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SYNERGISTIC ACTIVITY OF *CALENDULA OFFICINALIS* PETAL EXTRACT WITH CEFOTAXIME ON ESBL PRODUCING *ESCHERICHIA COLI*

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ABSTRACT

Extended spectrum beta lactamase (ESBL) production in Gram negative bacteria is a potent clinical concern, especially in Escherichia coli, making it highly resistant to most of the antibiotics used. Calendula officinalis, a medicinal plant, has been traditionally used in healing skin infections. The present study evaluates the antibacterial and the synergistic effect of the methanolic Calendula officinalis extract (CMe) on ESBL producing Escherichia coli strains isolated from skin and soft tissue infections (SSTIs), along with Cefotaxime. The isolates were confirmed for ESBL production by phenotypic confirmatory disc diffusion test and E-test. The screening for antibacterial activity of CMe was performed using the disc diffusion technique. The minimum inhibitory concentration (MIC) of Cefotaxime and CMe was determined using agar dilution technique and was estimated to be in the range of 100-400 µg/ml and 5-7% (50 - 70 mg/ml) respectively, against the selected isolates. The fractional inhibitory concentration (FIC) index was calculated by the checkerboard method and synergistic interaction was observed in 47% (8/17) Escherichia coli isolates. The rutin content in the methanolic Calendula extract was found to be 1.18 % (w/w) by High-performance liquid chromatography (HPLC) analysis. These results suggest that C. officinalis methanolic petal extract can aid in controlling infections caused by ESBL producers.

KEY WORDS

Calendula officinalis, ESBL, E-test, FIC index, HPLC, MIC, Synergy

INTRODUCTION

Skin is one of the largest organs in the body, performing numerous vital functions. Skin and soft tissue infections (SSTIs) are the infections of the entire skin layer, which includes subcutaneous and muscle tissue layers, and their respective fascia structures [1]. Most of the severe secondary skin infections, which can lead to sepsis, organ failure and death, generally belong to Gram negative bacteria. A study monitoring SSTIs during a 7-year period and encompassing three continents (Europe, Latin America, and North America) showed *E.coli* to be an important causative agent, since it was the third-most prevalent isolated species, preceded solely by *S.aureus* and *Pseudomonas aeruginosa.* [2]. *E.coli* is also one of the most common Gram negative bacilli likely to be encountered in SSTIs from India [3,4]. *E. coli* isolates from SSTI therefore merits detailed study, especially taking into account the striking decline in antibiotic susceptibility of pathogenic *E. coli* strains in recent years.

The Beta lactam antibiotics are the most commonly used drug for treatment of various Skin and soft tissue infections. [5]. A variety of molecular mechanisms for resistance to broad-spectrum beta-lactams have been reported, such as mutations of penicillin-binding proteins and alterations of membrane permeability, but the most common mechanism is attributed to the presence of beta-lactamases encoded by either

chromosomes or plasmids [6]. Extended spectrum beta lactamases (ESBLs) are plasmid mediated, clavulanate susceptible enzymes that hydrolyze penicillins, expanded-spectrum cephalosporins, monobactams and are commonly inhibited by beta lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam. Due to multi-drug resistance commonly seen in ESBL producers and their rapid dissemination across the globe, clinical management of severe skin infections caused by them, has become very complicated [7].

Plant products have been traditionally used in the history for therapeutic purposes. The use of traditional medicine to treat infections is equally emphasized nowadays by the World Health Organization (WHO) [8]. *Calendula officinalis L. (*Pot Marigold), a member of the *Asteraceae family,* encompass multiple pharmacological activities due to the presence of a diverse range of biologically active substances like carotenoids, flavonoids, terpenoids, coumarins, amino acids, carbohydrates and lipids [9]. *C.officinalis* extract exhibits anti-inflammatory, antioxidant, anticancer, wound healing, antibacterial and antifungal activity [10,11]. Since plant extracts provide unmatched availability of chemical diversity, they can also be combined with antibiotics to have an effective therapy, particularly against resistant pathogens [12].

Studies analyzing the combined antimicrobial activity of antibiotics and plant extracts can suggest if the latter can aid in reducing the dosages of antibiotics in treating infections. Thus, the aim of the present study was to evaluate the antimicrobial activity of *C. officinalis* methanolic petal extracts on ESBL producing *E.coli*, isolated from SSTIs and to investigate the synergistic effect of the extract and cefotaxime.

