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Evaluation of the Efficacy of Extracting Solvents on the Phytochemical, Antibacterial and Synergistic Effects of *Bryophyllum pinnatum* L. and *Citrus aurantifolia* SW.

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ABSTRACT: The aim of this research was to assess the efficacy of different extracting solvents on the phytochemical, antibacterial and synergistic effects of *Bryophyllum pinnatum* and *Citrus aurantifolia*. Four bacterial species: *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa* were used as test organisms and the antibacterial activity of the extracts was determined by the agar-well diffusion method. Synergistic antibacterial activity of the aqueous extract ranged from 0 (no effect) to 24.0 ± 0.6 mm, the synergistic antibacterial activity of the ethanolic extract ranged from 11.3 ± 0.9 mm to 23.5 ± 1.1 mm. Synergistic antibacterial activity of the methanol extract ranged from 16.7 ± 0.3 mm to 27.3 ± 0.6 mm while the synergistic antibacterial activity of the acetone extract ranged from 8.7 ± 0.9 mm to 22.7 ± 0.9 mm. Larger zones of inhibition were observed in the methanolic extract of the synergy than the other extracting solvents. Their antibacterial activity was compared with that of standard antibiotic and it was observed that the extracts compared well with the antibiotic. The phytochemical analysis of the extract was also carried out and results reveal the presence of phytochemical constituents which conferred antibacterial property on the plants. From the foregoing, the methanolic extract of the synergy is considered more effective in the treatment of infections caused by the test organisms than the other extracts from other extracting solvents.

Keywords: *Bryophyllum pinnatum*, *Citrus aurantifolia*, Synergy, Antibacterial, Phytochemical constituents.

Introduction

A medicinal plant is any plant which in one or more of its organs contain substances that can be used for the synthesis of useful drugs (WHO, 1977). According to the World Health Organization, medicinal plants would be the best source to obtain a variety of drugs. Therefore, such plants should be investigated to better understand their properties, safety and efficacy (Nascimento *et al.,* 2000). There are several published reports describing the antimicrobial activity of various crude plant extracts either in single or in combinations (Igoli *et al.,* 2005). It is estimated that there are about 2.5 million species of higher plants and the majority of these have not yet been examined for their pharmacological activities. Herbal extracts are fast becoming popular as natural antimicrobial preservatives or additives (Cox *et al.*, 2010).

Medicinal plants contains biologically active chemical substances such as saponins, tannins, essential oil flavonoids, alkaloids and other chemical (Sofowora, 1996) which have curative properties. These complex chemical substances of different composition are found as secondary plant metabolite in one or more of these plants (Kayode and Kayode, 2011).

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In recent years, drug resistance to human pathogenic bacteria has been commonly reported from all over the world (Piddock and Wise, 1989; Singh *et al*., 1992; Mulligen *et al*., 1993; Davis, 1994; Robin *et al*., 1998). However, the situation is alarming in developing as well as developed countries due to indiscriminate use of antibiotics. Therefore, alternative antimicrobial strategies are urgently needed, and thus this situation has led to a re-evaluation of the therapeutic use of ancient remedies, such as plants (Mandal *et al*., 2010; Basualdo *et al*., 2007).

Plant based antimicrobials represent a vast untapped source. The use of plant extract for medicinal treatment has become popular especially now when people are beginning to realize that the effective life span of antibiotics is limited and over prescription and misuse of traditional antibiotics are causing microbial resistance (Alam *et al.,* 2009; Harborne and Baxter, 1995). Antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world (Grosvenor *et al.,* 1995; Ratnakar and Murthy, 1995; David, 1997; Saxena, 1997; Nimri *et al.,* 1999; Saxena and Sharma, 1999). Presently, nearly 30% or more of the modern pharmacological drugs are derived directly or indirectly from plants and their extracts dominate in homeopathic or ayurvedic medicines (Murugesan *et al.,* 2011; Jabeen *et al*., 2007; Banso, 2009; Ahameethunisa and Hopper, 2010).

Citrus aurantifolia (Lime) is a small fruit from the Citrus family; it comes either sour or sweet naturally. Sour limes possess a greater sugar and citric acid content than lemons and have an acidic and tart taste (Bina *et al*., 2010). The nutritional profile of lime including carbohydrates, sugar, soluble and insoluble fiber, sodium, vitamins, minerals, fatty acids, and amino acids. They also contain unique flavonoid compounds that have antioxidant and anti-cancer properties. While these flavonoids have been shown to stop cell division in many cancer cell lines, they are perhaps most interesting for their antibiotic effects (Tomotake, 2006). *C. aurantifolia* exhibits bioactive activities for colds, fevers, sore throats, sinusitis, bronchitis and asthma (Khan *et al.,* 2012).

