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In-vitro antibiogram of *Bryophyllum pinnatum* extracts with or without potassium aluminum sulphate against some pathogenic bacteria

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Abstract
In-vitro antibiogram of *Bryophyllum pinnatum* leaf extracts with or without potassium aluminium sulphate (Alum) were investigated by disc diffusion method (DDM) and agar well diffusion method (AWDM) respectively. The leaves of *B. pinnatum* was dried in an oven at 45^{o}C for 14days and pulverized with a blender. Alum, methanolic and aqueous leaf extracts of *B. pinnatum* were reconstituted in 100ml of sterile distilled water respectively. Antibacterial activity of different percentage concentrations (0.5, 1.0, 1.5 and 2.0% (w/v) of the leaf extracts with or without alum were evaluated by measuring diameter of inhibition zones (DIZs) respectively. Aqueous leaf extract with alum had the highest mean DIZ values which ranged from 17.5-29.5mm, methanolic leaf extract with alum 0-22.0mm and leaf extracts without alum 0-8.5mm at 2.0% concentrations respectively. There were progressive increases in DIZs against test bacteria (*Bacillus subtilis*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Salmonella typhi*) with increasing concentrations of leaf extracts with and without alum although certain discrepancies were observed with aqueous leaf extract, methanolic leaf extract with and without alum respectively. Demonstration of antibacterial activity on Gram negative and positive test bacteria by leaf extracts with or without alum suggests broad spectrum activity. Thus, this study reveals that the potassium aluminum sulphate (Alum) potentiated the inhibitory activity of *B. pinnatum* leaf extracts and should be used topically or otherwise as a novel antibacterial agent to combat infections caused by these pathogens.

Keywords: alum, antibiogram, broad spectrum, *b. pinnatum*, leaf extracts

Introduction
Byophyllum pinnatum (Lam), is a perennial herb growing widely and used in folklore medicine in tropical Africa, India, China, Australia, Madagascar, Asia, Hawaii and tropical America [1, 2, 3] and synonymous with *B. calycinum* and *Kalanchoe pinnatum* [4]. The plant belongs to the family *Crassulaceae* and commonly called resurrection plant, miracle leaf, divine plant, life plant and wonder of the world [5]. It has been widely accepted as a herbal remedy in virtually all parts of the world and distributed globally but growing luxuriantly in the rain forest [6, 7]. It has been reported to contain a variety of bioactive compounds such as triterpenes, lipids, alkaloids, flavonoids, steroids, phenols, glycosides, bufadienolides, cardienolides and organic acids [3, 8]. In ethnomedicine, it has been employed as remedy against; respiratory tract infections, allergies, diarrhea, otitis, burns, gastric ulcers, skin ulcers, convulsion, general debility and a variety of other infections/diseases [3, 9]. Marketed preparations and patents from *B. pinnatum* extracts also have been reported [10, 11], bacterial and fungal infections [12, 13].

Potassium aluminum sulphate (Potash alum/alum) refers to any of a group of a hydrated double salts resulting from the hydration of the sulphate of a singly charged cation (K^{+}) and the sulphate of any one of a number of triply charged cation (Al^{+}). It has been used topically to perform deodorant, antibacterial and astringent actions in lotions, cream and gel respectively [14, 15]. It has a variety of applications in foods, cosmetics and in water purification processes [16, 17, 18], for the treatment of burns and ulcers in the oral cavity with anticariogenic effect [19].

It is becoming quite apparent that the use of single drug/antimicrobial agent does not produce the desired effects, especially where organisms exhibit multidrug resistant traits so a combination of drugs/chemical agents often exhibit more profound effects or synergism surpassing individual performance.

