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Original Article

EVALUATION OF PHYTOCHEMICALS AND SYNERGISTIC INTERACTION BETWEEN PLANT EXTRACTS AND ANTIBIOTICS FOR EFFLUX PUMP INHIBITORY ACTIVITY AGAINST *SALMONELLA ENTERICA SEROVAR TYPHIMURIUM* STRAINS

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ABSTRACT

Objective: Traditional antibiotics are increasingly suffering from the emergence of efflux related multidrug resistance (MDR) amongst pathogenic bacteria that led to novel approaches to control microbial infections being investigated as potential alternative treatments. Synergism between natural sources and antibiotics has received much attention and efforts have been put in to identify compounds that can act as efflux pump inhibitors.

Methods: Methanol and ethyl acetate extracts of important medicinal plant species widely used in traditional remedies for various ailments were screened for their synergism with ciprofloxacin and tetracycline antibiotics using the agar well diffusion method. Phytochemical analysis was also done by standard methods. In addition, to evaluate the potential of synergistic extracts as efflux pump inhibitors against *Salmonella enterica* serovar *Typhimurium* (*S. Typhimurium*) strains, including wild-type (NKS70), overexpressed AcrAB (NKS773) as well as against knockout TolC (NKS174), berberine uptake and ethidium bromide inhibition assays were done.

Results: *In vitro* synergistic activity were confirmed for methanolic extracts of all five plants, namely, *Allium sativum* (Amaryllidaceae), *Syzygium aromaticum* (Myrtaceae), *Berberis aristata* (Berberidaceae), *Rhus cotinus* (Anacardiaceae), and *Phyllanthus emblica* (Phyllanthaceae). Agar well diffusion method confirmed the greatest synergistic activity of *P. emblica* with used antibiotics. The phytochemical analysis of medicinal plants showed that the terpenoids and reducing sugar were found to be present in all synergistic extracts. Phytochemicals have great potential as antimicrobial agents. Further efflux inhibition assays confirmed maximum efflux pump inhibition through *Phyllanthus emblica* extract against *S. Typhimurium* when extracted with methanol solvent.

Conclusion: It is hypothesized that phyto compounds present in these plants might be following the same mechanism of action responsible for synergistic interaction as well as efflux inhibition. These data provide bioactive compounds for possible clinical utility as efflux inhibitors.

Keywords: Antibiotics, Efflux pump inhibition, Medicinal plants, *S. Typhimurium*, Synergistic activity

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INTRODUCTION

Salmonella enterica serovar *Typhimurium* (*S. Typhimurium*) is a facultative anaerobic Gram-negative foodborne pathogen under the family Enterobacteriaceae. They cause various diseases in humans and animals, from gastroenteritis to bacteremia and typhoid fever [1]. Multidrug-resistant bacteria resist a broad range of antimicrobials thereby reducing the treatment options and hence increasing the mortality rate day by day in developing countries where the use of antibiotics is high. This problem of antimicrobial resistance is of great concern. In the past few years, rapid and widespread emergence of resistance to newly introduced antimicrobial agents indicates that even new families of antimicrobial agents will have a short life expectancy [2]. One way of prolonging antibiotic efficiency or potentiating the activity of antibiotics against drug-resistant pathogens is by blocking drug efflux pumps with efflux pump inhibitors (EPIs), attractive compounds known to reverse multidrug resistance and prevent the development of resistance in clinically relevant bacterial pathogens [3-6]. Therefore, researchers are increasingly making efforts that lead to the development of better drugs against MDR microbe strains with the use of herbal products [7]. Tyagi *et al.* [8] reviewed a number of herbal plants that has been used as a drug in the form of crude extracts and extensively used for their antimicrobial possessions. Different plant extracts exhibit variation in their activity against uropathogenic *E. coli*, Enteropathogenic *E. coli*, Enterotoxigenic *E. coli*, *S. Typhimurium*, *K. pneumoniae* and *P. aureginosa* [9].

