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COMBINATION OF AGERATUM CONYZOIDES LEAF EXTRACTS WITH ANTIBIOTICS AGAINST STAPHYLOCOCCUS AUREUS AND PSEUDOMONAS AERUGINOSA ISOLATED FROM WOUND INFECTION

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Abstract

There has been increasing prevalence of bacterial resistance to commonly used antibiotics. The present study investigates the synergistic action of *Ageratum conyzoides* leaf extracts and antibiotics against *Pseudomonas aeruginosa* and *Staphylococcus aureus* isolated from wounds.

The disc diffusion method was used. Extracts were diluted to concentrations ranging from 3.125 to 5.0 mg/ml.

The results indicated that ethanolic extract of *Ageratum conyzoides* showed antibacterial activity with zone of inhibition of 10.33 mm and 11 mm for *Staphylococcus aureus* and *Pseudomonas aeruginosa* respectively. Antibiotics were applied in combination with ethanolic extract of *Ageratum conuyzoides* and produced zones of inhibition higher than that of the individual antibiotics and plant extract. Ethanolic extract + ciproflaxicin and ethanolic extract + erythromycin produced inhibition of 29 mm and 30 mm for *Staphylococcus* aureus and *Pseudomonas aeruginosa* respectively. The result revealed that combination of *Ageratum conyzoides* extract and antibiotics tested could be useful in treating wound infections.

Introduction

The management of infected wounds is a challenge in terms of rational antimicrobial use especially with the presence of a wide variety of antimicrobial drugs. Similarly, a lot of concern has been generated worldwide over bacterial drug resistance, leading to cheap drugs being replaced with more effective and expensive ones[1]. Sule and Olusanya[2] have reported the increasing prevalence of bacterial resistance to most of the commonly used antibiotics. At present, clinical isolates of *Staphylococcus aureus* are multiple drugs resistant to three or more agents such as ciprofloxacin, erythromycin, clindamycin, gentamicin, linezolide and vancomycin[3]. Global resistance rate of Streptococcus pyogenes isolates are as high as 80% for erythromycin and 50% for penicillin[4]. Infections with *Pseudomonas aeruginosa* are particularly challenging since the bacterium exhibits inherent tolerance to several antimicrobial agents and can acquire additional resistant mechanism turning ineffective all current antimicrobial drugs. In many poor countries, the high cost of such replacement drugs is prohibitive, with the result that some disease can no longer be treated in areas where resistance to first line drugs is widespread[5]. Faced with such a challenge, there is need to develop alternative approaches in addition to the search of new antimicrobial compounds.

Plants are valuable source of medicinal compounds that contain broad spectrum of biological activity[6]. Approximately 25-

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50% of current pharmaceuticals is derived from plants and show less side effects than the systemic drugs. Normally, during their life cycle, plants encounter various infectious agents such as viruses, bacteria, fungi and other parasites and synthesize a variety of secondary metabolites capable of destroying the infectious agents. For example, single and combine plant extract of Rhusconaria and Thymus vulgaris were observed to be highly effective against multiple drug resistant Pseudomonas aeruginosa[7].

Ageratum conyzoides has been known since ancient times for its curative properties. It has been utilized for treatment of various ailments and largely, for its antibacterial properties against bacterial infections. In Africa, *Ageratum conyzoides* is used to treat fever, colic, rheumatism, headache, pneumonia, wounds and burns[8]. Pharmacological investigation by Durodola[9] verified that ether and chloroform extracts of *Ageratum conyzoides* has inhibitory activities against in vitro development of *Staphylococcus aureus.* An in-vitro study of methanolic extracts of whole plants has shown antibacterial activity against Bacillus subtilis, Escherichia coli, *Pseudomonas aeruginosa* and *Staphylococcus aureus*[10].

