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Enhancement of Activity of Antibiotics by Plant Extracts

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Bio-enhancers are molecules without drug activity that augment the bioavailability of drugs in combination therapy. The aim of this study was to assess the bio-enhancing activity of bioactive fractions obtained from plant extracts on commonly used antibiotics. The effect of ethyl acetate extract of *Cleome rutidosperma* leaves on the antibacterial activities of amoxycillin and cloxacillin against *Staphylococcus aureus* NCIM 2614 and *Pseudomonas aeruginosa* NCIM 2653, respectively, was studied. Moreover, the antimicrobial activities of extracts of different parts of *C. rutidosperma* were evaluated against *S. aureus*, *P. aeruginosa*, *Klebsiella pneumoniae*, *Salmonella typhi, Candida albicans,* and *Aspergillus niger*. Hot ethyl acetate leaves extract (HEALE) and antibiotics were tested individually and in combination. HEALE showed maximum activity against test organisms; the minimum inhibitory concentration (MIC) of HEALE was 600 μg/mL. The test organisms were found to be multidrug-resistant (MDR) strains. However, HEALE or HEALE in combination with antibiotics did not lead to the development of resistance. Phytochemical and chromatographic analyses of HEALE revealed the presence of antimicrobial and bio-enhancing constituents. Such findings herald the possibility of a potentially active bio-enhancer of plant origin.

Key words: *Cleome rutidosperma*, Amoxicillin, Cloxacillin, HEALE, Bio-enhancing agent.

Infectious diseases caused by bacteria and fungi affect millions worldwide, and antibiotics are used against these infectious agents. Antibiotics are bactericidal or bacteriostatic, and their bioactivities have transformed the ability of humanity to treat many infectious diseases. The annual global human consumption of antibiotics is estimated to be above 250 million doses. However, the widespread use of antibiotics promotes the spread of antibiotic resistance leading to multidrug resistance (MDR)[1,2]. The prevalence of unutilized drug in the body acts as a selection pressure facilitating the emergence of drug resistance and resulting in the failure of antibiotics[3,2].

Only a miniscule percentage of the concentration of antibiotic in a given dosage reaches the target microbes. This is attributed to the following reasons: (1) less absorption in the gut membrane when taken orally (2) restricted uptake by target microbe or (3) operation of efflux pump leading to indiscriminate extrusion of the therapeutic molecules[2]. Thus,. However, the unutilized drug persists as a load in the body and environment[4].

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Use of bio-enhancers is an efficient method to combat with this problem because bio-enhancers in combination with antibiotics can cause almost 200-fold increase in activity[5,2].

Bio-enhancers are molecules that do not possess drug activity but promote and augment biological activity or bioavailability or uptake of drugs in combination therapy. Generally, molecules like glycosides, flavonoids, coumarins, and essential oils are responsible for bio-enhancing properties[1,2,3,5,6]. Some examples of bio-enhancers are plant extracts, urine distillates, or some Ayurvedic preparations[2,5]. Bio-enhancers do not exert any selection pressure and therefore, do not lead to emergence of resistant mutants. They can, in fact, reduce drug dosage and minimize their ill effects. Thus, they delay the process of resistance development, leading to an increase in the lifespan of novel and existing antibiotics.

Bio-enhancers should be non-toxic, effective at low concentrations, synergistic, and easy to formulate, and, most importantly, should be able to enhance uptake/absorption and activity of the drug molecules. The judicious use of strategic concentrations of drugs with specific bio-enhancers will improve drug availability for effectively controlling infectious organisms.

The aim of this study was to assess the bio-enhancing activity of bioactive fractions obtained from plant extracts on commonly used antibiotics. These plant extracts can be highly inhibitory to selected pathogens, and may provide better alternatives and/or supplements to the conventional antibacterial or antifungal agents.

MATERIAL AND METHODS

Preparation of plant extracts

Plant parts, like leaf, stem, roots, fruits and flowers collected from the different places were washed, shade dried, and pulverized to obtain a coarse powder[7], which was filtered through a 1-mm sieve. Extracts were prepared by Soxhlet extraction by using solvents like ethanol, methanol, and ethyl acetate[8].

Phytochemical analysis

Chemical tests specific for glycosides, tannins, saponins, flavonoids, and alkaloids were performed on hot extracts, by using previously described methods[9,10,11].

Activity-guided fractionation of HEALE

Activity-guided fractionation of the extract was performed using petroleum ether, chloroform, benzene, ethyl acetate, acetone, ethanol, methanol, and water to get the final fraction, which was further used for bioassays.

High-performance thin layer chromatography (HPTLC) analysis

Samples were spotted on 20X 10 Silica F-654 thin layer chromatography (TLC) plates by using CAMAG Linomat 5 TLC sample applicator and visualizer (Camag, Switzerland). The plates were immersed in a suitable mobile phase and then visualized under 366 nm, 254 nm, and white light. Derivatization was performed with different agents; the plates were dried under hot air, and observed under 366 nm, 254 nm, and white light.

