In Vitro Combine Interactions of Antimicrobial Agents with Plant Extract against Multidrug-Resistant Bacterial Strains

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The in vitro interaction between n-hexane extract (nhexEXT) of root of Adiantum capillus-veneris and certain known antimicrobial drugs i.e. Oxacillin, Ceftazimide, Ceftriaxone, Ofloxacin, Meropenem, Erythromycin, Cefuroxime, Cefoxitin, Cefotaxime and Ampicillin was evaluated. The study was carried out against ten bacterial strains (Staphylococcus aureus, S.epidermidis, Salmonella typhi, Klebsiella pneumoniae, Shigella dysentriae, Proteus vulgaris, Pseudomonas aeruginosa, Providencia species, Citrobacter freundii and Escherichia coli isolated from urine, pus and blood samples. Both disc diffusion and well diffusion methods were used to determine antimicrobial activity of plant extract in combination with antibiotics. Antimicrobial sensitivity indicated that Meropenem was the most effective antibiotic with zone of inhibition (ZI) of 25-33 mm among all tested antibiotics followed by Ofloxacin (10-26.5 mm), Ceftriaxone (8-20 mm), while Oxacillin showed no activity against almost all bacterial strains. The study showed that most bacterial strains were resistant to most of the antibiotics used, ranging from 20-60%. The methanolic extract (nhexEXT) of A. capillus-veneris used alone was active against most of the bacterial isolates with maximum activity against E. coli with 16 mm ZI. The study also indicated that there was an increased activity in case of combination of nhexEXT with antibiotics. The combined effects of plant extract with antibiotics were synergistic against most of the bacterial strains. The nhexEXT showed maximum synergistic effect with Ceftazimide with ZI of 42 mm followed by Meropenem (40 mm) and Ceftriaxone (28 mm) against multidrug-resistant (MDR) bacterial strains. The data suggests that plant extract could be used as alternative to antibiotics. These results give scientific backing that combination between plant extract and antibiotics would be useful in fighting the emerging drug-resistant bacterial pathogens.

**Keywords:** Adiantum capillus-veneris, antibacterial activity, synergistic effects, antagonistic effect, medicinal plant

INTRODUCTION

Antibiotic resistant bacteria have been a source of ever increasing therapeutic problems (Sheikh et al., 2003). Continued mismanagement and indiscriminate usage of commonly prescribed antibiotics result in the emergence of MDR bacteria (Cohen and Auxe, 1992). Antibiotics resistant bacteria can be found in all different ecological niches. Selective pressure of misuse of antibiotics mainly in hospitals, agriculture and animal farming, goes in favor of bacteria by developing new genes responsible for the antibiotic resistance (Kummerer, 2004). Liquid manure of animals as well as human excretion has also led to dissemination of resistant bacteria in the environment (Reinhalter, 2003).

The emergence and spread of microbes that is resistant to cheap and effective first choice antibiotics has become a common occurrence. Faced with this problem, there is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structure and novel mechanisms of action because there has been an alarming increase in the incidence of new and re-emerging infectious diseases, appearance of undesirable side benefits.
effects of certain antibiotics, as well as the increasing development of resistant to the antibiotics in current clinical use (Cowan, 1999). Therefore, actions must be taken to control the use of antibiotics, to better understand the genetic mechanisms of resistance and to continue studies to develop new drugs.

There are different approaches to cure and control the infections caused by MDR bacterial strains. One of which is by isolation of active phytochemicals that can help to prevent the spread of infection. Another method is to formulate new synergistic combination using different commercially available antibiotics, or to combine an antibiotic with active phytochemicals having antimicrobial properties. It has been found that some medicinal plants produce MDR inhibitors which enhance the activities of antibiotics against MDR bacterial strains (Stermitz et al., 2000).

Several in vitro studies have reported synergistic effects resulting from the combination of different antibiotics with different plants extracts against MDR bacterial strains (Betoni et al., 2006). According to Estimone et al. (2006) the herb-drug interaction between tea extract and penicillin G against Staphylococcus aureus showed that the interaction was mainly additive. Sibanda and Ocoh (2008) showed potential of synergy between acetone extracts of Garcinia kola and Amoxicillin, Ciprofloxacin, Tetracycline and Chloramphenicol against MDR pathogenic microorganisms.