The use of single antibiotics does not often produce the desired effective inhibitory effects and to overcome this, a combination of drugs, often exercise their synergistic effect which surpasses their individual performance. The synergistic effect from the association of antibiotics with plant extracts against resistant bacteria offers a new choice for the treatment of infectious diseases. Therefore, this study was undertaken to investigate the synergistic activity of ethanol and aqueous extracts of *Ageratum conyzoides* with some antibiotics against *Staphylococcus aureus and Pseudomonas aeruginosa* isolated from wounds infection.

Materials and Methods

Plant Collection and Identification

Fresh leaves of *Ageratum conyzoides* (common name: Goat weed) were collected around the University of Benin, Benin City. Contaminant were hand-picked and then washed with clean water to remove sand. The plant materials were identified by the Department of Plant Biology and Biotechnology, University of Benin, Benin City.

Specimen Collection

Clinical isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolated from wound infection were obtained from the stock culture of the Microbiology Laboratory of University of Benin Teaching Hospital Benin, Nigeria. Viability test of the isolates were carried out by resuscitating the organism into a nutrient broth and thereafter sub-cultured into nutrient agar plates and incubated at 37 ^{o}C for 24 h. Standard culture based and biochemical reaction methods were used for the confirmation of the test bacteria.

Preparation of Plant Extract

The leaves of *Ageratum conyzoides* were separated, the plant were cleaned with sterile distilled water; oven dried and finely ground using a grinder mill. Twenty gram (20 g) of the fine powder from *Ageratum conyzoides* was soaked in 200 mL of ethanol and distilled water placed in different conical flasks and allowed to stand for 24 h. The content of each conical flask containing plant materials and the solvent were filtered after 24 h using

Whatman filter paper, supernatant of each plant extract was transferred into conical flask. The conical flask containing plant extract filtrates were allowed to dry completely to obtain a solvent free dried residue using water bath evaporator. The plant extracts residues were resuspended in 0.5 mL dimethhylsulphoxide (DMSO) for subsequent analysis.

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) was determined using the disc diffusion. Filter paper was made into disc using sterile puncher. The discs were sterilized in a hot air oven at 160 °C for 1 h. Extracts were diluted to concentrations ranging from 3.125 to 5.0 mg/mL (for a mixture of the plant extract and the antibiotic). The sterilized discs were soaked in different concentration of the plant extract and the antibiotic for 24 h. Solidified nutrient agar plates were inoculated with test organisms and the soaked discs were placed aseptically on the inoculated plates. The nutrient agar plates were incubated at 37 °C for 24 h. The MIC was read as the least concentration that inhibited the growth of the test organism.

Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration (MBC) was determined by first selecting tubes that showed no growth during MIC determination; a loop full from each tube was sub-cultured onto extract-free agar plates, incubated for further 24 h at 37°C. The least concentration at which no growth was observed was noted as MBC.

Antibiotic Susceptibility Testing

Single disc diffusion method were employed as described by Bauer et al.[11] was used to examine bacterial susceptibility to antimicrobial agents. The antibiotic sensitivity discs used were norfloxacin (10 μg), chloramphenicol (10 μg), ciprofloxacin (10 μg) erythromycin (30 μg), levofloxacin (20 μg), gentamicin (10 μg), ampiclox (20 μg), rifampicin (20 μg), amoxicillin (20 μg), and streptomycin (30 μg) (Becton Dickson, USA). Single bacterial colonies from overnight culture were suspended in 5 ml normal saline. The surface of Muller Hinton agar plates was evenly inoculated; the antibiotics discs were applied on the surface of the inoculated agar plates with sterile forceps. Each disc was gently pressed down onto the agar to ensure complete contact with the agar surface; plates were incubated at 37 °C for 24 h. After incubation, the plates were examined and the diameter of the zones of inhibition was measured. Susceptibility data were interpreted according to the Clinical and Laboratory Standard Institute[12].

