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Interactions Between Leaf Extracts of Ageratum conyzoides and Antibiotics Against Clinical Strains of Pseudomonas aeruginosa and Staphylococcus aureus

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Abstract
The antibacterial effects of Ageratum conyzoides aqueous and alcoholic leaf extracts against Staphylococcus aureus and Pseudomonas aeruginosa were determined using spread plate, disc diffusion and tube dilution procedures. The antibiogram profiles of both S. aureus and P. aeruginosa were determined using disc diffusion method. The synergistic activity of the mixture of 200 mg/ml leaf extract concentrate and the respective antibiotics; Ciprofloxacin, Norfloxacin and Septrin against P. aeruginosa and S.aureus was evaluated using both spread plate and disc diffusion methods. The aqueous leaf extract elicited an inhibitory zone diameter of inhibition ranging from 6mm to 10mm for S. aureus; 7mm to 12mm for P. aeruginosa. P. aeruginosa and S.aureus exposed to the methanolic leaf extract displayed zones which ranged from 9mm to 15mm and 10mm to 16mmrespectively. S. aureus was resistant to all antibiotics except for Norfloxacin, Septrin and Ciprofloxacin, while P. aeruginosa was susceptible to ciprofloxacin alone. The synergism between the leaf extracts and the selected antibiotics showed greater inhibitory zones against the test isolates as against exposure to only the selected antibiotics. The results indicated the potential health benefits of the use of antibiotics in combination with decoctions from known medicinal plants.

Keywords: Disc diffusion method, antimicrobial activity, A. conyzoides, synergistic action, methanolic extract
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Introduction

Aside food, plants are often used as medicine (Iwu, 1986; Ogunkule and Ladejobi 2006). Plants utilized for medicinal purposes are known as medicinal plants (Soforowa, 1986). Medicinal plants are known to display a wide range of biological and pharmacological activities such as anti-inflammatory, antibacterial and antifungal properties (Okwu, 2003). Extracts, syrup, infusions and concoctions prepared from different part of these plants are utilized in the treatment of different ailments which include; typhoid fever, anemia and malaria (Okwu, 2003). The efficacy of medical plants against ill health is possibly due to certain biological active compounds such as nutrients and phytochemicals which have physiological actions on other living organisms (Okwu and Okwu 2005). Ageratum conyzoides belong to the family and order Asteraceae and Eupatoriae respectively. A.conyzoides has been utilized for the treatment of burns and wound, headaches, pneumonia, inflammation, asthma, spasmodic and stomach ailments, gynecological diseases, leprosy and other skin disease and also as an analgesic (Kamboj and Saluja 2008). Ageratum conyzoides is commonly called Billy goat weed or Goat weed and in parts of south Western Nigeria, it is called "Imiesu" (Ming, 1999). Durodola (1977) reported that the methanolic and aqueous extracts of A. conyzoides exhibited inhibitory activities against the in vitro development of S.aureus. The steady increase in bacterial resistance to existing drugs is a serious problem and as such, there is an urgent need to search for new classes of antibacterial substance especially from natural sources. Sometimes, the use of a single antibiotic does not produce the desired effective inhibitory effects and to overcome this, a combination of drugs often exercises their synergistic effect which surpasses their individual performance. The synergistic effect may be due to certain formation of complexes

which become more effective in the inhibition of a particular microorganism. The synergistic effect from the association of antibiotics with plant extracts against drug resistant bacteria can lead to new choices for the treatment of infectious diseases. The present study was undertaken to investigate the synergistic antibacterial activity of methanolic, ethanolic and aqueous extract of A.conyzoides with some antibiotics on P. aeruginosa and S. aureus.