MATERIALS AND METHODS

The present study was conducted in a tertiary care hospital and was approved by the local ethics committee of the institution.

Bacterial strains

A total of 59 non-duplicate *Escherichia coli* strains isolated from skin and soft tissue infections of patients from our tertiary care hospital were selected for the study after the identification by standard laboratory methods.

Antimicrobial susceptibility Test (AST)

The antimicrobial susceptibility was determined by Kirby-Bauer disk diffusion method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines using commercially available antimicrobial discs (HiMedia, Mumbai, India). The following antibiotics were used- Ampicillin (10 µg), Amikacin (10 µg), Ciprofloxacin (5 µg), Gentamicin (10 µg), Amoxyclav (30 µg), Ceftazidime (30 µg), Cefoxitin (30 µg), Imipenem (10 µg), Piperacillin-Tazobactam (100/10).

All the isolates which were resistant to Ceftazidime as per the CLSI susceptible breakpoints were further screened for confirmation of ESBL production [13].

Phenotypic Confirmatory Disc diffusion test (PCDDT)

Ceftazidime (30 µg) - Ceftazidime/Clavulanic acid (30/10, HiMedia, Mumbai, India) were used for ESBL detection. If there was ≥5 mm increase in the inhibition zone diameter of Ceftazidime/Clavulanic acid versus Ceftazidime alone, the isolate was considered as an ESBL producer [13].

E-test

ESBL detection strips (HiMedia, Mumbai, India) are drug-impregnated strips in which upper half contains a concentration gradient of three antibiotics; Ceftazidime, Cefotaxime and Cefepime plus Clavulanic and lower half contains of Ceftazidime, Cefotaxime, and Cefepime in a concentration gradient in a reverse direction. The strain was reported and confirmed as ESBL producer as per the application sheet supplied by the manufacturer [14].

A standard reference strain of *Escherichia coli* (ATCC 25922), susceptible to all antimicrobial drugs tested, and ESBL positive control strain *Klebsiella pneumoniae* ATCC 700603 were used as a quality control for antimicrobial susceptibility test, phenotypic confirmatory disc diffusion test and the E-test. These phenotypically confirmed ESBL producers were further utilized in the study as test strains.

Preparation of methanolic *Calendula* extract

Fresh *Calendula officinalis* flowers were purchased from local market. The petals were separated, washed and dried in shade. Ten grams of dried and grounded petals were transferred into a flask containing 150 ml of the solvent methanol. Extract of *Calendula officinalis* petals was obtained by maceration in methanol for 1 week. The macerate was filtered, and the solvent was evaporated. The dried powder obtained after solvent evaporation was dissolved in 50% Dimethyl sulfoxide (DMSO) to obtain a concentration of 500 mg/ml. The prepared extract was stored at 4°C for further use in the study after sterility testing of the extract [10,15].

High-performance liquid chromatography analysis (HPLC)

The High-performance liquid chromatography system consisted of a Shimadzu LC-2010 CHT model (Shimadzu, Tokyo, Japan), with a C18- packed with silanised octadecylsilyl silica gel, 0.5µ size, 250 x 4.6 mm (Merck) stainless steel column. 20 µl of the prepared sample was injected into the HPLC column for the analysis. The elution was carried out at a flow rate of 1.2 ml/min using the gradient proportion of 100% acetonitrile and buffer as the mobile phase. For preparation of buffer, dissolve 0.136 g of anhydrous potassium dihydrogen orthophosphate (KH2PO4) in 900 ml of HPLC grade water and add 0.5ml of Orthophosphoric acid. Make up the total volume to 1000 ml with HPLC grade water, filter through 0.45µm membrane and degas in a sonicator for 3 minutes. (Natural Remedies, Bangalore, India).

Antibacterial activity of *Calendula*

Antibacterial activity of *Calendula officinalis* methanolic petal extract (CMe) was carried out by disc diffusion method using Mueller Hinton agar [15]. For the positive control, a disc of Cefotaxime/clavulanic acid (30/10 µg) and for negative control, a disc impregnated with Dimethyl sulfoxide (DMSO), were placed on the inoculated Mueller-Hinton agar [16].