Bryophyllum pinnatum (*Kalanchoe pinnatum* or *Bryophyllum calycinum*), belongs to the family crassulaceae, and it is commonly known as sprouting leaf. It is found in tropical Africa, India, China, America and Australia (Devbhuti *et al.,* 2012; Gill, 1992). The leaves and leaf juice have been used traditionally as anti-inflammatory, antipyretic, antimicrobial, anti-oxidant, antitumour, antidiabetic, anti-ulcer, antiseptic, hypocholosterolemic, and cough suppressant (Ali *et al.,* 2013). The leaves and bark are not sweet, astringent to the bowels, analgesic, and useful in diarrhea and vomiting (Quazi *et al.,* 2011).

Traditional preparation of medicinal plants for antimicrobial activities have been extensively explored in the West African regions (Adesuyi *et al*., 2012; Dunford *et al*., 2000; Mboto *et al*., 2009; Mythilypriya *et al*., 2007). Combined therapy is traditionally used to increase antimicrobial activity and reduce toxic effects of agents (Tallarida, 2001). This study was carried out to assess and compare the antimicrobial efficacy of *Bryophyllum pinnatum* and *Citrus aurantifolia* and their individual activity using different extracting solvents.

Materials and Methods

Collection of plant materials:

Fresh *B. pinnatum* and *C. aurantifolia* leaves were obtained from Benin City, Edo State, Nigeria and identified in Department of Plant Biology and Biotechnology of the University of Benin, Benin City, identification was confirmed with appropriate literature (Akobundu and Agyakwa, 1998; Keay, 1989). The leaves were air dried, ground and made into a fine powder using laboratory mortar and pestle. The powdered leaves were kept in a sterile air-tight container to avoid contamination.

Preparation of extract:

Fifty grammes each of dried pulverized leaf powder was dissolved in 500 ml each of distilled water (to make aqueous extract) for 24 hrs and centrifuged at 3000 rpm to enable paper diffusion of the active ingredients into the extraction medium. Filtration was later carried out using Whatman's (No. II) filter paper and the filtrate was evaporated to dryness using steam water bath at 100 ^{o}C. This procedure was carried out with ethanol, methanol and acetone to obtain ethanol extract, methanol extract and acetone extract respectively. The extracts were now stored at 4 ^{o}C in a refrigerator. Combination of both plants was used in the synergistic assessment.

Test organisms

Bacterial cultures of the test organisms (*Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli* and *Pseudomonas aeruginosa)* were obtained from the Department of Medical Microbiology, University of Benin Teaching Hospital, Benin City, Nigeria. Their identity was confirmed using cultural, morphological and biochemical

test as previously described (Cheesebrough *et al*., 2002). They were maintained on nutrient agar slants at 4 ºC. These test bacteria have been previously described (Prescott *et al*., 2008; Akinnibosun *et al*., 2008a, b).

Phytochemical screening of the extracts:
Phytochemical screening of the extracts was carried out according to methods described by Odebiyi and Soforowa, (1978) and Trease and Evans, (1989).

Determination of Antibacterial Activity
The crude extracts were screened for antibacterial activity by determining the zone of inhibition against the test organisms by agar-well diffusion method. Sterile Mueller-Hinton agar plates were inoculated with prepared inoculum with sterile cotton swab. Wells were made in the inoculated media plate using sterile cork borer (6 mm in diameter). Then 50 µl of the extract was transferred into the well using micropipette. The control was also placed in the separate well at the same time. After incubation at 37 ºC for 24 hrs, the plates were viewed for the zone of inhibition, which is suggested by clear areas without growth around the well.

