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Synergistic Effect of *Aloe vera* and *Curcuma longa* Extracts in the Inhibition of Drug-Resistant *E. coli*

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The excessive and inappropriate application of antibiotics has led to the emergence of drug resistant bacteria. The present study was focused on isolation of bacterial strains which have gained resistance to cefixime, a third generation drug. The isolate VITSSA4 was isolated from sewage treatment plant and was found to show resistance to cefixime at a concentration of 10,000 mgL^{-1}. Further effort was made to develop an effective drug, by using combination of medicinal plant extract of *Aloe vera* and *Curcuma longa*. It was evident that the methanolic extract of *Aloe vera* and *Curcuma longa* was effective in inhibiting the growth of the isolate VITSSA4. The phenotypic and genotypic characterization revealed that the isolate VITSSA4 was showing 99.9% similarity to *E. coli*. Thus antibacterial activity of the drugs can be enhanced by *Aloe vera* and *Curcuma longa* extract.

Key words: Cefixime, Multi drug resistance, Synergism, *Aloe vera, Curcuma longa.*

Emergence of drug resistant bacteria is one of the world's most pressing health problems during the 21st century. Increased use of antibiotics has led to the microbial resistance towards the antibiotics. Most of the microbial strains have already gained resistance against the first and second generation drugs[1] and thus third generation drugs are often used to treat drug resistant bacteria. The presence of multidrug resistant bacteria has been wide spread[2]. There is a rapid increase in the spread of drug resistance bacteria due to the transfer of the resistance genes through horizontal gene transfer, conjugation, transduction and transformation. Most of the genes responsible for the drug resistance are located on the transmissible plasmids[3].

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Usage of high dosage antibiotics like Cephalosporin etc., to treat MDR pathogens causes subsequent side effects in the host. In order to reduce the side effects of commercial antibiotics, there is a need for an alternative method to suppress to the growth of antibiotic resistant bacteria by using naturally available antibiotic compounds[4]. In present scenario people are interested in using bacteria and this has raised to the need of an alternative method of suppressing the growth of these bacteria, without causing any damage. The use of naturally available antimicrobial compounds to increases the antibiotic susceptibility of resistant bacteria[4] is preferred. Nowadays people are interested in using natural antibacterial and antimicrobial compounds in spices and various kinds of herbs[5].

Plants like *Aloe vera* and *Curcuma longa* shown a good antibacterial and antimicrobial activity. *Aloe vera* is used as a medicinal plant since the start of the civilization. It reduces inflammation and pain and its sap is used in healing

wounds. Various phyto-compound analysis of *Aloe vera* has shown its antimicrobial activity[6].

Aloe vera contains various vitamins, saponins, minerals, sugars, phenolic compounds. Aloe plant also consists of anthraquinones and its derivative compounds have an analgesic effect and these compounds are verified as antimicrobial agents[7].

Curcuma longa or turmeric also shows antibacterial activity. Curcuminoides is the major component of turmeric other than that sesquiterpenes has also been isolated from rhizome of *Curcuma longa*. Antibiotics acts on bacteria either by blocking peptidoglycan or protein synthesis of the cell wall. Turmeric has shown antibacterial activity against many bacteria[8]. By using both the antibiotics and the plant extract a synergistic effect will be produced which delay the emergency of bacteria resistance. This alternate approach has been evaluated by taking various plant extracts and by well diffusion method and by minimum inhibitory concentration test[9].

Our study was encompassed on the usage of third generation drug in combination with *Aloe vera* and *Curcuma longa* extracts at various concentrations to assess the antibacterial capability, since the extracts are natural in origin the risk of side effects can be lowered, in comparison with the commercially available drugs.

MATERIALS AND METHOD

Sample Collection

Untreated sewage sample was collected from VIT sewage treatment plant, VIT University, Vellore, TN, India, using a sterile screw capped bottles and processed immediately.

Collection of medicinal plants

For the study, a wild strains of *Aloe was obtained* from Brahmapuram, Vellore, TN, India and *Curcuma longa* (turmeric) from Vellore, TN, India.

Isolation of antibiotic resistant bacteria from untreated sewage water

The raw sewage sample was serially diluted and 0.1ml of the sample was spread onto SSA plates supplemented with antibiotic Cefixime (1000 mgL^{-1}) and incubated at 30°C for 48h under aerobic condition. The morphologically distinct colonies were selected, purified and maintained in glycerol stock[10].

Phenotypic Characterization

Isolates were further characterized morphologically by gram's staining, capsule staining, hanging drop and biochemical tests like an idol, methyl red, Voges Proskauer, citrate utilization test, TSI, catalase, oxidase test[11-12].