Materials and methods

Plant Collection and Identification: Fresh leaves of A.conyzoides were collected from the surroundings of University of Benin, Edo State, Nigeria. They were washed with clean water to remove sand and properly identified and authenticated with reference to the available Herbarium sheets at the Department of Plant Biology and Biotechnology, University of Benin, Benin, and Nigeria

Test Organisms: Cold stored agar slant cultures of already identified P. aeruginosa and S. aureus were obtained from the Medical Microbiology laboratory of the University Benin Teaching Hospital (U.B.T.H), Benin City, Nigeria. Viability test of each isolate was carried out by resuscitating the organisms in buffered peptone broth and thereafter sub cultured into nutrient agar medium and incubated at 37°C for 24 h. The probable identity of the clinically sourced isolates was further confirmed by exposing the cultures to an array of standard biochemical tests which included coagulase production and oxidase test as described by Collins et al. 2004) and Sharma (2009) The results of the biochemical reactions elicited by the test isolates were compared with standard identification keys as described by Collins et al. (2004).

Preparation of plant extract: A.conyzoides leaves were separated manually and cleaned with sterile distilled water; air dried, oven dried and finely ground using a grinder mill. Twenty grams of the fine powder from A.conyzoides leaves were placed in 250ml of each solvent (95% ethanol, 95% methanol, and distilled water), placed in different conical flasks and refluxed at 50^0C for 60mins. The extracts were filtered through Whatman filter paper No 1 and were evaporated to dryness using a hot air oven at 40^0C. The residues obtained were dissolved in 1% dimethyl sulphoxide (DMSO). The weights of the extracts were determined and stored below ambient temperature.

Antibacterial Assay: Disk diffusion method using Müeller-Hinton agar plates was used to demonstrate the antimicrobial properties of the crude extracts. A suspension of the test bacterial isolates compared to 0.5 Macfarland standard was seeded on the Mueller - Hinton agar plates. 10 mm discs were cut from Whatman No.1 filter paper and sterilized in the oven at 160 □C for 2 h. The disks were then impregnated with the extract by soaking in the extract for 24 h of different concentrations of the extracts (i.e., 25, 50, 100 and 200 mg/ml respectively). Each of the disc contained approximately 200 mg/ml hot distilled water, ethanolic and methanolic extract. Using sterilized forceps, each disc was recovered from the extract and applied aseptically unto the agar plates already inoculated with a pure culture of the test organisms and the plates were incubated overnight at 37 ° C. Growth was determined by measuring the diameter of the zone of inhibition.

Minimum Inhibitory Concentration (MIC): The MIC of the potent extracts was determined according to the macro broth dilution technique (Baron and Finegold, 1990). Standardized suspension of the test organism was inoculated into a series of sterile tubes of nutrient broth containing two-fold dilutions of leaf extracts and incubated at 37^0C for 24 h. The MICs were recorded as the least concentration that inhibited the growth of the test organisms.

Minimum Bactericidal Concentration (MBC): The minimum bactericidal concentration (MBC) of the respective leaf extracts was determined by procedure described by Asowata et al. (2013). Aliquots (1 ml) were taken from MIC tubes with no visible growth and sub-cultured on freshly prepared Nutrient agar plates and later incubated at 37 for 24 h. The MBC was taken as the concentration of the extract that did not show any growth on a new set of agar plates.

Antibiotic Susceptibility Test: Single disc diffusion method as described by NCCLS, (2004) was used to examine the susceptibility of the bacterial isolates to the antimicrobial agents. The antibiotic sensitivity discs utilized were Cephalexin(20µg), Norfloxacin(10µg), Amoxicillin(10µg), Septrin(25µg), Erythromycin(10µg), Ciprofloxacin(5µg), Gentamicin(10µg), Nitrofurantoin(10µg), Augmentin(10µg), Ofloxacin(10µg), Chloramphenicol(30µg) were used (Becton Dickson, USA). The zone diameter for individual antimicrobial agents was then translated into resistance and susceptibility categories according to the NCCLS (2004).

Synergistic Action Assay: The synergistic effect of the combination of the 200 mg/ml leaf extract concentrate and the respective antibiotic discs was determined using the procedure described by Toroglu (2007). The commercially

available antibiotic disc were respectively immersed in 2 ml of the 200 mg/ml leaf extract concentrate and the saturated disc were impregnated on Mueller Hinton agar plates seeded with lawns of the standardized test bacterial isolates.

