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***Euphorbia graminea* Jacq. (Euphorbiaceae): The antimicrobial assessment of the extract and fractions of the leaves**

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ABSTRACT

This study was carried out as a result of the belief that certain medicinal plants have antimicrobial activity against pathogenic bacteria and fungi species, hence the *in vitro* antimicrobial activity of the extract and fractions of *Euphorbia graminea* was performed against bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*) and fungal (*Candida albican*) non-clinical isolates. The methanol extract of *Euphorbia graminea* was fractionated via solvent-solvent partitioning and vacuum liquid chromatographic techniques and the corresponding fractions were tested for phytochemicals and were biologically tested against the organisms employing the agar well diffusion method. While the extract and partitioned fractions were tested at concentrations between 4.69-300 mg/mL, the vlc subfractions were tested at 12.50-200 mg/mL against the organisms. The MIC of the active vlc subfractions was also tested (9.38-37.5 mg/mL). The alkaloids as well as glycosides, tannins, terpenes and steroids were detected among the extracts and fractions of *E. graminea*. The extract showed mild activities against the test organisms with the highest zone of inhibition of 7.00 mm recorded at 300 mg/mL against *S.aureus*. The activities of both extract at 300 mg/mL, showed notable increase against the organisms used, with zones of inhibition of the aqueous extract been 10.50 and 9.50mm and for the chloroform extract, 16.50 and 13.05mm (*S. aureus* and *E. coli* respectively). The vlc sub-fraction 6-8 (C) among the other subfractions was more potent against *S. aureus*, *E. coli* and *C. albican* as 16.50, 12.00 and 0.50 mm zones of inhibition were recorded at 100 mg/mL. The zones of inhibition against these organisms later increased to 20.00, 18.50 and 15.00 mm at 200 mg/mL. This study has highlighted the fact that the plant *E. graminea* has antimicrobial activity which occurs more in the partitioned chloroform and its chromatographic vacuum liquid subfraction (6-8) that gave the highest activity. However, in order to isolate the active biological components and to determine their safety in drug production, further studies are needed.

Keywords: Antimicrobial, *Euphorbia graminea*, fractionation, chromatography, chloroform, phytochemicals

Introduction

The rising trend in antibiotic resistance has become a global health challenge, hence there is need for the discovery of new antibiotics which are inexpensive and effective for the treatment of microbial diseases (Li and Webster, 2018).

Antimicrobial resistance has been a major problem facing the therapeutic use of different collections of antimicrobials, causing their efficacy to decline and fall continuously, creating a need for new

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antimicrobial drugs to be discovered (Chopra *et al.*, 2002).

Over the years, plants have served as a valuable source of natural products for the maintenance of human health, especially during the last decade (Atanasov *et al.*, 2015). The World Health Organization has estimated that medicinal plants would be the best way to receive a range of medicines and that about 80 percent of people from developing countries would use conventional medicine, which has medicinal plant derived compounds (WHO, 2019).

Discovering the therapeutic benefits of plants is an old concept. For several centuries people have tried to ameliorate specific illnesses with different plant extracts and formulations (Pan *et al.*, 2013). The focus on plants with antimicrobial activities has been reinvigorated because of prevailing problems that come through the use of antimicrobial drugs (Manandhar *et al.*, 2019).

Euphorbia graminea Jacq (Euphorbiaceae) is a plant previously described as a new specie of the Euphorbia genus which became noticeable in parts of Nigeria in mid-2000 (Aigbokhan and Ekutu, 2012). The plant is an annual plant growing 15-30 cm tall, but often taller and sometimes splitting from the base and becoming distally branched spirally. The leaves appear to be alternate and may be ovate or oblong, elliptic to linear with full margin (Vincent, 2013).

The scarcity of data on *E. graminea* in literature suggests its present introduction into the Nigerian vegetation. The plant has not really been considered in Nigeria for the management of several diseases, but its uses may not be as exceptional from other species of Euphorbia.