Different mobile phases and derivatizing reagents were used to identify alkaloids, coumarins, flavonoids, glycosides, saponins, tannins, and essential oils.

Antimicrobial assays of plant extracts

Agar well method: A single colony of bacterial test strains was grown overnight in Mueller–Hinton (MH) broth at 200 rpm at 35°C. For fungal cultures, spore suspensions in sterile water containing Tween-80 were used as inoculums. The bacterial cultures were diluted with 0.85% NaCl and surface spread on MH agar plates; the similar protocol was followed for fungal cutures with Sabouraud agar. The wells contained (1) different extracts of plant and/or its different fractions alone or (2) antibiotics, or (3) combinations of antibiotics and plant extracts, as well as controls. The zones of inhibition were measured after incubation.

Tube dilution method: The minimum inhibitory concentrations (MICs) of (1) different extracts of plant and/or its different fractions alone or (2) antibiotics, or (3) combinations of antibiotics and plant extracts were determined. HEALE was concentrated in a vacuum evaporator from 100 mL to 5 mL and then dry concentrated by evaporating the solvent; the residue was used for MIC. MIC was determined by standard broth dilution method, in accordance with NCCLS guidelines in MH broth supplemented with serial concentration of HEALE, ranging from 200 to 1000 µg/mL. After incubation, MIC values were determined. Tubes free from HEALE were included as controls.

Identification of MDR test cultures

Cultures (optical density ($O.D._{530\ nm}$) = 0.1) were surface spread on sterile MH agar plates, and octodiscs (HiMedia, India) were placed on the agar surface. The zones of inhibition were measured after incubation. If the culture was resistant to more than 3 antibiotics, then the culture was termed as an MDR culture.

Growth curve studies

The test cultures were incubated under different experimental systems, in nutrient medium, under shaker conditions. The systems were as follows: nutrient broth (NB) + culture + antibiotic, NB + culture + antibiotic + extract, NB + culture + extract, and NB + culture + solvent. Controls and blanks were also set up. After incubation, $O.D._{530\ nm}$ was measured, and growth curves were plotted.

RESULTS AND DISCUSSION

Phytochemical analysis

The results of the chemical tests are listed in Table 1.

The tests revealed the presence of medicinally active constituents like alkaloids, tannins, flavonoids, saponins, essential oils, phlobatannins, coumarins, and glycosides in the leaves extract; alkaloids, tannins, and essential oils, in stem extract; and alkaloids, essential oils, and flavonoids, in fruits extract. Anthraquinones and starch were absent, and essential oils were present in all extracts.

HPTLC analysis

The results have been tabulated in Table 2. Alkaloids, coumarins, flavonoids, glycosides, saponins, tannins, and essential oils were present in HEALE.

Antimicrobial assays of plant extracts

Although all extracts showed inhibitory effects (diameter of zones of inhibition: 10–24 mm/ 60 µL of extract) against all test cultures, HEALE was highly active against *P. aeruginosa* and *S. aureus*. HEALE showed good activity against almost all the test cultures.

Candida albicans showed resistance to most of the extracts but complete inhibition was observed only with HEALE. Similar inhibition pattern was obtained in the case of *Aspergillus niger*. The largest zones of inhibition were observed with ethyl acetate and ethanol extracts of leaves against all test cultures.

Enhancement of antibiotics against test cultures

The antibiotic activity was enhanced by halving the concentrations of extracts and antibiotics. Agar well method showed that halved concentration of antibiotics (5 µg/mL) with ethyl

Table 1. Results of phytochemical analysis of different extracts of *Cleome rutidosperma*

Extracts: Secondary metabolites	LEA	SEA	FEA	LEA	SEA	FEA	LCE	SCE	LME	LCEA	SCEA	FCEA
Glycosides	-	-	-	+	-	-	-	-	-	+	-	-
Tannins	+	+	-	+	+	-	-	-	-	+	-	-
Saponins	+	-	-	-	-	-	-	-	-	+	-	-
Flavonoids	+	-	-	+	-	-	+	-	+	+	-	+
Alkaloids	+	+	-	+	-	-	+	+	-	+	-	+
Anthraquinones	-	-	-	-	-	-	-	-	-	-	-	-
Coumarins	+	-	-	-	-	-	+	-	-	-	-	-
Starch	-	-	-	-	-	-	-	-	-	-	-	-
Essential oils	+	+	+	+	+	+	+	+	+	+	+	+
Phlobatannins	-	-	-	-	-	-	-	-	-	+	-	-

Legends:
Key: + = Positive - = Negative
LE, Leaves ethanol extract; SE, Stem ethanol extract; FE, Fruit ethanol extract; LEA, Leaves ethyl acetate extract; SEA, Stem ethyl acetate extract; FEA, Fruit ethyl acetate extract; LCE, Leaves cold ethanol extract; SCE, Stem cold ethanol extract; FCE, Fruit cold ethanol extract; LME, Leaves cold methanol extract; SCM, Stem cold methanol extract; FCM, Fruit cold methanol extract; LCAE, Leaves cold ethyl acetate extract; SCEA, Stem cold ethyl acetate extract; and FCEA, Fruit cold ethyl acetate extract.