Natural products play a major role in drug discovery by providing bioactive scaffolds with activity against a variety of targets in infections. Efflux pump inhibitors (EPIs) isolated from natural sources are the best strategy of efflux system inhibition, which has emerged from the last few decades. Reports for novel and already known natural chemical entities which are active against all of the major microbial efflux systems have populated the chemical space as well as the literature [10, 11]. Medicinal plants contain some organic compounds such as tannins, alkaloids, flavonoids, carbohydrates, terpenoids and steroids, which produces definite physiological action on humans [12, 13] and *in vitro* found to have antimicrobial properties [14]. There is a vast array of medicinal plants used singly or in combination with antibiotics that confer synergistic effects in the treatment of various ailments. The combination of an antibiotic with an efflux pump inhibitor (EPI) would be expected to re-establish susceptibility of the bacteria to antibiotics that at present cannot be used any longer. The mode of action of combination therapy significantly differs from that of the same drugs acting individually. Therefore the selection of an appropriate combination is crucial and essential which requires understanding the potential interaction between the plant extracts and antimicrobial agents. Antimicrobial agents are exported out of the bacterial cell through efflux pumps that serve to protect it from building up to toxic levels and led to the development of resistance to antimicrobials [15-19].

In Enterobacteriaceae family, overproduction of the AcrAB-TolC efflux pumps confers clinically relevant resistance to many antimicrobial agents, including ciprofloxacin and tigecycline [20] and are associated with innate multidrug resistance resulting in major clinical problem, so there is a need to understand their physiology to reveal their interesting perspectives uses in combination therapy along with existing antibiotics that lead to the development of inhibitors.

Identification of new efflux pump inhibitors (EPIs) is expected to hasten the development of effective adjuvant therapies to existing antibiotics. The EPIs can effectively increase the intracellular concentration of the drug to the level essential for its activity and hence reduce the minimal inhibitory concentration required for the antibiotic to kill the resistant organisms. Plants have evolved to effectively fight off infections by producing an array of special chemicals. We were specifically interested in identifying EPIs from plant sources. Therefore, in the present study, the plant extracts that have been shown to act synergistically with antibiotics such as ciprofloxacin and tetracycline were analyzed for their phytochemicals that have a major role in the identification and development of effective efflux pump inhibitors against *Salmonella Typhimurium*.

MATERIALS AND METHODS

Selection of plant material

Fresh leaves of *Berberis aristata* (Berber dance) and *Rhus cotinus* (Anacardiaceae), Fruits of *Phyllanthus emblica* (Phyllanthaceae), flower bud of *Syzygium aromaticum* (Myrtaceae) and bulb of *Allium sativum* (Amaryllidaceae) selected on the basis of traditional applications and pharmacological reports were collected from surroundings and local market into plastic zip lock bags with appropriate labelling and got identified from Botany department of Shoolini University of biotechnology and Management Sciences, Bajhol, India. Voucher specimens have been submitted to the herbarium of the University (mentioned in tables).

Preparation of plant extracts

The collected samples were carefully washed under running tap water to remove debris and dust particles, followed by washing with 0.1% mercuric chloride to remove the contamination and after that washed with distilled water and shade dried for 4-5 d. The dried plant materials were ground to powder and stored in airtight containers. 70% methanol and ethyl acetate were used as extraction solvents. 10 g of powdered sample was soaked in a conical flask containing 100 ml of solvents with occasional shaking followed by keeping all the flasks on a rotary shaker at 200 rpm and filtered through a sterilized Whatman No. 1 filter paper after 48 h. The plant residue was re-extracted with the addition of solvents, and after 24 h, it was filtered again. Combined filtrates were concentrated to dryness at 40 °C on a rotary evaporator. The dried extract, thus, obtained was sterilized by overnight UV-irradiation, checked for sterility on nutrient agar plates and stored at 4 °C in a refrigerator for further use [21]. The dried extracts were reconstituted to 5% in Dimethyl sulphoxide (DMSO) for further use.

Microbial strains and culture conditions

Three strains of *Salmonella enterica* serovar *Typhimurium* NKS70 (wild type), NKS174 (TolC knockout) and NKS773 (AcrAB over expressive) were used as test organisms in the present study. All three strains were a generous gift given by Kunihiko Nishino, Associate professor of Osaka University (Japan). All strains were maintained in 30% (v/v) glycerol at-80 °C until required. The pure bacterial cultures were maintained on bismuth sulphite agar and Luria-Bertani medium (LB media were obtained from Acumedia and Agar from Oxoid). Each bacterial culture was further maintained by subculturing on the same medium and stored at 4 °C until use. Cell growth (optical density) was assessed with a spectrophotometer at 600 nm. Cells were used for experiments in the mid-log growth phase (optical density at 600 nm, ~0.4 to 0.8 or 10^8/ml).

Chemicals used

Fehling solution A, Fehling solution B, hydrochloric acid (HCl), acetic anhydride, methanol, ethyl acetate, ethanol, sodium hydroxide (NaOH), iodine, potassium iodide, chloroform, concentrated sulphuric acid, DMSO, and ferric chloride were obtained from Sigma-Aldrich Chemical Co. (India). All the chemicals and solvents used were of standard analytical grades.