Our previous work on this plant showed a remarkable antioxidant and antiproliferative potentials against human breast (MCF-7) and lung (NCI-H460) cancer cell lines (Ikpefan *et al.*, 2020). The current study takes a further look on the antimicrobial activity of the extract and its various solvent fractions with the aim of identifying the bioactive fractions of the extract as well as

assessing the degree of activity against selected pathogenic bacteria.

Materials and Methods

Collection and Preparation of Plant Materials

The leaves of *E. graminea* were collected at Ekosodin, Benin city, Edo state and were properly identified by Dr. Henry Akinnibosun of the Department of Botany, University of Benin, Benin City. At the Forest Research Institute of Nigeria (F.R.I.N), the plants identity was authenticated and a voucher collection number (FHI 109024) was obtained.

Processing of plant Material

The leaves were air dry for 48 h and further dried in the oven (Teltech, LO001) maintained at 40°C. The dried leaves were grinded coarsely using a laboratory electric milling machine (Chris Norris, England) and the powdered preserve in an air tight container.

Extraction and fractionation of the extract

A total of 2.5kg of the leaves powdered material of *E. graminea* was extracted by Soxhlet technique using 95% methanol. The liquid extract was then concentrated to slurry under vacuum at 40 °C using rotary evaporator and the yield documented. The 150 g of the extract was dissolved in equal amount of water and methanol (1:1, 300 mL) and partitioned exhaustively with chloroform (500 mL × 4). The chloroform as well as the aqueous layers were collected and concentrated and were subjected to antimicrobial studies. Having shown a higher activity over the aqueous fraction, 45g of the chloroform fraction was subjected to vacuum liquid chromatographic (VLC) using 800 mL of ; C₆H₁₄ (100 %), C₆H₁₄- CH₃COOC₂H₅ (1:1; 1:3), CH₃COOC₂H₅ (100%), CH₃COOC₂H₅-CH₂Cl₂ (1:1, 1:3), CHCl₂ (100 %), CH₂Cl₂-CH₃OH (1:1,1:3) and CH₃OH in increasing polarity which gave ten (10) fractions which were bulked into 3-5, 6-8, 9-10 sub-fractions using analytical thin layer chromatographic aluminium plate made up of silica

gel GF₂₅₄. Fractions 1 and 2 were negligible. The bulked fractions were subjected to sensitivity test against selected microorganism.

Phytochemical screening

This was carried on the extract and fractions of *E. graminea* using previously described methods (Sofowora, 1993; Trease and Evans, 2002; Khandelwal, 2006).

Collection and processing of isolates

The research organisms used are human infectious bacteria and fungi obtained from stock cultures of the Pharmaceutical Microbiology Laboratory of the Faculty of Pharmacy, Delta State University, Abraka. *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albican* were used. The bacteria and fungi isolates were stock on nutrient agar and potato dextrose agar respectively at 40 °C and were subsequently sub-cultured for purification prior to biochemical identification. The identities of these organisms were identified using standard biochemical test technique previously described by Monika *et al.*, (2017). The method of preparation of the inoculum used was according to the method of Mahesh and Satish (2008).

Antimicrobial Sensitivity test of the extract and fractions of *Euphorbia graminea*

The antimicrobial susceptibility test was performed using Jimoh *et al.*, (2020) method by which the suspensions of bacteria and fungi cells (108 cfu / mL) were uniformly streaked on solidified nutrient and dextrox agar plates. A volume of 200 µl from each of the extract and partitioned fractions working concentrations (4.69-300 mg / mL) as well as vlc fractions (12.5-200 mL) was inoculated into wells (6 mm in diameter) already prepared. The experiment was carried out in three (3) replicates. Positive tests were used for Ciprofloxacin (200mg/100 mL) and Clotrimazole (20µg / mL).

Determination of Minimum Inhibitory Concentration (MIC) of active fraction C

The (MIC) of the active vlc bulked fraction C (6-9) against the isolates (mg/mL) was carried out adopting the method of Sonibare *et al.*, (2016) with slight modification in concentrations used.

Statistical analysis

The Graphpad software was used and all experimental data were presented as Mean ± Standard Error of Mean of the results taken. The level of statistical significance was done using One-way Anova with $p \leq 0.05$ or $p > 0.05$ considered statistically significant and not significant respectively.