Antibacterial Assay

Susceptibility screening test using agar well diffusion method, each microorganism was suspended in nutrient broth and diluted to 10^6 colony forming units per ml (cfu/mL). They were spread (inoculated) onto the surface of agar plates which were then dried. Four millimeter agar wells were cut from the agar using sterile cork-borer and 0.1 ml of the plant extract and the antibiotics were inoculated into the wells. After incubation at 37 °C for 24 h, the plates were examined for any zones of inhibition.

Combination Action Assay

The antibiotics disc were impregnated with the *Ageratum conyzoides* leave extract and then dried. The pure culture of Staphylococcus aureus and *Pseudomonas aeroginosa* were subculture on nutrient agar media in pre-sterilized petri dish. The *Ageratum conyzoides* extract and

antibiotics disc were freshly inoculated into agar plates carefully. The plates were incubated at 37 °C for 24 h to test efficacy of both leaf extract and antibiotics against the wound isolates.

Result

The plant extracts showed varying antimicrobial activity. The aqueous leaf extract showed no activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus.* The ethanol leaf extract revealed an average mean zone diameter of inhibition of 11 mm ± 1.0 for *Pseudomonas aeruginosa* and 10.33 mm ± 1.53 for Staphylococcus aureus (table 1).

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the ethanol extract is presented in table 2. The MIC and MBC was 3.125 and 6.25mg/mL for both Staphylococcus aureus and *Pseudomonas aeruginosa* respectively.

The antibiotics sensitivity patterns of *Staphylococcus aureus* and *Pseudomonas aeruginosa* from wound infection is presented in table 3. Result showed that *Staphylococcus aureus* was highly resistant to all antibiotic except for ciprofloxacin and erythromycin while *Pseudomonas aeruginosa* was only susceptible to ciprofloxacin and gentamicin.

The synergistic action of *Ageratum conyzoides* extracts with antibiotics on the wound isolates is given in table 4. Results revealed that the combination of *Ageratum conyzoides* leaf extract plus the individual test antibiotics produced zones of inhibition higher than that of the individual antibiotics and extract.

Discussion

Herbal medicine has been shown to have genuine utility and about 80% of rural dwellers depend on its efficacy for their primary health care[5]. Medicinal plants contribute an effective source of both traditional and modern medicines. *Staphylococcus aureus* is a normal flora of the skin and the leading cause of both surgical and accidental wound infection[13]. *Pseudomonas aeruginosa* is frequently present in small numbers as normal flora of the intestine and skin of humans. These bacteria are widely distributed in nature and are commonly present in moist environment in the hospital and are pathogenic only when introduced into area devoid of normal defenses such as when mucus membrane and skin are disrupted by direct tissue damage[14].

The results obtained in the study revealed the antimicrobial efficacy of ethanolic extract of *Ageratum conyzoides* leaves with mean zone of inhibition of 11 mm ± 1.0 and 10.33 mm ± 1.53 for *Pseudomonas aeruginosa* and *Staphylococcus aureus* respectively (table 1). There was significant difference in the antibacterial activity between the aqueous and ethanolic extracts of *Ageratum conyzoides* ($p<0.05$). This could suggest that the active ingredients of the plant extract are only soluble in organic solvents. Junaid et al.[15] reported organic solvents such as hexane as the best solvents for extracting plant metabolites, due to high non-polar compounds.

This study showed that ciprofloxacin alone could cause a zone diameter of inhibition of 10 mm and 20 mm against *P. aeruginosa* and *S. aureus* respectively (table 3) but produced 24 mm and 29 mm zones of inhibition when combined with ethanol extract of *Ageratum conyzoides* (table 4). Also, gentamicin was 13 mm and 0 mm zone of inhibition on *P. aeruginosa* and S. aureus respectively, but produced 30 mm and 7 mm against P*seudomonas aeruginosa* and *Staphylococcus aureus*