Minimum Inhibitory Concentration (MIC)

The MIC of CMe and Cefotaxime was determined by the Agar dilution method. For MIC of CMe, dilutions were prepared by mixing CMe with sterile Mueller Hinton Agar to get final concentrations ranging between 2.5 mg/ml (0.25%) – 70 mg/ml (7%). For MIC of Cefotaxime, dilutions were prepared by mixing Cefotaxime with sterile Mueller-Hinton Agar to get final concentrations ranging from 25 µg/ml – 500 µg/ml. A plate of Mueller-Hinton Agar with DMSO served as a control. The MIC was recorded as the lowest concentration of CMe and Cefotaxime at which visible bacterial growth was completely inhibited [17].

Determination of Synergistic activity by Checkerboard assay using Agar dilution method

Checker board assay by Agar dilution method was used to determine the synergistic interaction of various concentrations of Cefotaxime and CMe. Two-fold serial dilution agar plates were prepared by mixing CMe in the range of 0.25%- 2% along with 12.5-200 µg/ml of Cefotaxime. The results obtained were used to calculate Fractional inhibitory concentration (FIC) indices. Visible growth after 24 h of incubation at 37°C was checked and the MIC in combination for Cefotaxime and CMe were determined for all strains. The FIC index (∑FIC) was calculated as follows:

∑FIC= FIC A+ FIC B, where

FIC A = MIC of Cefotaxime in combination/ MIC of Cefotaxime alone

FIC B = MIC of CMe in combination/ MIC OF CMe alone

A minimum FIC index of ≤0.5 indicates synergy, while a FIC index >2 indicates antagonism. If the minimum FIC index was > 0.5 and ≤ 1, the effect of the combination was classified as additive. If the minimum FIC index was >1 and ≤2, the effect of the combination was classified as indifference [17,18].

RESULTS

The AST studies of 59 *E.coli* strains revealed that carbapenems and aminoglycosides were the most effective antibiotics against the ESBL producers. 81.16% of the isolates were resistant to Ampicillin. No isolates were resistant to carbapenems – Imipenem and Meropenem, making it the most effective antibiotic group against *E.coli*. All the isolates exhibited a higher resistance rate towards quinolones in comparison with carbapenems and aminoglycosides (figure 1). Resistance to Ceftazidime was indicative of ESBL production, which was further confirmed by PCDDT and E-test. Amongst the 59 E.coli strains isolated from skin and soft tissue infections; 28.81% (17/59) isolates were found to be ESBL producers, and these were used as test strains further in the study.

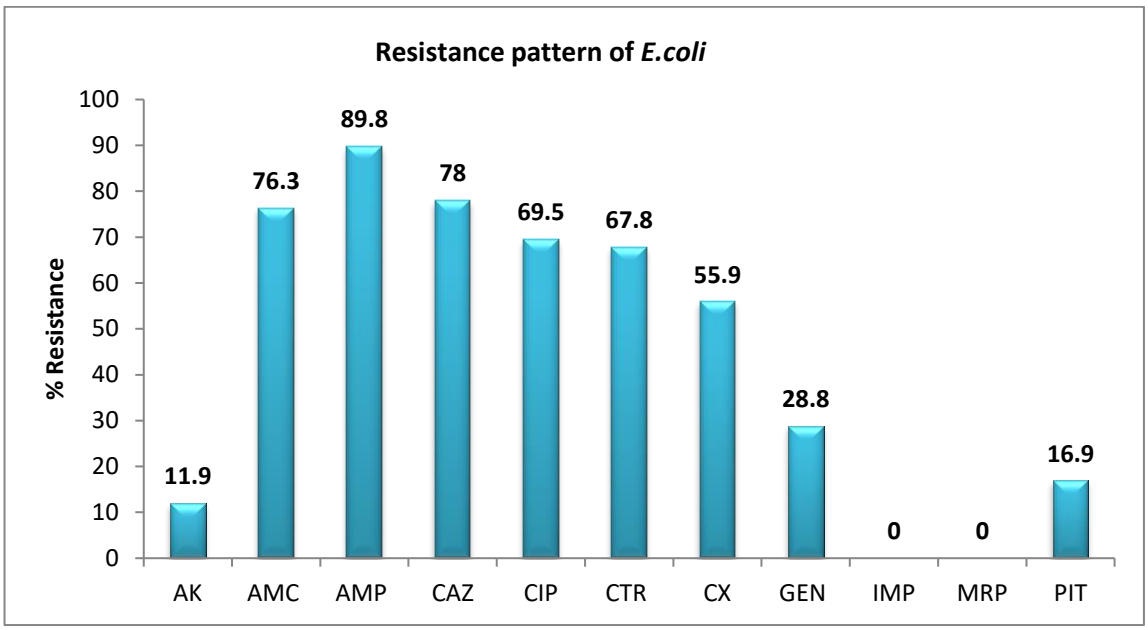

Figure 1- Rate of antibiotic resistance amongst *E.coli* strain (n=59)