Results

Yield of the extract and fractions.

The 2.5kg of the powdered sample of *Euphorbia graminea* yielded 208.55g of the extract which implies to 8.34 %. While the solvent-solvent partitioning of 150 g of extract gave 76.87 (3.07 %) and 55.25g (2.21 %) of the aqueous and chloroform fractions, the vacuum liquid chromatographic fractionation of the chloroform fraction (45 g) recorded 1.28, 5.10, 12.10 and 14.80 g for bulked fractions 2 (A), 3-5 (B), 6-8 (C) and 9-10 (D) respectively.

Preliminary Phytochemical Screening

The preliminary phytochemical investigation of the extract and fractions of *Euphorbia graminea* reveal the presence of alkaloids, tannins, saponins, flavonoids, antraquinones, cardiac glycosides, terpenes, and steroids (Table 1).

Results of sensitivity test of the extract and fractions of *E. graminea*

The sensitivity test of the extract and fractions were observed to be concentration dependent. The activity of the extract against the test organisms were insignificant compared to the control drugs used (Table 1, Plate 1). The aqueous and

chloroform fractions similarly exhibited a concentration-based activity with the *S. aureus*, *E. coli* and *C. albican* more susceptible. For example, at 150 mg/mL, the aqueous fraction recorded 9.10, 7.50 and 7.00 mm against *S. aureus*, *E. coli* and *C. albican* which later increased to 10.50, 9.50 and 9.00 mm at the maximum concentration of 300 mg/mL. The activity of the chloroform fraction followed the same pattern as the aqueous but recorded higher activities against all three organisms (Table 4). Among the vlc bulked fractions, bulked fractions 6-8 (C) recorded the highest activity against all organisms (Table 5).

The extract and fractions sensitivity test were observed as being dependent on concentration. The extract 's activity against the test organisms was marginal compared to the control drugs used (Table 2, Plate 1). The zones of inhibition were higher for *S. aureus*. Likewise, the aqueous and chloroform fractions showed a concentration-based activity with the more sensitive recorded against *S. aureus*, *E. coli*, and *C. albican*. For instance, the aqueous fraction reported 9.10, 7.50 and 7.00 mm against *S.*

aureus, *E. coli* and *C. albican* at 150 mg / ml but later increased to 10.50, 9.50 and 9.00 mm at the maximum concentration of 300 mg / ml. The activity of the chloroform fraction followed the same pattern as the aqueous but recorded higher activity against all three organisms (Table 4) with *S. aureus* been susceptible at all concentration used. Among the vlc bulked fractions, bulked fractions 6-8 (C) recorded the highest sensitivity against *S. aureus* than the others. For instance, at 50 mg/mL while other fractions were resistant, fractions 6-8 (C) recorded 11.50, 8.10, 5.00, 4.50 and 5.00 mm zones of inhibition against *S. aureus*, *E. coli*. *P. aeruginosa* and *C. albican* respectively which later increased to 20.50, 18.50, 12.50, 15.00 and 11.50 mm at 200 mg/ml against the corresponding organisms (Table 5). The active fraction C recorded lower MIC values against *S. aureus* and *E. coli* compared to the other organisms (Table 6).

Table 1: The preliminary screening of the methanol extract of *Euphorbia graminea*

Phytochemical groups	Extract	Partitioned fractions		VLC bulked fractions			
		Aqueous	Chloroform	2 (A)	3-5(B)	6-8 (C)	9-10(D)
Alkaloid	++	+	-	-	-	++	+
Anthraquinones	++		+++		-	+++	-
Cardiac glycoside	++	++	+	-	-	+	+
Flavonoids	+	+	++	-	-	++	-
Saponins	+	++	-	-	+	++	+
Steroids	+	-	++	+	++	++	-
Tanins	+++	+	++	-	-	++	-
Terpenes	+	-	++	+	+	+++	+

Key: +++: appreciable amount; ++: moderate amount; +: minute amounts; -: not detected
A-D = Bulked vacuum liquid chromatographic fractions of the extract.