Adiantum capillus-veneris is commonly known as maiden hair fern belonging to kingdom Plantae and family Adiantaceae, distributed throughout the world and in different areas of Nigeria. It is traditionally used to treat infectious diseases (Singh et al., 2008). It can be used as expectorant, diuretic, as hair tonic and in chest disease (Piyali et al., 2005). It has various phytochemicals like alkaloids, tannins, steroids, flavonoids, saponins, terpenoids, cardiac glycosides and reducing sugar, and also have antibacterial and antifungal activity (Ishaq et al., 2014).

Adiantum capillus-veneris has many traditional uses globally. It is used for blood cleaning, cough, menstrual and respiratory problems in Arizona. In Brazil it is used for asthma, bronchitis, cough, digestion, excessive mucous, flu and hair loss. Likewise, in India, it is used for asthma, boils, bronchial disease and cold diabetes (Ansari and Ekhlasi, 2012).

Therefore, the present study was designed to investigate the antibacterial activity of methanolic extract of A. capillus-veneris and their interaction with ten commonly used antibiotics against MDR bacterial strains isolated from clinical samples.

MATERIALS AND METHODS

Plant material collection and extraction

The A. capillus-veneris was collected from different areas of Abajah in Nwangele LGA Imo State. The plant was identified in the department of Botany, Imo State university, while processed in microbiology laboratory. The roots of A. capillus-veneris are used as plant sample for extract preparation which were first thoroughly washed with water and soaked in detergent to remove the microbial load on the surface of plant sample. These were then shade dried and ground to homogenous powder (Sood and Sharma, 2010). One hundred gram of powder was soaked in flask containing 1-liter n-hexane for 24 h at 25 ºC and then filtered through Whatman No. 1 filter paper (Cseke et al., 2006). The filtrate was collected in separate flask and the same process was repeated for three times. The filtrates, that is, crude extract obtained was concentrated in rotary evaporator. For the isolation of pure extract, the isolated crude extract was resuspended in a minimum required volume of corresponding solvent and placed on the water bath (60 ºC) for the evaporation of extra solvents. The extract was then preserved in separate containers for further experimentations at 5 ºC, according to previous method of Deveeka et al. (2013).

Collection and identification of bacterial strains

Ten different bacterial strains used in this study were isolated from different clinical samples like urine, wound pus, and blood which were collected from the main laboratory of Imo State University Teaching Hospital, St John’s. Laboratory Owerri and University of Benin teaching Hospital. The isolated bacterial strains were subcultured on differential and selective media e.g. MacConkey, SS agar, MSA and CLED agar and were identified by their specific morphological and biochemical characteristics (Collee and Marr, 1996).

Assessment of drug resistance pattern of test bacterial strains

The Kirby-Baur method described by Benson (2002) was used to study antimicrobial sensitivity tests. Disc diffusion method was used for measurement of the antimicrobial activity of antibiotic discs on Mueller-Hinton agar. The organisms were tested against ten commonly used antibiotics. Antibiotics used were Oxacillin (OX) (1µg), Ceftazimide (CAZ) (30 µg), Cefotaxime (CTX) (30 µg), Ceftiraxone (CRO) (30 µg), Cefuroxime (CXM) (30 µg), Meropenem (MEM) (10 µg), Ofloxacin (OFX) (5 µg), Cefoxitin (FOX) (30 µg), Ampicillin (AMP) (10 µg) and Erythromycin (E) (15 µg) and the process was repeated thrice. All the plates were incubated at 37 ºC for 24 h.
**Antimicrobial activity of plant extract**

Well diffusion method of Janovska et al. (2003) was followed with some modifications for the antimicrobial activity of plant extracts. One mg of plant extract was dissolved in 1 mL of dimethyl sulfoxide (DMSO) (1 mg/1 mL). Pre-autoclaved Nutrient agar plates were inoculated with a $10^{-5}$ dilution of bacterial culture, using sterile cotton swabs to achieve uniform lawn of growth and sterile corn borer was used to bore wells in the agar. Sixty microliters of extract were introduced through micropipette aseptically into specifically marked wells in the agar plates. All cultured plates were incubated at 37 °C for 24 h and the process was repeated thrice.