Synergy of plant extracts with antimicrobial agents/standard antibiotics against microbial pathogens either to reduce bioburden (by virtue of their susceptibility to the microbes) or induction of other cytological effects have reported [20, 21, 22, 23]. However, there is paucity of information/study on the antibiogram of *B. pinnatum* leaf extracts and alum. Since these agents have been reported to possess antimicrobial activity it becomes imperative to potentiate their effects by combining them. Therefore, the present study was to investigate the antibiogram of *B. pinnatum* leaf extracts with and without alum on some bacterial pathogens from clinical samples using disc diffusion and agar well diffusion methods.

Materials and methods
Collection and identification of plant leaves
The leaves of the plant (Figure 1) grown in Zaakpon community, Bori, were collected during the rainy season between the months of July and August, 2015. They were identified as *Bryophyllum pinnatum* by Dr. W.N Barabe (Plant taxonomist) in the Department of Science Laboratory Technology, Ken Saro-Wiwa Polytechnic, Bori, Rivers State.

Fig 1: Live *Bryophyllum pinnatum* plant with glabrous, serrated simple leaves.

Preparation of crude *B. pinnatum* leaf extracts

B. pinnatum leaves were washed and dried in an oven (Genlab) at 45°C for 14days. The dried leaves were pulverized using sterile ceramic mortar and pistle and stored in sterile sample bottles at room temperature[24].

Methanol extraction was carried out by dissolving 50g of the dried pulverized leaves in 250ml of methanol solvent in a sterile beaker[25]. After 24hr, the resultant solution was filtered using muslin cloth and then Whatman No.1 filter paper the filtrate was kept in a water bath at 37℃ to ensure complete evaporation of methanol before storage in the refrigerator at 4℃. Similar method was used for aqueous extraction but the filtrate was maintained in water bath at 80℃ for 48h for drying/evaporation. These congealed samples were further reconstituted with sterile distilled water to form *B. pinnatum* methanol and aqueous leaf extracts based on the required percentages respectively.

Reconstitution of *B. pinnatum* leaf extracts and Alum

A 0.5g of *B. pinnatum* leaf extracts of both methanol and aqueous samples as well as alum (Analytical grade, Vickers Laboratories, Ltd, England) were reconstituted in 100ml sterile distilled water to obtain 0.5% (w/v) concentration and repeated to obtain 1.0%, 1.5% and 2.0% respectively.

Concentrations of *B. pinnatum* leaf extracts with Alum

Different concentrations of both *B. pinnatum* aqueous or methanolic leaf extracts with alum (BAWA and BMWA) were made at a ratio of 1:1 respectively. The potency of these concentrations were compared with standard antibiotic discs, ciprofloxacin (CP = 10µg, Abtek Biologicals Ltd., Uk.).

Source of test pathogenic bacteria

Test pathogenic bacteria consist of *Bacillus subtilis* and *Staphylococcus aureus* (Gram-positive), *Salmonella typhi* and *Klebsiella pneumoniae* (Gram- negative) were obtained from stock cultures of clinical samples of Medical Laboratory Department, Ebony Hospital, Ebony/Orazi street, off Rumuola Road, Obio/Akpor Local Government Area, Rivers state, Nigeria.

Susceptibility test of *B.pinnatum* leaf extracts with or without Alum

In-vitro antibiogram was performed by the disc diffusion method (DDM) and agar well diffusion method (AWDM) [26, 27]. About 10µL of each of the bacterial suspensions from the overnight culture, following adjustment to 0.5 McFarland turbidity standards were spread-plated on Mueller Hinton agar (MHA (Titan Biotech Ltd. Bhiwadi-301019, Rajasthan, India.) and allowed to dry for 2 to 5 minutes [28]. Filter paper discs, made as described by Ochei and Kolhatkar [29] were impregnated with various concentrations of aqueous and methanolic leaf extracts with or without alum respectively. These discs and commercially supplied ciprofloxacin, positive control was placed on the surface-dried inoculated MHA with sterile forceps. For agar well diffusion method, equal volume of the bacterial suspensions was spread-plated onto surface-dried MHA. Thereafter, four (4) wells of 6.0mm diameter equidistant of from each other were made on the agar plates using sterile cork borer and 0.1ml concentrations of aqueous leaf extract- or methanol leaf extract with or without alum were dispensed into the wells including (10µg/ml) of the reconstituted CP antibiotic positive control respectively. Duplicate plates were incubated at 37℃ for 24hours. The diameter of inhibition zones (DIZs) were measured with a transparent ruler and expressed in millimeters (mm). The mean values of DIZ were calculated and compared with ciprofloxacin. Interpretation of results was based on the zones of inhibition [29, 30, 31].