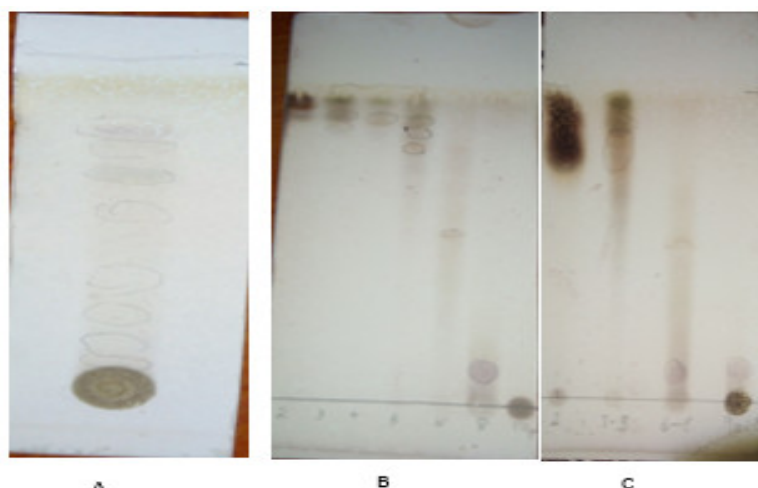


Figure 1: Chromatogram of the extract (A), vlc fractions before (B) and after bulking (C)

Table 2: The effects of the extract on the organisms

Organisms	Concentrations (mg/mL)/ Zones of inhibitions						
	300	150	75	37.5	18.75	9.38	4.69
S.A.	7.00±0.03	6.00±0.00	4.50±0.00	-	-	-	-
E.C.	5.00±0.01	5.02±0.05	2.00±0.00	-	-	-	-
P.A.	3.00±0.01	-	-	-	-	-	-
C.A.	3.50±0.50	-	-	-	-	-	-

Control: Ciprofloxacin ^b (20 mg/mL) = 37.33±1.20 mm, Clotrimazole ^f (20µg/mL) = 16.5±0.50mm. S.A.; *Staphylococcus aureus*, E.C.; =*Escherichia coli*, P.A.; =*Pseudomonas aeruginosa*, C.A. =*Candida albican*. All values were statistically different from control ^{b,f}. (p<0.05). b= Bacteria control drug; f= fungal control drug

Table 3: The effects of the aqueous fraction on test organisms

Organisms	Concentrations (mg/mL)/ Zones of inhibitions						
	300	150	75	37.5	18.75	9.38	4.69
S.A.	10.50±0.00	9.10±0.00	7.50±0.00	5.50±0.15*	-	-	-
E.C.	9.50±0.00*	7.50±0.00*	4.00±0.00*	-	-	-	-
P.A.	7.00±0.01*	6.00±0.00*	3.00±0.00*	-	-	-	-
C.A.	9.00±0.00**	7.00±0.00**	3.00±0.00**	-	-	-	-

Ciprofloxacin ^b (20 mg/mL) = ;38.50 ±1.72 mm; Clotrimazole ^f (20µg/mL) = ;16.25 ±0.25 mm . *. (antibacterial), ** (antifungal); All values were statistically different from control ^{b,f}. (p<0.05). b= Bacteria control drug; f= fungal control drug.

Table 4: The effects of the chloroform fraction on test organisms

Organisms	Concentrations (mg/mL)/ Zones of inhibitions						
	300	150	75	37.5	18.75	9.38	4.69
S.A.	16.50±0.21	12.60±0.26	10.00±0.02	8.50±0.92	6.00±0.11	5.00±0.26	3.50±0.16
E.C.	13.05±0.10	10.50±0.31	6.00±0.12	5.10±0.10	-	-	-
P.A.	9.50±0.01	6.00±0.50	4.50±0.00	-	-	-	-
C.A.	11.50±0.20*	9.00±0.50*	6.00±0.00*	4.00±0.00*	-	-	-