Results and Discussion

Microbial resistance to several antibiotics is becoming a source of challenge and concern to public health. In view of the increasing rate of antimicrobial drug resistance ravaging not only the African continent but the world at large, alternative, effective and affordable substitutes are essential if bacterial infections are to be properly controlled. Phytochemicals are secondary metabolites of plants known to exhibit diverse pharmacological and biochemical effects on living organisms (Jindal *et al.* 2012). Many plants containing alkaloids and flavonoids have diuretic, anti-inflammatory and analgesic effects. Alkaloids are capable of reducing headache-associated with hypertension. It has been reported that alkaloids can be used in the management of cold, fever and chronic Catarrh. Flavonoids are known for their antioxidant activity and hence they help to protect the body against cancer and other degenerative diseases (Jindal *et al.* 2012). Tannins are known to exhibit antiviral, antibacterial and antitumor activities. Saponin is used as hypercholesterolemia, hyperglycaemia, antioxidant, anticancer, anti-inflammatory and weight loss. The presence of these phytochemicals (steroids, tannins, reducing sugars, flavonoids, alkaloids, saponins and cardiac glycosides) in *B. pinnatum* and *C. aurantifolia* (Tables 1 – 3) supports their use as medicinal plants. These chemical constituents could be responsible for their antibacterial activity (Gill, 1992). Different plant parts contain a complex of chemicals with unique biological activity, which is thought to be due to toxins and secondary metabolites, which act as attractants or deterrents (Fisher, 1991). Over the years, these bioactive principles have been exploited in tradomedical practice for the treatment of various ailments (Adebanjo *et al.,* 1983).

Table 1: Phytochemical constituents of *Bryophyllum pinnatum* leaf extract using different extracting solvents.

Constituents	Aqueous extract	Ethanol extract	Methanol extract	Acetone extract
Saponins	+	+	+	+
Flavonoids	+	+	+	+
Steroids	_	+	+	+
Alkaloids	+	+	-	+
Tannins	_	+	+	_
Cardiac glycosides	_	+	+	+
Reducing sugars	+	+	+	+

Key:
+ = Present
- = Absent

Table 2: Phytochemical constituents of *Citrus aurantifolia* leaf extract using different extracting solvents.

Constituents	Aqueous extract	Ethanol extract	Methanol extract	Acetone extract
Saponins	+	+	_	_
Flavonoids	+	+	+	+
Steroids	+	+	+	_
Alkaloids	+	+	+	_
Tannins	+	+	+	+
Cardiac glycosides	+	+	+	_
Reducing sugars	+	+	+	+

Key:
+ = Present
- = Absent

Table 3: Phytochemical constituents of *Bryophyllum pinnatum* and *Citrus aurantifolia* synergy using different extracting solvents.

Constituents	Aqueous extract	Ethanol extract	Methanol extract	Acetone extract
Saponins	+	+	+	+
Flavonoids	+	+	+	+
Steroids	+	+	+	+
Alkaloids	+	+	+	+
Tannins	+	+	+	+
Cardiac glycosides	+	+	+	+
Reducing sugars	+	+	+	+

Key:
+ = Present
- = Absent

Antibiotic resistance of pathogenic bacteria to current synthetic drugs has necessitated the investigation into new, safe, efficient and cost-effective antibacterials as alternative agents for controlling the infectious diseases (Khan *et al.,* 2004). The extent of sensitivity of the test organisms to the plant fractions was assessed by measuring the zone of inhibition after 24 hrs incubation. Table 4 shows the antibacterial activity of *B. pinnatum* leaf extract using different extracting solvents. The results revealed that the ethanol extract of *B. pinnatum* was most effective against the test organisms than the other extracting solvents. This is in agreement with the observations of Ammara *et al*., 2009, who concluded that the stronger extraction capacity of ethanol could have been responsible for the higher antibacterial activity. *S. aureus* showed the highest susceptibility (17.3 ± 1.2 mm) to *B. pinnatum* ethanol extract, while *P. aeruginosa* showed the least susceptibility (8.3 ± 0.9 mm).

Table 4: Antibacterial activity of *Bryophyllum pinnatum* leaf extract using different extracting solvents (Zone of inhibition in mm)

	Zone of Inhibition (mm)			
Test organisms	**Aqueous extract**	**Ethanol extract**	**Methanol extract**	**Acetone extract**
S. aureus	11.5 ± 0.9	17.3 ± 1.2	12.7 ± 0.6	10.7 ± 0.9
E.coli	9.0 ± 0.6	12.7 ± 0.9	9.3 ± 0.9	7.0 ± 0.6
K. pneumoniae	0	10.0 ± 1.2	6.7 ± 0.9	4.7 ± 0.9
P. aeruginosa	0	8.3 ± 0.9	5.0 ± 1.2	5.0 ± 1.2

Table 5 shows antibacterial activity of *C. aurantifolia* leaf extract using different extracting solvents. The results revealed that the methanol extract of *C. aurantifolia* was most effective against the test organisms than the other extracting solvents. This explains the reason for the highest antibacterial activity of *C. aurantifolia* using methanol as the extracting medium. The stronger extraction capacity of methanol for *C. aurantifolia* could have been responsible for the higher antibacterial activity. The biologically active components in the plant could have been enhanced in the presence of methanol. *S. aureus* showed the highest susceptibility (25.3 ± 0.9 mm) to *C. aurantifolia* ethanol extract, while *P. aeruginosa* showed the least susceptibility (12.7 ± 0.9 mm). The aqueous extract had the least effect on the test organisms.