Determination of MIC range of the isolates

For the determination of minimal inhibitory concentration of the drug, 2% of 0.5 O.D seed cultures was inoculated in LB broth supplemented with different concentrations of Cefixime, ranging from 1000 – 12000 mgL^{-1}, incubated at 120rpm for 24h at 30°C. The plating was performed from all concentrations and effective strain was identified[13].

Growth Kinetics

Growth kinetics was performed using 50ml of LB broth with 2% of the seed culture. Un-inoculated LB broth served as control. The optical density was recorded for every 30 minutes of time interval at 600nm and plotted on a graph[14].

Solvent extraction from the medicinal plants

Various solvents like Methanol [polar] and Chloroform [Non polar]) were taken in Erlenmeyer flasks and 150gm of *Aloe vera* extract was added to them and left on shaker operated at 250rpm for 24h at RT. Solvents were collected and concentrated using hot water baths which were pre-set to the boiling point of the solvents. Freshly collected *Curcuma longa*, was powdered and same procedure was employed to get the extract[15-16].

Conjugation method

In vitro conjugation was performed to check the exchange of transmissible plasmids between the Donor (Isolate) and the receptor (*Lactobacillus*)

Equal amounts of Donor and receptor culture were taken and mixed on Nutrient agar plate containing the nitrocellulose paper. The inoculum was spread evenly all over the nitrocellulose paper by using a sterile glass rod. The plates were incubated at 37°C for 24h. The nitrocellulose sheet would help increase the conjugation efficiency. After the incubation individual colony was picked and streaked on to MRS media containing 1000 mgL^{-1} and 2000 mgL^{-1} antibiotic concentration[17].

Combination therapy

Combination therapy was assayed using cefixime along with the medicinal plant extracts of *Aloe vera and Curcuma longer* used in different

concentrations (i.e. [Antibiotic + *Aloe vera* extract], [*Aloe extract* + Turmeric extract], [Turmeric extract + Antibiotic]) to check their antibacterial activity.

A standard protocol of the well diffusion method was followed to check the antibacterial activity. MH agar was prepared and streaked with a sterile swab dipped in overnight culture in 3 different directions to obtain a lawn culture. Wells were made using sterile cork borer. The wells were prepared in such a way that it could hold 0.005ml of the sample. Individual wells were loaded with Solvent alone, stock extract, extract + antibiotic, two different extracts. Same procedure was employed for polar extracts and non-polar extracts.

Molecular characterization using 16S rRNA sequencing

Bacterial strains were characterized using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') DNA was extracted from cells and the 16S rRNA sequence was determined by the fluorescent dye terminator method using the sequencing kit (ABI Prism Big dye terminator cycle sequencing ready reaction kit v.3.1). Products were run on an ABI13730XL capillary DNA sequencer (ABI Prism 310 genetic Analyzer, Tokyo, Japan). The aligned sequences were computed using ClustalW software and sequence homologies were determined using BLASTn search to create an evolutionary distance matrix[18].

RESULTS

Isolation of antibiotic resistant bacteria from untreated sewage water

Upon incubation different colonies were observed on the SSA plate supplemented with cefixime. Six different isolates were purified and named as VITSSA1, VITSS2, VITSSA3, VITSSA4, VITSSA5 and VITSSA6. All the isolates were maintained in glycerol stock.

Morphological and Biochemical characterization

All the isolates were found to be Gram negative and motile (Table 1). The biochemical results of VITSSA1, VITSSA2, VITSSA5, and VITSSA6 were found to be *Salmonella* VITSSA3 and VITSSA4 were found to be *E. coli*[19]

MIC

MIC was performed for all the isolates and it was found that VITSSA03 and VITSSA04 were resistant to cefixime even at 10,000 mgL^{-1} concentrations. Previous report on the resistance of *E. coli* to drugs ampicillin, erythromycin, penicillin and bacitracin [4] but resistance to third generation drugs has not been reported. Hence the present study is the first report on bacteria showing resistance at the high concentration (Fig. 1).

Growth Kinetics

Growth kinetics was performed for both the effective isolates and it was found that both VITSSA03 and VITSSA04 were showing similar patterns, i.e, they reached log phase in 2h and stationary after 5h respectively (Fig. 2).