Ciprofloxacin^b (20 mg/mL) = 38.50 ±1.72 mm; Clotrimazole^f(20µg/mL= 16.25 ±0.25 mm
 S.A. =*Staphylococcus aureus*, E.C. =*Escherichia coli*, P.A. =*Pseudomonas aeruginosa*, C.A. =*Candida albican*.
 *All values were statistically different from control^{b,f} (p<0.05)

Table 5: The effects of the vlc bulked fractions on the test organisms

Organisms	Fractions	Concentrations (mg/mL)/Zones of inhibitions (mm)				
		200	100	50	25	12.5
S.A.	2	-	-	-	-	-
	3-5	-	-	-	-	-
	6-8	20.5±0.18	16.5±0.91	11.5±0.42	10.00±0.21	7.50±0.50
E.C.	9-10	7.11± 0.21	5.0±0.86	-	-	-
	2	-	-	-	-	-
	3-5	-	-	-	-	-
	6-8	18.50±0.50	12.00±0.62	8.10±0.05	6.00±0.10	4.00±0.07
P.A.	9-10	5.50±0.50	3.10±0.09	-	-	-
	2	-	-	-	-	-
	3-5	-	-	-	-	-
	6-8	12.50±1.72	7.0±1.5	5.0±0.	-	-
C.A.	9-10	-	-	-	-	-
	2	-	-	-	-	-
	3-5	-	-	-	-	-
	6-8	15.00±0.50	10.50±0.52	4.50±0.61	2.50± 0.01	-
	9-10	-	-	-	-	-

Ciprofloxacin (20 mg/mL) = 39.63 ±0.38 mm; Clotrimazole (20 µg/mL= 16.25 ±1.13 mm
 S.A. =*Staphylococcus aureus*, E.C. =*Escherichia coli*, P.A. =*Pseudomonas aeruginosa*, C.A. =*Candida albican*