AK- Amikacin, AMC-Amoxyclav, AMP- Ampicillin, CAZ- Ceftazidime, CIP-Ciprofloxacin, CTR – Ceftriaxone, CX- Cefoxitin, GEN-Gentamicin, IMP-Imipenem, MRP – Meropenem, PIT-Piperacillin-Tazobactam

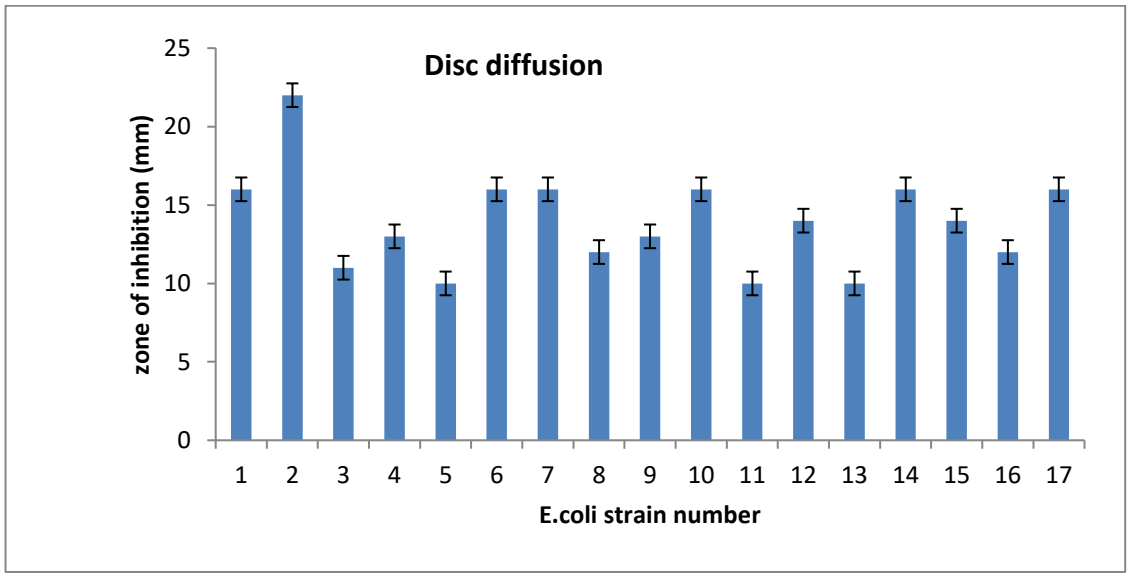

Figure 2: Mean inhibition zone sizes of CMe against ESBL producers by disc diffusion method. (Data are expressed as mean ± SEM, n=3) (Error bars with Standard error)

Amongst the two beta lactamase inhibitors, Piperacillin – Tazobactam combination was more effective than Amoxyclav against all the isolates. All the ESBL producers were also found to be multidrug-resistant i.e. they were resistant to 3 or more than 3 groups of antibiotics.

The estimation of active ingredient, Rutin was carried out by HPLC analysis and was found to be 1.18% (w/w). Primary screening for in-vitro antibacterial activity of CMe was carried out by disc diffusion method. The extract showed activity against all the 17 isolates producing ESBL from SSTIs, the average zone of inhibition (ZOI) was in the range of 10–22 mm with a mean of 13.94±3.11 mm against the test strains. No zone of inhibition was seen for DMSO used as control. (Figure 2).

The MIC of CMe extract and Cefotaxime was determined by the Agar dilution method. The MIC of CMe extract against all the 17 test strains was found to be in the range of 5 - 7% (50 - 70 mg/ml), with a mean of 6.53%

(65.3mg/ml), as revealed in Table 1. The MIC of Cefotaxime was obtained in the range 100-400µg/ml, with a mean of 300µg/ml (Table 2). Growth of all isolates was observed on DMSO control plates.