Antimicrobial agents and antibiotics

Two antibiotics (tetracycline and ciprofloxacin were purchased from Himedia Laboratory, India) were evaluated for synergism with extracts of five medicinal plants. Zones of inhibition were determined in accordance with the procedures of the National Committee for Clinical Laboratory Standard [22]. Bacterial strain was considered susceptible to antibiotics if the inhibition zones were ≥13 mm.

Evaluation of the synergistic activity of plant extracts with antibiotics

Synergistic activity of a plant extracts of 5 medicinal plants with two above-mentioned antibiotics were investigated using the agar well diffusion method [23]. Petri plates containing 30 ml Mueller-Hinton agar medium were kept for solidification followed by spreading 100 µl (10^6 CFU/ml) of the microorganism (24 h broth culture). Four wells of uniform diameter of 6 mm made after solidification, using sterile aluminum borer. Four wells (6 mm diameter) were punched in the Mueller Hinton agar and the combination of 40 µl of 100 mg/ml plant extracts and antibiotics were added in a well along with antibiotic and plant extract alone in other two wells. The negative control was DMSO. After incubation for 24 h at 37 °C, the plates were observed for the synergistic activity, and diameter of the zone of inhibition for each extract was measured in terms of a millimeter (mm). The experiment was repeated in triplicate for each extract.

Qualitative tests for phytochemicals

Screening of the extracts of above five selected synergistic medicinal plants for various phytochemical constituents carried out using standard methods [24-29]

Test for alkaloids (Wagner's test)

A fraction of the extract was treated with 3-5drops of Wagner's reagent [1.27g of iodine and 2g of potassium iodide in 100 ml of water] and observed for the formation of a reddish brown precipitate (or coloration).

Test for flavonoid (Alkaline reagent test)

Treat the extract with dilute NaOH, followed by the addition of dilute HCl. A yellow solution with NaOH turns colorless with dilute HCl.

Test for saponins (Frothing test)

Saponins were tested by dissolving one-half grams (0.5 g) of the crude extract in a test tube containing 3 ml of hot distilled water and then the mixture was shaken vigorously for one minute and persistent foaming observed indicated the presence of saponins.

Test for terpenoids (Salkowki's test)

1 ml of chloroform was added to 2 ml of each extract followed by a few drops of concentrated sulphuric acid. A reddish brown precipitate produced immediately indicated the presence of terpenoids.

Reducing sugars (Fehling test)

The extracts were shaken with distilled water, filtered and boiled with a few drops of Fehling's solution A and B for a few minutes. Orange/red color indicates the presence of reducing sugars.

Test for phenols

A small amount of the extract was treated with 2 ml of ethanol and a few drops of ferric chloride solution added to it. The blue color indicates the presence of phenols.

Screening of crude plant extracts for efflux pump inhibitory activity

Berberine uptake assay

Screening of crude plant extracts for efflux pump inhibitory activity was done by berberine uptake assay [30]. Serial 2-fold dilutions of berberine and a plant extract were mixed in each well of a 96-well microtiter plate. Each row (and column) contained a fixed amount of one agent and increasing amounts of the second agent. The plate presents a pattern in which every well contain a unique combination of concentrations between the two molecules. The concentrations of berberine (row) range from 30 to 0.5 µg/ml (89-1.5 µM), while plant

extracts (column) concentrations range from 15 to 0.015 µg/ml. Berberine without plant extracts was used as negative control. CCCP was added into the culture along with berberine as a positive control. Cells were added to each well at a final concentration of 5 × 10^6 CFU/ml, and plates were incubated at 37 °C for 24 h. Growth was assayed by absorption at 600 nm with a microtiter plate reader. An optical density less than 0.04 revealed no bacterial growth.

Ethidium bromide efflux inhibition assay

The ethidium bromide efflux inhibition assay was also performed for the confirmation of efflux pump inhibitory activity [31]. 175 µl of bacterial inoculum were added to each well of 96 well microtiter plate. 5 µl of test compound were added to columns 1-10. 20 µl of 100 µM EtBr were added to each well. 5 µl of positive control (CCCP) was added to the eleventh column. 5 µl of negative control (DMSO) without plant extract was added to column 12. Plates were immediately placed in Fluoroscan Ascent and fluorescence of the accumulated EtBr was measured for 30 min with 5 min of the interval at 530 nm excitation and 600 nm emission. Sterile microtiter plates were used for this assay.