Table 2. Results of HPTLC analysis to identify the important constituents of HEALE

Phytochemical constituents	Mobile phase	Derivatizing reagent	Results
Alkaloids	Toluene : ethyl acetate : diethylamine (7:2:1)	Dragendorff's reagent	Positive
Coumarins	Chloroform (10 mL)	Vanillin-sulphuric acid reagent	Positive
Flavonoids	Ethyl acetate : formic acid : glacial acetic acid : water (10:0.5:0.5:1.3)	Anisaldehyde sulphuric acid reagent	Positive
Glycosides	Ethyl acetate : methanol : water (10:1.4:1)	Alcoholic potassium hydroxide	Positive
Saponins	Chloroform:acetic acid : methanol : water (6.4:3.2:1.2:0.8)	Anisaldehyde sulphuric acid reagent	Positive
Tannins	Toluene : ethyl acetate : formic acid (6:4:0.3)	5% ferric chloride solution	Positive
Essential oils	Toluene : ethyl acetate (9.3:0.7)	Vanillin-sulphuric acid reagent	Positive

acetate extracts and doubled concentration of antibiotics (10 µg/mL) exhibited identical zones of inhibition. This clearly indicates the enhancement of antibiotic activity by plant extracts. The MIC of HEALE for *S. aureus* and *P. aeruginosa* was found to be 600 mg/mL.

Identification of MDR test cultures

S. aureus was found to be resistant to ampicillin, nalidixic acid, cephelexin, and gentamicin; P. aeruginosa, to ampicillin, nalidixic acid, augmentin, trimethoprim, cephelexin, and nitrofurantoin; and *Salmonella typhi* and *Klebsiella pneumoniae*, only to 2 antibiotics. This proves that S. aureus and P. aeruginosa strains used in the study were MDR strains.

Growth curve studies

HEALE (1 µL/mL) in combination with antibiotic (10 mg/mL) showed very good inhibitory effect against *S. aureus*. Moreover, HEALE (6 µL/mL) showed similar inhibitory effect against *S. aureus*. On the other hand, HEALE (3 µL/mL) in combination with antibiotic (10 µg/mL) showed very good inhibitory effect against *P. aeruginosa*. HEALE (6 µL/mL) showed similar inhibitory effect against *P. aeruginosa*. The cultures in the control and solvent-containing systems showed normal growth with all stages.

In both cases, cultures in the control and solvent-containing systems showed normal growth with all stages, i.e., lag phase, log phase, stationary phase, and decline phase. The system containing amoxicillin (10 µg/mL) showed a slightly longer lag phase followed by a continuous increase in optical density, indicating that amoxicillin has no effect on the cultures.

The cultures grown in presence of amoxicillin (10 µg/mL), extract, and combination of both were further used for the sensitivity test against amoxicillin (10 µg/ml), extract, and their combination.

It was noted that *S. aureus* developed resistance to amoxicillin in few hours, indicated by colonies growing in the zone of inhibition, but not to HEALE. This proved that HEALE contains compounds, which enhances the activity of antibiotics and limits the resistance of the pathogen.

CONCLUSION

Indiscriminate use of antibiotics generates MDR in many clinically significant bacteria. In this study, we tried to identify compounds that could deal with this drug resistance.

Qualitative phytochemical and HPTLC analyses of HEALE showed the presence of important medicinal constituents like glycosides, flavonoids, coumarins, and other essential oils.

HEALE showed antimicrobial activity against the following test organisms: *P. aeruginosa, S. aureus, S. typhi, K. pneumoniae, C. albicans*, and *A. niger*. Moreover, the HEALE-antibiotic combination significantly enhanced antibiotic activity.

The growth curve studies of *S. aureus* and *P. aeruginosa* showed that HEALE and HEALE-antibiotic (1:1) combination is cidal against *S. aureus* and *P. aeruginosa*, and that these organisms acquire antibiotic resistance after 4 h, thus indicating the bio-enhancing activity of HEALE.

In the susceptibility studies, the cultures incubated for 4 h with 5 systems were tested for their susceptibility to the extract and antibiotics. HEALE resulted in an increase in the inhibition of test organisms, while antibiotics resulted in the development of resistance in test organisms. Our findings indicate the bio-enhancing activity of HEALE. Thus, *C. rutidosperma* leaves extract has high antibiotic-activity enhancing property.

Further studies involving the isolation, identification, characterization, and elucidation of the structures of the bioactive constituents of HEALE are required. Toxicological studies of HEALE will prove useful in promoting the commercial utilization of this bio-enhancer.

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