Statistical analysis

All data were obtained from at least two replicated experiments and the mean values estimated.

Results

Antibiogram of different concentrations of *B.pinnatum* aqueous leaf extract with alum (BAWA) and without (BAWOA) using DDM are presented in Tables 1 and 2. At 2.0% concentration, *K. pneumoniae* had the highest mean DIZ value of 22.0mm followed by *B. subtilis* and *Salmonella typhi* 20.0mm respectively. *S. aureus* had the least 18.0mm (Table 1). Generally, there was progressive increase in mean DIZ values on test pathogenic bacteria with increasing concentrations of BAWA but not so with BAWOA (Table 1). In Table 2, there were no/zero inhibitory effects with *B.pinnatum* methanolic leaf extract with alum (BMWA) or without (BMWOA) on test bacteria irrespective of different concentrations. This phenomenon contrast sharply with the rest of the tabulated data obtained in this study. Similar trends of increased DIZs were observed with increasing concentrations of leaf extracts with or without alum by agar well diffusion method (AWDM) but DIZ values were markedly lower for extracts without alum (Tables 3 and 4). *B. subtilis* had the highest mean DIZ value of 29.5mm followed by *K. pneumoniae* 21.0mm, the least being *S. aureus* 17.5mm at 2% concentration of BAWA but very low or zero mean DIZ values for BAWOA (Table 3). However, there was marked disparity in mean DIZ profiles of *B. pinnatum* methanolic leaf extract with alum (BMWA) or without (BMWOA) at 2.0% concentrations (Table 4) in comparison with that obtained by

DDM (Table 2). Generally, *B. pinnatum* leaf extract concentrations with alum had the largest inhibitory effects on test pathogens than those without alum (Tables 1, 3 and 4). However, ciprofloxacin had the highest and consistent DIZ range of 18.0-41.0mm or inhibitory activity on test pathogenic bacteria.

Table 1: Antibiogram of different concentrations (%) of *B pinnatum* aqueous leaf extracts with alum (BAWA) or without alum (Bawoa)

Diameter of inhibition (DIZ (mm)									
Bacterial pathogens	**Conc. of BAWA**				**BAWOA (%)**				**CP**
	0.5	**1.0**	**1.5**	**2.0**	**0.5**	**1.0**	**1.5**	**2.0**	**10µg**
Bacillus subtilis	15.0	16.0	17.0	20.0	-	-	-	-	18.0
K. pneumoniae	11.5	15.5	19.0	22.0	-	-	-	-	41.0
S. aureus	10.0	14.0	15.0	18.0	-	-	-	-	39.0
Salm. typhi	11.0	13.0	15.0	20.0	-	-	-	-	29.0

* = Disc diffusion method used; CP = Ciprofloxacin (control); ― = No/zero inhibition zone

Table 2: Antibiogram of different concentrations (%) of *B pinnatum* methanolic leaf extract with alum (BMWA) or without alum (Bmwoa)

Diameter of inhibition (DIZ (mm)									
Bacterial pathogens	**Conc. of BAWA**				**BAWOA (%)**				**CP**
	0.5	**1.0**	**1.5**	**2.0**	**0.5**	**1.0**	**1.5**	**2.0**	**10µg**
Bacillus subtilis	-	-	-	-	-	-	-	-	18.0
K. pneumoniae	-	-	-	-	-	-	-	-	41.0
S. aureus	-	-	-	-	-	-	-	-	39.0
Salm. typhi	-	-	-	-	-	-	-	-	29.0