**Combined plant extract and antibiotics**

The combine activity of plant extract and antibiotics was calculated by combining different antibiotics and extract using agar well method. The plates were incubated for 24 h at 37 °C and synergistic and antagonistic effects were measured and compared with individually used extract and antibiotics (Okore, 2009).

**RESULTS**

**Drug resistance profile of the test bacterial strains**

Different bacterial species were isolated from different sources like S. aureus and *Salmonella typhi* were isolated from blood samples, *Escherichia coli*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Shigella dysenteriae*, *Proteus vulgaris* and *Providencia* species from urine while *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* were isolated from wound pus. The bacterial strains were tested for antibiotics sensitivity profile against 10 frequently used antibiotics. Most of the tested bacterial strains were found to be resistant to the used antibiotics. *E. coli* and *S. typhi* were found to be the most resistant bacterial strains (60%) to all tested antibiotics, followed by *S. dysenteriae*, *K. pneumoniae* and *Providencia* (50%), *S. aureus* and *P. aeruginosa* (40%), *C. freundii* (30%), *S. epidermidis* and *P. vulgaris* (Figure 1).

![Figure 1: Percentage of antibiotic sensitivity of MDR bacterial strains.](image)

Amongst all used antibiotics MEM showed maximum effect against all tested bacterial strains. The maximum ZI was shown by *Providencia* species and *P. aeruginosa* (34 mm) followed by *E. coli* and *S. dysenteriae* (33 mm), *P. vulgaris* (32.5 mm), *C. freundii* (32 mm), *S. aureus* and *S. epidermidis* (31 mm), *S. typhi* (30 mm), and *K. pneumoniae* (25 mm). The most effective antibiotics were OFX, CTX and CRO (Figure 1 and Table 1).
Table 1: Drug resistance profile of test bacterial strains.

Antibiotics disc with ZI (mm), while (--) representing resistance

<table>
<thead>
<tr>
<th>Microorganisms</th>
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<td>OX</td>
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</table>

Table 2: Antibacterial activity of plant extract.

Antibacterial activity of extract of *Adiantum capillus-veneris* against MDR bacterial strains

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>MDR</th>
<th>E. coli</th>
<th>S. epidermidis</th>
<th>S. dysenteriae</th>
<th>S. typhi</th>
<th>P. vulgaris</th>
<th>Providencia species</th>
<th>S. aureus</th>
<th>P. aeruginosa</th>
<th>K. pneumoniae</th>
<th>C. freundii</th>
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</table>

Extracts with zone of inhibition (ZI) representing sensitivity in millimeter (mm)

Table 3: Combined activities of antibiotics with methanolic extract of *Adiantum capillus-veneris*.

Combined ZI in (mm) of plant extract with antibiotics

<table>
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<th>Microorganisms</th>
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<td>E. coli</td>
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<td>S. dysenteriae</td>
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-- No ZI; I, Indifferent; s, Synergistic effect; a, Antagonistic effect; ND, Not done.

Assessment of antibacterial activity of plant extract

The extracts of *Adiantum capillus-veneris* showed little effect against tested bacterial strains. The extract showed maximum ZI against *E. coli* (16 mm), followed by *S. epidermidis* and *C. freundii* (12 mm), *S. dysenteriae* and *S. aureas* (10 mm), *Providencia* species (8 mm), *P. aeruginosa* (7 mm), while showed resistance to *S. typhi*, *P. vulgaris* and *K. pneumoniae* (Table 2).