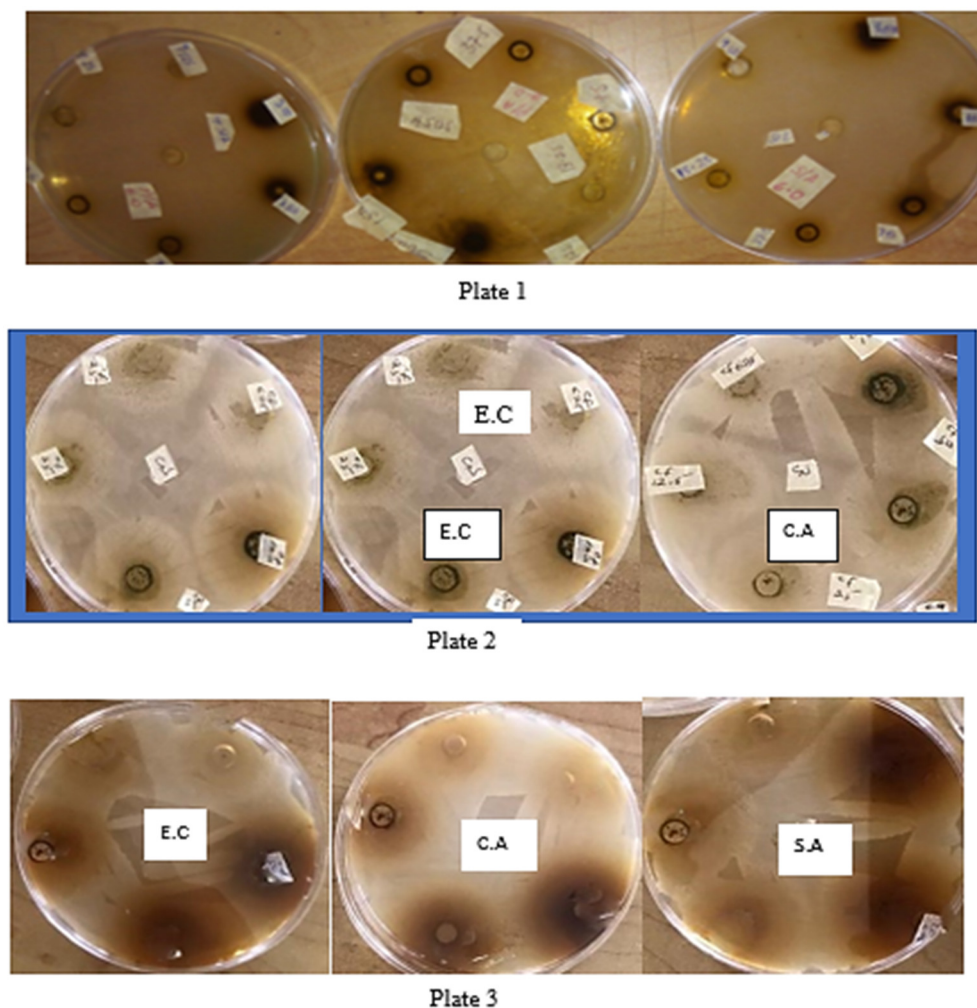


Figure 2: The effects of the extract (plate 1), the partitioned fractions (plate 2) and active vlc sub-fractions (plate 3) of *E. graminea* on the organisms. S.A. = *Staphylococcus aureus*, E.C. = *Escherichia coli*, P.A. = *Pseudomonas aeruginosa*, C.A. = *Candida albican*.

Table 6: Minimum inhibitory concentration (MIC) of active vlc fraction 6-8.

Organisms	Fractions	Concentrations (mg/ml)/Zones of inhibitions (mm)					
		75	37.5	18.75	9.38	4.69	2.34
<i>S. aureus</i>	6-8	-	-	-	-	+	+
<i>E. coli</i>		-	-	-	+	+	+
<i>P. aeruginosa</i>		-	-	+	+	+	+
<i>C. albican</i>		-	-	+	+	+	+

Key: - = +: growth; -: No growth

Discussion

The global threat to the emergence of drug-resistant species has continued to rise and has resulted in several medical practitioners failing to treat multiple diseases (Aslam *et al.*, 2018). The effects of the extract and fractions of *E. graminea* on selected pathogens commonly associated with various infections, with high mortality and disease risk in healthcare sector was evaluated in this study.

The phytochemical screening results showed the distribution of various phytochemical groups among the extract and the respective fractions. There have been reports of antimicrobial properties of some of these phytochemical classes. For example, the flavonoids have been reported to be effective against a wide range of microorganisms as they easily disrupt microbial membranes (Tsuchiya *et al.*, 2003). The activity of the extract against the test organisms was relatively weak which corroborates the earlier work by Mezouar *et al.*, (2014) where a very poor antibacterial activity was demonstrated by the extract of *B. vulgaris* against all species of organisms tested.

The extract's liquid-liquid partitioning was observed to further improved the activity against the test organisms. For example, at 150 mg/mL, the extract showed inhibition zones of 6.00 and 5.00 mm against *S. aureus* and *E. coli*, while at the same concentrations the aqueous and chloroform fractions gave 9.00 and 7.50 mm as well as 12.00 and 10.50 mm respectively. The higher chloroform fraction antimicrobial activity is consistent with past findings reported by Mills-Robertson *et al.*, (2012).

The higher inhibition zones by the chloroform fraction could be as a result of the high concentrations of the active secondary metabolites and probably the diffusing capacity of chloroform fraction into the cells of the organisms. Further fractionation of the

chloroform fraction involving the use of vacuum liquid chromatography was observed to maximize the activity as the vlc sub-fraction 6-8 among the other fractions, recorded the highest activities against the organisms. The higher activity of this fraction could be as a result of the concentration of the active compounds in them.

The MIC determination for the most active vlc subfraction further demonstrated the fraction's potency particularly against *S. aureus* and *E. coli*. The lower MIC range for *S. aureus* and *E. coli* recorded by the fraction suggests the presence of a potential antimicrobial agent making it possible to reduce the growth of the bacteria and fungi organisms, thus, it may be used as an antimicrobial

Conclusion

This study provides the foundation for further studies involving more bacterial and fungi with emphasis on clinical species. Further investigations into the nature of these compounds responsible for these activities are needed to determine its safety and the drug development prospects.

Conflict of interest: None

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