Table 5: Antibacterial activity of *Citrus aurantifolia* leaf extract using different extracting solvents (Zone of inhibition in mm)

	Zone of Inhibition (mm)			
Test organisms	**Aqueous extract**	**Ethanol extract**	**Methanol extract**	**Acetone extract**
S. aureus	11.3 ± 0.9	22.7 ± 0.9	25.3 ± 0.9	15.3 ± 0.9
E. coli	8.0 ± 0.6	16.7 ± 0.9	22.0 ± 0.6	12.0 ± 0.6
K. pneumoniae	0	12.7 ± 0.9	17.0 ± 1.2	8.0 ± 0.6
P. aeruginosa	0	11.0 ± 1.2	12.7 ± 0.9	5.3 ± 0.9

Table 6 shows antibacterial activity of *Bryophyllum pinnatum* and *Citrus aurantifolia* synergy using different extracting solvents. The results revealed that the methanol extract of *Bryophyllum pinnatum* and *C. aurantifolia* synergy was most effective against the test organisms than the other extracting solvents. This explains the reason for the highest antibacterial activity of the synergy using methanol as the extracting medium. The stronger extraction capacity of methanol could have produced greater active constituents responsible for the higher antibacterial activity of the synergy which had broad antibacterial spectrum (Bankole, 1992). *S. aureus* showed the highest susceptibility (27.3 ± 0.6 mm) to methanol synergy extract, while *P. aeruginosa* showed the least susceptibility (16.7 ± 0.3 mm). All the test organisms were susceptible to all the extracting solvents except the aqueous extract. *K. pneumoniae* and *P. aeruginosa* were particularly resistant to the aqueous extract. This could be due to the inability of the aqueous extract to fully extract all the bioactive ingredients. The synergy of *B. pinnatum* and *C. aurantifolia* leaf extracts gave higher zones of inhibition that neither *B. pinnatum* extract nor *C. aurantifolia* extract could give. This showed that both leaf extracts acted synergistically against the test isolates The results of this synergy is supported by Prekesh *et al.*, 2006a and Dawoud *et al.*, 2013. The additive and synergistic effects of phytochemicals enhanced the antibacterial effect of the synergy extract (combined) (Matchimuthu *et al.*, 2008) According to Cain *et al*. (2003), synergistic activity suggest different mode of action of the combining components. The extract synergy compared

well with the standard antibiotic which also acted as positive control (Table 7). The synergy, therefore has shown potential antibacterial effect against the test bacteria.

Table 6: Antibacterial activity of *Bryophyllum pinnatum* and *Citrus aurantifolia* synergy using different extracting solvents (Zone of inhibition in mm)

	Zone of Inhibition (mm)			
Test organisms	**Aqueous extract**	**Ethanol extract**	**Methanol extract**	**Acetone extract**
S. aureus	17.0 ± 0.9	23.5 ± 1.1	27.3 ± 0.6	22.7 ± 0.9
E.coli	24.0 ± 0.6	18.3 ± 0.7	26.7 ± 0.9	17.3 ± 0.6
K. pneumoniae	0	15.6 ± 0.8	20.3 ± 0.9	11.6 ± 0.8
P. aeruginosa	0	11.3 ± 0.9	16.7 ± 0.3	8.7 ± 0.9

Table 7: Zone of Inhibition (mm) of Standard antibiotic (Ciprofloxacin) against the test bacteria (Positive control)

	Zone of Inhibition (mm)
Test bacteria	**Ciprofloxacin**
S. aureus	26.0
E.coli	33.0
K. pneumonia	24.0
P. aeruginosa	29.0

Conclusion

The results from this study have shown that combinations of extracts demonstrated synergistic and additive effects on microorganisms. This synergy is better as microbial tolerance is less likely to develop against substances having more than one type of mode of action. Differential antimicrobial activity of the extract against different bacteria was due to the presence of different active phyto-compounds which made the test organisms to be susceptible. It is therefore recommended that the synergistic use of the plant extracts be encouraged to prevent drug resistance and treat emerging and re-emerging diseases caused by the test organisms.

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