* = Disc diffusion method used; CP = Ciprofloxacin (control); ― = No/zero inhibition zone

Table 3: Antibiogram of different concentrations (%) of *B pinnatum* aqueous leaf extract with alum (BAWA) or without alum (Bawoa)

Diameter of inhibition (DIZ (mm)									
Bacterial pathogens	**Conc. of BAWA**				**BAWOA (%)**				**CP**
	0.5	**1.0**	**1.5**	**2.0**	**0.5**	**1.0**	**1.5**	**2.0**	**10µg**
Bacillus subtilis	11.0	13.0	20.5	29.5	4.0	4.8	5.8	8.0	18.0
K. pneumoniae	10.0	15.0	15.5	21.0	4.0	5.0	5.5	7.0	41.0
S. aureus	9.0	12.0	15.0	17.5	-	-	7.5	8.0	39.0
Salm. typhi	13.0	16.0	18.0	20.0	-	-	-	4.0	29.0

** = Agar well diffusion method used; CP = Ciprofloxacin; ― = No/zero inhibition zone

Table 4: Antibiogram of different concentrations (%) of *B pinnatum* methanolic leaf extract with alum (BMWA) or without alum (Bmwoa)

Diameter of inhibition (DIZ (mm)									
Bacterial pathogens	**Conc. of BAWA**				**BAWOA (%)**				**CP**
	0.5	**1.0**	**1.5**	**2.0**	**0.5**	**1.0**	**1.5**	**2.0**	**10µg**
Bacillus subtilis	-	11.0	13.5	14.0	5.5	6.8	7.5	8.5	18.0
K. pneumoniae	11.5	15.5	19.0	22.0	4.3	4.5	5.0	6.5	41.0
S. aureus	-	-	-	-	-	-	8.0	8.0	39.0
Salm. typhi	-	-	-	-	-	-	4.5	4.8	29.0

** = Agar well diffusion method used; CP = Ciprofloxacin; ― = No/zero inhibition zone

Discussion

High level mean DIZ profiles with *B. pinnatum* aqueous leaf extract with alum (BAWA) on test pathogenic bacteria indicates synergism which becomes apparently visible by DDM and AWDM (Tables 1 and 3). This however, suggests more efficacious and beneficial impacts than those of methanolic extract with or without alum (Tables 2 and 4). A similar phenomenon also has been reported with aqueous leaf extracts of guava [23]. Furthermore, the inhibitory effects of this compound on Gram negative and positive test bacteria demonstrate broad spectrum antibacterial activity. The ability of alum to boost antibacterial activity may be linked with astringency and formation of sulphuric acid in solution thus increasing the acidity of the microcosm [18, 20]. Inhibition of bacteria such as *S. aureus*, *B. subtilis*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, etc., by alum and its combination with phytochemicals has been reported earlier [15, 17, 20]. The zero/no or fluctuations in inhibition of bacteria may be attributed to the crude nature of extracts to mask the active ingredients or to factors not clearly understood, even though antimicrobial activity of two flavonoids from *B. pinnatum* leaves has been reported recently by Okwu and Nnamdi [13]. Although, similar occurrence had been reported with guava leaf extracts [23]. The susceptibility profiles of ciprofloxacin (a fluoroquinolone) on test bacteria indicate its clinical success and should continue to be used as a firstline therapy for enteric and other pathogenic bacterial infections.

Conclusion

Bryophyllum pinnatum aqueous leaf extract with alum exhibited the best and most beneficial results on test bacteria. This leaf extracts with alum depicted synergism and broad spectrum antibacterial activity. Such novel combinations should be used to treat infections/diseases cause by these pathogens. Ciprofloxacin should be the best standard antibiotic drug of choice for therapy and management of patients infected with these bacterial pathogens.

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