RESULTS

The zone diameters were found to increase when extracts were used in combination with antibiotics. The combination was found to be more potent than either of the two. Differences in diameter of inhibition zone ≥ 4 mm between t he combined effect of medicinal plants with ciprofloxacin and tetracycline and antibacterial activity of ciprofloxacin and tetracycline alone revealed the synergistic activity of that plant extract. Inhibition zones are represented in millimeter (mm).

Table 1 presents the synergistic activity of plant extracts with ciprofloxacin. Among methanolic extracts, *R. cotinus* showed a maximum zone of inhibition alone, but in combination with antibiotics, *P. emblica* showed maximum synergism and was found to be very effective with antibiotics. Minimum or no zone of inhibition was observed in plant extract of *B. aristata.* Similarly, all methanolic extracts and ethyl acetate extracts of only *A. sativum* and *P. emblica* showed their synergistic activity with tetracycline, are displayed in table 2.

In NKS70 strain, methanolic leaf extract of *B. aristata* had no antimicrobial activity alone, but enhances the activity of ciprofloxacin with an inhibition zone of 27±0.5 and therefore, found to be a synergistic enhancer. Methanolic *P. emblica* extract measured inhibition zone of 28±0.7 with ciprofloxacin, exhibited maximum synergistic activity. Among ethyl acetate extracts, combination effects of ciprofloxacin with *P. emblica* and *A. sativum* were 27.5±0.5 and 27±0.5 respectively, both exhibited maximum synergism. With the combined effect of tetracycline, the *P. emblica* methanolic extract produced inhibition zone of 24.3±0.8 and *A. sativum* formed 19.5±0.5 of inhibition zone, which revealed their maximum synergistic activities.

In NKS174 strain, a maximum zone of inhibition 33.6±0.9 and 28.6±0.5 were measured in the methanolic extract of *P. emblica* with ciprofloxacin and tetracycline respectively.

In NKS773 strain, the combined effect of ciprofloxacin with *P. emblica* formed inhibition zone of 26.5±0.6 and *A. sativum* measured 24±0.1, both exhibited best synergistic activities. Inhibition zone measured for *P. emblica* with tetracycline was 20.6±0.4.

On the basis of synergistic activities of *Allium sativum*, *Syzygium aromaticum*, *Berberis aristata*, *Rhus cotinus* and *Phyllanthus emblica,* methanolic extracts revealing maximum synergism were further selected for evaluation of their phytochemicals and efflux pump inhibitory activity.

Results obtained for the qualitative screening of phytochemicals are presented in table 3. The results revealed the presence of medically active compounds in the five plants studied. From this table, it is evident that alkaloids, phenols, terpenoids, flavonoids, reducing sugars and saponins were present in almost all the plants. Flavonoids were absent only in the fruit of *P. emblica*. Terpenoids and reducing sugars were present in all plant extracts. Saponins and phenols were present in all except *A. sativum*. Alkaloids were absent in the *S. aromaticum*, *A. sativum* and also in *R. cotinus.*

Berberine works as an efflux pump substrate and inhibits the growth of bacteria in the presence of an EPI. The experimental approach to detect EPI activity was to test the combined action of a plant extract with 30 µg/ml or 89 µM berberine added at a sub-inhibitory concentration. Extracts that inhibited cell growth in the presence of berberine and had no activity when added alone were likely to contain an EPI. Inhibition of cell growth in the presence of berberine exhibited by extracts was in the following order: *P. emblica* (M)>*A. sativum* (M)>*S. aromaticum* (M)>*R. cotinus* (M)>*B. aristata* (M) (Fig.1). Therefore, methanolic extract of *P. emblica* was found to be an active efflux pump inhibitor as it increases the efficacy of berberine.