The antimicrobial synergistic activity of Cefotaxime and CMe was evaluated in terms of FIC index obtained from Checkerboard assay by Agar dilution method. In the current study, 47% (8/17) isolates exhibited synergistic association, whereas 41% (7/17) isolates exhibited additive association and 11.76% (2/17) isolates exhibited indifference. No antagonistic activity was observed against any of the isolates (Table 3).

Table 1: MIC of CMe against *E.coli* strains producing ESBL(n=17)

Concentration of CMe (mg/ml)	40	50	60	70	80
No. of strains inhibited	-	2	4	11	-
Percentage of strains inhibited	-	11.8	23.5	64.7	-

Table 2: MIC of Cefotaxime against *E.coli* strains producing beta-lactamase (n=17)

Concentration of Cefotaxime (µg/ml)	25	50	100	200	300	400	500
No. of strains inhibited	-	-	2	3	5	7	-
Percentage of strains inhibited	-	-	11.8	17.6	29.4	41.2	-

Table 3: Combined effect of Cefotaxime and CMe against *E.coli* strains ESBL (n=17).

Type of Association	*E.coli* strains
No. of strains exhibiting Synergy (FIC ≤ 0.5)	8
No. of strains exhibiting Additive (0.5 <FIC ≤ 1)	7
No. of strains exhibiting Indifference (1< FIC ≤ 2)	2
No. of strains exhibiting Antagonism (FIC >2)	-

DISCUSSION

Gram-negative bacilli producing ESBL generally result in chemotherapeutic failure due to their multi-drug resistance nature. Hence, making it imperative to study their occurrence and antibiotic resistance pattern. The occurrence of ESBL production in *E.coli* isolates, in the current study was similar to a study conducted in Mumbai, by Shinde et al [19]. In this study, carbapenems were the most effective antibiotics for strains which produced ESBL, followed by aminoglycosides, Amikacin and Gentamicin, which is well corroborated with the study of Shinde et al [19].

Antibacterial activity of CMe was studied using disc diffusion test and the MIC was determined. The extract inhibited all the strains indicating an effective antibacterial activity against *E.coli* isolates from SSTIs producing ESBL. Our results are in agreement with the findings of previous studies against non ESBL producers [16,20]. The methanolic extract of the flower is a rich source of polyphenols and flavonoids [21]. The content of rutin found in methanolic extract in the present study was in accordance with previous study of Martins et al. [22].

With the continuous and unmonitored use of antibiotics, resistant microorganisms have emerged as a great threat. There are various mechanisms due to which microbes usually do not become resistant to herbs, like they may act synergistically with antibiotics to kill microbes, herbs may inactivate or destroy enzymes produced by bacteria which degrade antibiotics, or they may inhibit the action of efflux pumps making bacteria unable to remove antibiotics from their body, etc. [23]. In-vitro studies confirming a synergistic interaction between a plant extract and an antibiotic could be a possible indication for successful combination antimicrobial therapy. There is paucity of data regarding synergistic studies of *Calendula officinalis*. To the best of our knowledge, there is no documented study showing synergistic interaction between Cefotaxime and CMe against ESBL producing *E.coli* isolates from SSTIs. However, synergistic association between Cefotaxime and CMe against *Klebsiella* isolates from SSTIs has been reported by Shah and Wiliamson in a previous study [15].

In the present study, the combination of CMe and Cefotaxime exhibited at least a fourfold reduction in their respective MICs in 47% of the test isolates and a twofold reduction in 41% of the test isolates. The antagonistic effect was not observed in any of the isolates, indicative of a positive association between the

two antimicrobials. If a combination of a plant extract and an antibiotic can cause the reversal of the antibiotic resistance or lower its prescribed amount in therapy, then this combination could potentially improve the outcome for patients with severe infections.

CONCLUSION

The above study suggests that *C. officinalis* methanolic petal extract can prove a great prospective as an antimicrobial compound against ESBL producing *E.coli* isolates from SSTIs. Natural drugs from the plants are gaining popularity because of several advantages such as often having fewer side-effects, better patient tolerance, being relatively less expensive and acceptable due to a long history of use. Clinical trials are required to provide more conclusive proof of its efficacy.

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CONFLICT OF INTEREST STATEMENT:

The authors declare that they have no competing interests with whomsoever.

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