Combined effect of plant extract with antibiotics

The combined effects of plant extract with antibiotics were different from individually used antibiotics and extract of *Adiantum capillus-veneris*. The maximum antibiotics showed synergistic effects, some were antagonistic while some of them showed neither synergistic nor antagonistic effects against test MDR bacterial strain. CXM combined with mEXT showed synergistic effects against most of the bacterial strain. Its Maximum ZI was against *P. vulgaris* (30 mm), *S. aureus* (17 mm), *P. aeruginosa* and *S. epidermidis* (16 mm), *S. dysenteriae* (15 mm), *S. typhi* and *Providencia* species (10 mm), while there were no significant effects against *K. pneumoniae* and *C. freundii*. Another antibiotic CTX showed good effect in combination with plant extract against all tested organisms. The maximum combined ZI of CTX with plant extract was observed against *S. aureus* (25 mm), followed by *E. coli* and *P. aeruginosa* (22 mm), *S. dysenteriae* and *S. epidermidis* (21mm) *Providencia* species and *C. freundii* (18 mm) *S. typhi* and *P. vulgaris* (17 mm) and *K. pneumoniae* (15 mm). Other antibiotics that were used in combination with mEXT are shown in Table 3.
DISCUSSION

The ongoing emergence of antibiotic resistance has diverted the attention of researchers towards the medicinal plants and search of new and effective drugs. In this connection, Yang et al. (2010) stated that, in recent years, human pathogenic microorganisms have developed multiple drug resistance and causing nosocomial infections. Moreover, it was suggested that plant extracts can be further developed into antibiotic medicine due to their proven antimicrobial activity.

The present study has shown that methanolic extract of roots of A. capillus-veneris in combination with antibiotics inhibited the growth of test MDR bacterial strains at high level than were tested separately. This effect was synergistic or additive for the most of the test bacterial strains. Synergistic and additive interactions are a result of a combined effect of active compounds from plant extract and antibiotics. The plant extracts contain a great number of different compounds (phenol, flavonoids, tannins, coumarins, alkaloids and terpenoids) which have an impact on growth and metabolism of microorganisms (Cowan, 1999).

In the present study 10 bacterial strains were used which were multidrug-resistant to most of the given antibiotics. The results showed that E. coli and S. typhi were the most resistant strains (60%) among all the tested bacterial strains. This result goes in line with the studies in other countries of the world where 95-100% MDR E. coli was reported (Dimah, 2012). Moreover, (67%) MDR E. coli was seen in the study of Ogunleye et al. (2008). In India 10% MDR S. typhi was reported (Nagshetty et al., 2010). Similarly, 50% MDR Providencia species evaluated in our study is almost in line with the study of Tumbarello et al. (2004) who reported 75% MDR Providencia species. We have also investigated that S. dysenteriae, S. aureus, P.aeruginosa, C. freundii, S. epidermidis and P. vulgaris are rather more MDR than what was found in other regions of the world.

Several studies on A. capillus-veneris revealed its potency against MDR bacterial strains. For example E.coli, S. epidermidis, S. dysenteriae, Providencia species, P. aeruginosa and C. freundii were sensitive to methanolic extract of root of A. capillus-veneris, while K. pneumoniae, S. typhi and P. vulgaris were resistant. This study proved to be almost in accordance with the finding of Kumar and Nagarjan (2012) and Mahboubi et al. (2012).

Our result revealed that the combination of plant extracts and antibiotics could be useful in treatment of infectious diseases and useful in fighting emerging drug resistance problems. Synergistic effect was more pronounced in case of all the strains as evident from zone of inhibition of all the strains presented in Table 3. In all cases more than 15% increase in ZI was observed which is highly significant. Several studies evaluated the interactions of plants extract and certain antimicrobial agents and their impacts on MDR bacterial strains. Tiwari et al. (2005) reported that Chloramphenicol and Tea extract in combination inhibited the growth of S. dysenteriae, tea extract showed synergistic activity with Chloramphenicol and other antibiotics like Gentamycin, Methicillin and Nalidixic acid against tested bacterial strains. Voukeng et al. (2012) indicated that plant extracts contain chemicals that can modulate the activity of antibiotics against bacteria expressing MDR phenotypes.

Comparing with all these studies, the study concluded that the extract of roots of A. capillus-veneris have bioactive compounds that enhances the antimicrobial activities of antibiotics that have shown no effect or little effect against MDR bacterial strains.

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