Results

Table 1 showed the antibacterial activity of aqueous, methanolic and ethanolic A. conyzoides extracts against Staphylococcus aureus and Pseudomonas aeruginosa isolates. All the plant extracts showed varying antibacterial activity. Antibacterial activity was analyzed at different concentrations ranging from 25mg/ml to 200mg/ml. The aqueous leaf extract elicited an inhibitory zone diameter of inhibition ranging from 6mm to 10mm for Staphylococcus aureus; 7mm to 12mm for Pseudomonas aeruginosa. P. aeruginosa and S. aureus exposed to the methanolic leaf extract displayed zones which ranged from 9mm to 15mm and 10mm to 16mmrespectively (Table 1). S.aureus and P.aeruginosa exposed to the ethanolic leaf extract had inhibitory zones which varied from 8mm to 14mm and 6mm to 14mm respectively (Table 1). Table 2 revealed the minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) values of the leaf extract concentrates. The MIC readings for S. aureus and P. aeruginosa exposed to aqueous leaf extracts were 100 mg /ml and 200mg/ml respectively (Table 2). The MIC value of both test isolates exposed to varying concentrates of the ethanolic extract was100mg/ml respectively (Table 2). The MBC values of aqueous A. conyzoides leaf extract ranged from 50mg/ml for both S. aureus and P. aeruginosa respectively (Table 2), whilst the test bacterial isolates exposed to the methanolic extracts had a MBC value of 100mg/ml respectively (Table 2). The MBC values of the ethanolic leaf extract was 25 mg/ml respectively for both isolates . Table 3 showed the susceptibility pattern of S. aureus and P.aeruginosa exposed to the ten (10) antibiotics. The result revealed that S. aureus was resistant to all antibiotics except for Norfloxacin, Septrin and Ciprofloxacin, while P. aeruginosa were only susceptible to Ciprofloxacin. Table 4 showed the synergistic effect of both A.conyzoides leaf extract concentrate and antibiotics on the test bacterial isolates. Since the majority of the S. aureus and P. aeruginosa isolated were resistant to many antibiotics, only Ciprofloxacin, Septrin and Norfloxacin were used in the synergism assay (Table 4).S. aureus exposed to the combination of Ciprofloxacin, Septrin and Norfloxacin with 200 mg/ml of the respective leaf extract elicited inhibitory zones which ranged from 20 mm for the mixture of aqueous extract and Septrin to 30 mm for both ethanolic extract mixed with Norfloxacin and Septrin (Table 4).P. aeruginosa exposed to the combination of the antibiotics with 200 mg/ml of the respective leaf extract elaborated inhibitory zones which ranged from 13 mm for the mixture of aqueous extract and Septrin to 18 mm for methanolic extract mixed with Norfloxacin (Table 4).

Table 1: Antibacterial activity of A.conyzoides leaf extracts

Concentrations (mg/ml)	S.aureus			P.aeruginosa		
	Zone of Inhibition (mm)			Zone of inhibition (mm)		
	Aqueous	Methanol	Ethanol	Aqueous	Methanol	Ethanol
200	10	15	14	12	16	14
100	7	12	10	8	13	11
50	7	11	10	7	12	9
25	6	9	8	7	10	6

Table 2: Minimum inhibitory concentration and Minimum bactericidal concentration of A.conyzoides leaf extracts

Bacterial isolates	Aqueous		Methanol		Ethanol	
	MIC(mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC(mg/ml)	MIC (mg/ml)	MBC (mg/ml)
S. aureus	100	50	100	50	100	25
P.aeruginosa	200	50	100	25	100	25

Table 3: Antibiotic sensitivity patterns of S. aureus and P. aeruginosa isolates.

