*	–	–	–
Escherichia coli	–	–	–	–
Pseudomonas aeruginosa	–	–	–	–
Klebsiella pneumoniae	–	–	–	–

(-) No zone of inhibition; (n=3); *p < 0.01 when compared with standard drug.

Table 5: Antibacterial activity of aqueous dry leaf extract against test bacteria

Organism	300 mg/mL	150 mg/mL	75 mg/mL	Control (Solvent)
		Zone of inhibition (mm) ±SEM		
Staphylococcus aureus	–	–	–	–
Bacillus subtilis	14.75±0.50**	–	–	–
Escherichia coli	–	–	–	–
Pseudomonas aeruginosa	–	–	–	–
Klebsiella pneumoniae	–	–	–	–

(-) No zone of inhibition; (n=3); **P > 0.05 when compared with ethanol dry leaf extract.

Table 6: Antifungal activity of standard drug (Bifonazole) against test fungi

Organism	50 µg/mL	25 µg/mL	12.5 µg/mL	6.25 µg/mL
		Zone of inhibition (mm) ±SEM		
Aspergillus niger	31.00±2.00	19.30±0.30	18.50±0.50	15.50±0.70
Aspergillus flavus	–	–	–	–
Aspergillus fumigatus	–	–	–	–
Trichophyton rubrum	34.00±1.20	29.00±0.70	28.70±0.50	22.00±1.00
Mycobacterium globosa	–	–	–	–

(-) No zone of inhibition; (n=3).

Table 7: Minimum inhibitory concentrations of ethanol dry leaf extract of *Euphorbia milii* against susceptible bacteria

Assay (mg/ml)	Ethanol extract	
	Staphylococcus aureus	Bacillus subtilis
40	+	+
45	+	+
50	-	+
55	-	+
60	-	+
65	-	+
70	-	+
75	-	-
80	-	-
85	-	-
90	-	-
95	-	-
100	-	-

(+) Growth present;(-) Growth absent.

Results of phytochemical screening showed similarity between the ethanol and aqueous dry leaf extracts in terms of phytoconstituents. The results are similar to the works of Okwu [22], Edeoga *et al* [23] and Abdur *et al*, [24] who assessed the phytoconstituents of several medicinal plants including Euphorbia species.

The presence of bioactive constituents may have contributed to the antimicrobial activity of the extracts [25, 26]. Tannins have been reported to inhibit the development of microorganisms by precipitating microbial protein and making nutritional proteins unavailable for the organism [27]. Flavonoids form complexes with extracellular and soluble proteins which further complexes with bacterial cell walls leading to microbial death, more lipophilic flavonoids may also disrupt microbial membranes [28].

There was significant difference between the activity of levofloxacin and ethanol extract against *S. aureus and B. subtilis.* The aqueous dry leaf extract was not significantly different from the ethanol extract in the inhibition of *B. subtilis.* The activity of dry leaf extract on *S. aureus* is of interest because the organism is commonly found on the skin and is not easily eliminated especially from the deeper skin layers, sweat glands, sebaceous glands and the hair follicles by routine washing and scrubbing even with some antiseptics [7, 8]. Cream formulation of *Euphorbia milii* could therefore be useful in the treatment of skin infections in which *Staphylococcus aureus* is implicated. Some plant extracts have been reported to exhibit synergistic effect with antibiotics [29], combination of identified secondary metabolites with known antimicrobial compounds in formulations may enhance antimicrobial activity and help to overcome problem of antimicrobial resistance to existing drugs.

None of the extracts was active against any of the fungal isolates, which is contrary to the findings that *Euphorbia milii* is active against *Aspergillus flavus* [30]. The fungi organisms used in this study may have been resistant strains as even the reference drug was active only on two of the five fungi species.

The minimum inhibitory concentrations (MICs) of ethanol extract is consistent with the results of antimicrobial susceptibility that revealed the extract to be more potent against *Staphylococcus aureus* than *Bacillus subtilis.* This justifies the local use of the leaf for treatment of skin infections.

CONCLUSION

The results of this study show that ethanol and aqueous dry leaf extracts of *E. milii* have antibacterial properties that can be exploited in the management of diseases caused by susceptible organisms.

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