Table 1. Morphological and Biochemical Characterization

Isolates	Gram's staining	Hanging drop	Indole	Methyl red	VP test	Citrate	TSA
VITSSA01	-ve, rods	Motile	-	+	-	-	k/a
VITSSA02	-ve, rods	Motile	-	+	-	-	k/a
VITSSA03	-ve, rods	Motile	+	+	-	-	a/a
VITSSA04	-ve, rods	Motile	+	+	-	-	a/a
VITSSA05	-ve, rods	Motile	-	+	-	-	k/a
VITSSA06	-ve, rods	Motile	-	-	+	+	k/a

Table 2. Showing antimicrobial activity of Aloe vera extract along

Compound	Methanol	*Aloe vera* Extract in Methanol	Chloroform	*Aloe vera* Extract in chloroform	Antibiotic
Zone of inhibition (in mm)	0.5 mm	8 mm	1 mm	11 mm	No zone

Molecular characterization by 16S rRNA sequencing

Considering the MIC and growth kinetics results, the isolate VITSSA4 was found to be effective strain, thus it was sent for 16S rRNA sequencing as SSA1. The SSA1 was showing 99% similarity with *Escherichia/Shigella dysenteriae*. The sequence was further submitted in NCBI gene bank within an accession number KJ716460.1. A phonogram reflecting the relationship between the strains and candidate sequences of related strains obtained from the NCBI database is presented in (Fig. 4)[20-23].

Conjugation

Conjugation was performed between SSA1 (Donar) and *Lactobacillus* (Acceptor). After the incubation, 0.1ml of culture was taken and inoculated on to the Petri plate containing MRS media containing 1000 mgL^{-1}, 2000 mgL^{-1} antibiotic concentration. No growth was observed.

Antimicrobial activity of the extracts and solvents

Antimicrobial activity with methanolic and chloroform extracts of *Aloe vera* and *Curcuma longa* were tested against *E. coli*. Maximum zone of inhibition was found to be 11mm in *Aloe vera* extract and 9mm in *Curcuma longa* extracts of chloroform (Table 2, Table 3)[23-24].

Combination Therapy

Combinational therapy employing different combinations of plant extracts and antibiotic (Cefixime) was performed. Maximum zone of inhibition was observed when plant extracts were used in combination with the antibiotic (Table 4, Table 5).

DISCUSSION

Emergence of antibiotic resistant bacteria is one of the major concerns of this era[25-26]. New and powerful antibiotics are being discovered everyday, in order to treat this multidrug resistant bacteria and their infections. Use of powerful antibiotics has led to considerable amount of side effects and impairment to the human health[27]. Thus a newer approach is required to treat this multidrug resistant bacteria without causing any kind of damage to the host. In our study, we focused on some of the medicinal plants like *Aloe vera*, *Curcuma longa* which is well known for their antimicrobial activity[28-29].

Table 3. Showing antimicrobial activity of Curcuma longa

Compound	Methanol	*Curcuma longa* extract in Methanol	Chloroform	*Curcuma longa* extract in chloroform	Antibiotic
Zone of inhibition (in mm)	0.5 mm	7 mm	1 mm	9 mm	No zone

Table 4. Showing the antimicrobial activity of different combinations extracted using Methanol

Combination	*Aloe vera* extract + *Curcuma longa* extract	*Aloe vera* extract + Antibiotic	*Curcuma longa* extract + Antibiotic	*Aloe vera* extract + *Curcuma longa* extract + Antibiotic
Zone of inhibition (in mm)	10 mm	12 mm	11 mm	8 mm

Table 5. Showing the antimicrobial activity of different combinations extracted using Chloroform

Combinations	*Aloe vera* extract + *Curcuma longa* extract	*Aloe vera* extract + Antibiotic	*Curcuma longa* extract + Antibiotic	*Aloe vera* + *Curcuma longa* extract + Antibiotic
Zone of inhibition (in mm)	9 mm	11 mm	10 mm	9 mm

A total of six pathogenic drug resistant isolates were isolated from raw sewage. Based on morphological and biochemical results VITSSA1, VITSSA2, VITSSA5, VITSSA6 isolates were found to be *Salmonella*, and VITSSA3, VITSSA4 were found to be *E. coli*. All the isolates were assayed against 3rd generation antibiotic Cefixime which is known to be effective against gram-negative bacteria. Hickson, 2011 reported an exceptional activity of strains with an MIC90 value of 0.25 µgml^{-1} which reflects its high ²-lactamase producing amoxicillin-resistant strains. The isolates in the present study showed high resistance, i.e. 6000 mgL$^{-1,}$ 4000 mg L$^{-1,}$ 10,000 mgL^{-1}, 10,000 mgL^{-1}, 6000 mgL^{-1} and 4000 mgL^{-1}. Previously *E. coli* has been report to show resistance against drugs like ampicillin, erythromycin, penicillin and bacitracin[4] but this is the first report on an E. coli strain showing resistance to third generation drug. Based upon the MIC range and growth kinetics VITSSA4 was found to be an effective isolate showing a higher degree of resistance and maximum growth rate. It was analysed through 16S rRNA sequencing and identified as *E.coli* and the sequence was submitted in NCBI. The accession number of the sequence is KJ716460.1[31-33].