Antibiotics	Concentration (µg)	S.aureus Zone of inhibition (mm)	P.aeruginosa Zone of inhibition (mm)
Cephalexin (CEP)	20	2	0
Norfloxacin (NOR)	10	14	0
Amoxicillin (AM)	10	0	0
Septrin (SXT)	25	13	0
Erythromycin (ERY)	10	0	0
Ciprofloxacin (CPX)	5	16	5
Gentamicin (CN)	10	0	0
Nitrofurantoin (NB)	10	0	0
Augmentin (AUG)	10	0	1
Ofloxacin (OFL)	10	0	0

KEY: Cephalexin - CEP; Erythromycin – ERY; Augumentin – AUG; Norfloxacin –NOR ;Ciprofloxacin – CPX ;Ofloxacin - OFL Amoxicillin – AM; Gentamicin – CN; Chloramphenicol- CH Septrin – SXT; Nitrofurantoin – NB

Table 4: Synergistic action of A.conyzoides extracts with Norfloxacin, Septrin and Ciprofloxacin on bacterial isolates.

Extract +Antibiotics	S. aureus Zone of Inhibition(mm)	P. aeruginosa Zone of Inhibition(mm)
Aqueous + CPX(5 µg)	22	15
Aqueous+ STX(25µg)	20	13
Aqueous + NOR(10 µg)	24	17
Methanol + SXT(25µg)	25	16
Methanol+ CPX(5 µg)	26	16
Methanol+ NOR (10 µg)	27	18
Ethanol +NOR (10 µg)	30	15
Ethanol + CPX(5 µg)	28	15
Ethanol + STX(25µg)	30	17

KEY: Norfloxacin –NOR; Ciprofloxacin – CPX, Septrin – SXT

Discussion

The trend of increased resistance to antibiotics has been the main factor justifying the need to develop new antimicrobial agents. Cowan (1999) reported that some studies have focused on the antimicrobial agents and on the antimicrobial properties of plant-derived active principles, which have been used for a long time in traditional medicine to overcome infections. The results obtained in this study revealed the antibacterial efficacy of A.conyzoides leaf extracts. This trend is in line with the findings of Okworie et al.(2007). The methanolic and ethanolic extracts of A.conyzoides were more active against the test bacteria in comparison with the aqueous extract (Table 1). This phenomenon could indicate that the active phytochemicals present in the leaf were more soluble in methanol and ethanol as against water. However, Ijeh et al. (2005) and Junaid et al. (2006) reported that hexane was the best solvent for extracting plants phytochemicals, due its high non-polarity. Okwori et al.(2007) reported that active antimicrobial phytochemicals present in drugs often exercise their synergistic effect which surpasses their individual performance. There was observed differences between the MIC and MBC values as the MBC values was comparatively higher than the MIC data recorded for the respective extracts. These variations could imply that the MBCs values obtained from plating the various diluted extracts was more reliable and accurate in contrast to the MICs results which were obtained by visual observation using turbidity as an index. The test bacterial isolates exhibited multiple antibiotic resistance as S. aureus was resistant to seven antibiotics (Cephalexin, Amoxicillin, Erythromycin, Gentamycin, Nitrofuratin, Augmentin and Ofloxacin) whilst P. aeruginosa was susceptible to ciprofloxacin only (Table 3). Comparatively, the synergism between the leaf extracts and the selected antibiotics elicited greater inhibitory zones against the test isolates as against exposure to only the selected antibiotics (Table 3 and 4). The synergistic effect could be due to certain complex formation which becomes more effective in the inhibition of a particular microorganism either through the inhibition of cell wall synthesis or by

causing its lysis or death. This trend is in agreement with a report by Park et al.(2004) which indicated that the combination of antibiotic peptides with chloramphenicol inhibited the growth of S. aureus and P. aeruginosa.

Conclusion

The emergence of resistance to antimicrobial agents is a global public health problem, particularly for microbial pathogens causing nosocomial infections. Usage of antibiotics in combination with decoctions from known medicinal plants could potentially have health benefits and mitigate the scourge of active infections initiated by multiple drug resistant microbial pathogens.

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