respectively when combined with ethanol extract of *Ageratum conyzoides*. Stevanovic et al.[16] reported an increase in zone of inhibition from 23 mm to 30 mm when tetracycline was combined with leaf extract of Aegopodium podagranial. Park et al.[17] also reported that combination of antibiotic peptides with chloramphenicol inhibited the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Interaction between antibiotics and plant extracts depends upon species of microorganism, type and concentration of extract as well as the MIC value[16]. Studies have shown that the efficacy of antimicrobial agents can be improved by combining them with crude plant extract against different pathogens including Staphylococcus aureus, *Pseudomonas aeruginosa,* Escherichia coli, extended spectrum β-lactamase producing multiple E. coli and vancomycin resistant enterococci (Enterococcus faecalis)[18].

This synergistic effect may be due to certain complex formation which becomes more effective in the inhibition of particular specie of microorganisms either by inhibiting the cell wall synthesis or by causing its lysis or death. The combination of medicinal plant extracts and known antibiotics offer significant potential for the development of antimicrobial therapies and the treatment of several disease caused by microorganisms. The synergistic activity of plant extract is a positive interaction that is obtained when two agents in combination show inhibitory effect on targeted organism that is greater than the sum of their individual effect[19]. Synergy with chemotherapeutic agent is highly specified that is synergism with a particular extract may be effective with a specific drug and ineffective with other drug[20]. Several in-vitro studies have also reported synergistic effect with significant reduction in the MICs of the antibiotics resulting from the combination of different crude plant extracts against *S. aureus* strains[21]. The ability of plant extracts as potential antibiotics has been well known, so it is predicted that inhibition of drug efflux and alternative mechanisms of action could be responsible for the synergistic interaction between plant extracts and antibiotics[22]. Hence, research should be focused towards this direction to identify more medicinal plants which exhibit synergistic behaviour.

Conclusion

The emergence of resistance to antimicrobial agents is a global public health problem particularly in pathogens causing nosocomial infections when antibiotics are no longer effective by itself during therapeutic treatment. This could be overcome by the association of antibiotics with plant extracts to produce new choices for the treatment of infectious diseases.

Table 1: Antibacterial activity of ethanol leaf extract of *Ageratum conyzoides* on bacterial isolate

Test organism	**Inhibition zone diameter range (mm)**	**Mean zone diameter (mm)**
Pseudomonas aeruginosa	10 – 12	11 ± 1.0
Staphylococcus aureus	9 -12	10.33 ± 1.53

Table 2: Minimum inhibitory concentrations (MIC) and Minimum bactericidal concentrations (MBC) of leaf extracts of *Ageratum conyzoides*

Bacterial isolate	**Aqueous Extract**		**Ethanol Extract**	
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
S. aureus	-	-	3.125	6.25
P. aeruginosa	-	-	3.125	6.25

Table 3: Antibiotics sensitivity patterns of *Staphylococcus aureus* and *Pseudomonas aeruginosa* from wound infection.

Antibiotics	**Concentration (µg)**	***S. aureus* Zones of inhibition (mm)**	***P. aeruginosa* Zones of inhibition (mm)**
Norfloxacin	10	0	0
Chloramphenicol	10	0	0
Ciprofloxacin	10	20	10
Erythromycin	30	12	0
Levofloxacin	20	0	0
Gentamicin	10	0	13
Ampiclox	20	0	0
Rifampicin	20	0	0
Amoxicillin	20	0	0
Streptomycin	30	0	0

Table 4: Synergistic action of *Ageratum conyzoides* extracts with antibiotics on wound isolates.

Antibiotics + Ethanol Extract	*S. aureus* Zones of inhibition (mm)	*P. aeruginosa* Zones of inhibition (mm)
Norfloxacin	7	10
Chloramphenicol	6	8
Ciprofloxacin	29	24
Erythromycin	21	11
Levofloxacin	5	10
Gentamincin	7	30
Ampiclox	4	9
Rifampicin	6	11
Amoxicillin	5	10
Streptomycin	7	11

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