The effective strain VITSSA4 which was showing high resistance to 3rd generation antibiotic cefexime (10,000 mgL^{-1}) was surprisingly found to be susceptible to the crude extract of *Aloe vera* and *Curcuma longa* assayed through well diffusion method using MH agar. The crude methanolic and chloroform extract of *Aloe vera* showed effective inhibition against VITSSA4 respectively. However, when antibiotic disc was placed it failed to constrain the growth, this could

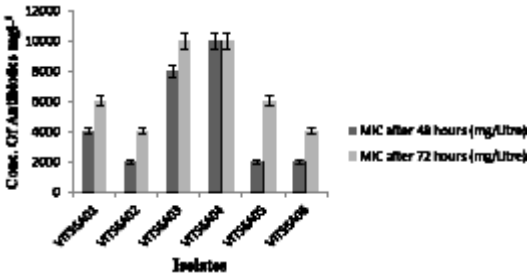

Fig. 1. MIC of all the 6 isolates obtained from the sewage sample

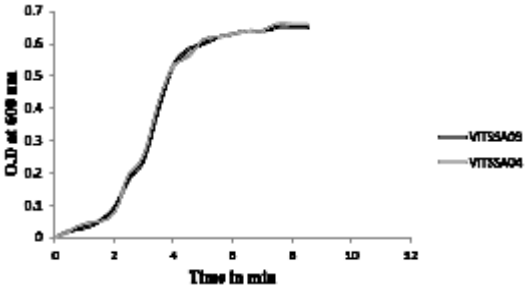

Fig. 2. Growth curve showing the growth pattern of VITSSA02 and VITSSA03

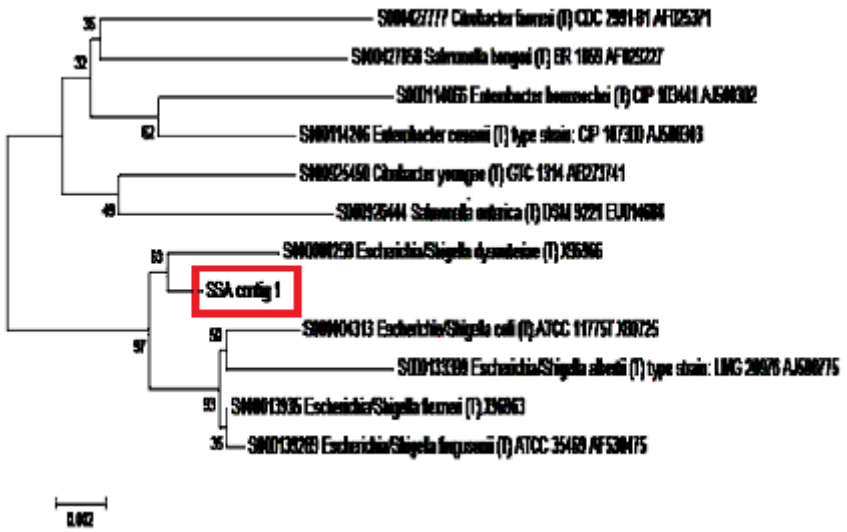

Fig. 3. Phonogram reflecting the relation between the strains

be of the fact that the compounds in crude extracts are plant derivatives which are from biological origin with minimum or no side effects in humans.

A newer plant consortium was used by combining the plant derivatives along with the 3rd generation drug cefexime as a combination therapy. Remarkable antimicrobial activity was observed with the combination (Crude extract + Antibiotic) was found to be high when compared to their individual antimicrobial activities. The plant derivatives somehow managed to boost the activity of the antibiotic to restrict the growth of the multidrug resistant bacteria isolate VITSSA4.

Therefore an effective formulation with *Aloe vera* and *Curcuma longa* can be prepared by as consortia with the commercial drugs for the treatment of multi-drug resistant *E. coli* and its infections.

CONCLUSION

Based on the above study we conclude that usage of third generation drug in combination with *Aloe vera* and *Curcuma longa* extracts at various concentrations can lower the risk of side effects and can be effectively formulated for the treatment of multi-drug resistant *E.coli*.

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