Table 1: Medicinal plants possessing synergistic activity with ciprofloxacin

S. No.	Plant name (voucher specimen no.)*		Methanolic extracts			Ethyl acetate extracts		
			NKS70 wildtype	NKS174 knockout	NKS773 over expressive	NKS70 wildtype	NKS174 knockout	NKS773 over expressive
			Diameter of zone of inhibition (in mm)					
1	*S. aromaticum* (flower buds) (SUBMS/BOT-S219) *	PE	12±0.0	13±0.0	13±0.0	10±0.0	10±0.0	10±0.0
		PE+A	20±0.5	25±0.5	18±0.5	18±0.1	23±0.5	12±0.4
		A	16±0.0	20±0.4	12±0.4	16±0.5	20±0.4	12±0.4
		DMSO	-	-	-	-	-	-
2	*A. sativum* (Bulbs) (SUBMS/BOT-S221) *	PE	12±0.0	13±0.0	12±0.0	12±0.0	11±0.0	11±0.0
		PE+A	27.5±0.5	30.5±0.4	24±0.1	27±0.5	30±0.2	22.5±0.3
		A	23±0.1	26±0.2	18±0.1	23±0.0	26±0.0	18±0.0
		DMSO	-	-	-	-	-	-
3	*R. cotinus* (leaf) (SUBMS/BOT-S184) *	PE	14.6±0.2	16.6±0.4	16±0.1	13±0.2	13±0.1	14±0.1
		PE+A	27.3±0.7	30.3±0.8	23.3±0.5	26±0.5	30±0.2	19±0.1
		A	23.6±0.4	26±0.5	18±0.1	24±0.2	28±0.5	17±0.4
		DMSO	-	-	-	-	-	-
4	*B. aristata* (leaf) (SUBMS/BOT-S108) *	PE	-	-	-	-	-	-
		PE+A	27±0.5	32.5±0.4	25±0.3	25±0.2	29±0.3	21.5±0.5
		A	23±0.2	28±0.1	21±0.1	24.5±0.3	28±0.1	20±0.2
		DMSO	-	-	-	-	-	-
5	*P. emblica* (fruits) (SUBMS/BOT-S170) *	PE	12±0.5	14±0.8	16±0.8	14.6±0.6	17±0.1	14.5±0.5
		PE+A	28±0.7	33.6±0.9	26.5±0.6	27.5±0.5	32±0.6	24.5±0.4
		A	23±0.5	28±0.5	20.5±0.1	23.5±0.3	28±0.5	20.5±0.1
		DMSO	-	-	-	-	-	-

A = Antibiotic (5µg/ml), DMSO = Dimethyl sulfoxide, PE = Plant extract, (-) = No inhibition zone (n = 3, Values are represented as mean±SD).

Table 2: Medicinal plants possessing synergistic activity with tetracycline

S. No.	Plant name (voucher specimen no.)*		Methanolic extracts			Ethyl acetate extracts		
			NKS70 wildtype	NKS174 knockout	NKS773 over expressive	NKS70 wildtype	NKS174 knockout	NKS773 over expressive
			Diameter of zone of Inhibition (mm)					
1	*S. aromaticum* (flower buds) *(*SUBMS/BOT-S219*)* *	PE	12±0.0	13±0.0	13±0.0	10±0.0	10±0.0	10±0.0
		PE+A	19±0.5	23±0.0	22±0.1	17.5±0.1	20±0.5	16±0.0
		A	15±0.1	17.5±0.5	11±0.0	15±0.1	17.5±0.5	11±0.0
		DMSO	-	-	-	-	-	-
2	*A. sativum* (bulbs) (SUBMS/BOT-S221) *	PE	12±0.0	13±0.0	12±0.0	12±0.0	11±0.0	11±0.0
		PE+A	19.5±0.5	23.5±0.5	16±0.0	18±0.6	22±0.6	17±0.8
		A	14.5±0.5	18.5±0.4	11±0.0	14±0.2	18±0.4	12±0.5
		DMSO	-	-	-	-	-	-
3	*R. cotinus* (leaf) (SUBMS/BOT-S184) *	PE	14.6±0.2	16.6±0.4	16±0.1	13±0.2	13±0.1	14±0.1
		PE+A	16.5±0.3	27±0.5	14.5±0.5	15±0.5	20±0.2	15±0.1
		A	12.5±0.4	22.5±0.5	10±0.1	13.3±0.2	22.5±0.5	10±0.4
		DMSO	-	-	-	-	-	-
4	*B. aristata* (leaf) (SUBMS/BOT-S108) *	PE	-	-	-	-	-	-
		PE+A	14.9±0.2	26±0.5	14±0.5	13±0.5	17±0.2	10±0.1
		A	11±0.1	22±0.5	10±0.4	13.3±0.2	18.6±0.5	10±0.4
		DMSO	-	-	-	-	-	-
5	*P. emblica* (fruits) (SUBMS/BOT-S170) *	PE	12±0.5	14±0.8	17±0.8	14.6±0.6	17±0.1	14.5±0.5
		PE+A	24.3±0.8	28.6±0.5	20.6±0.4	19.5±0.9	27.6±0.6	18.6±0.1
		A	19.0±0.6	23.6±0.4	14.6±0.4	15±0.1	23.3±0.6	14.1±0.2
		DMSO	-	-	-	-	-	-

A = Antibiotic (15µg/ml), DMSO = Dimethyl sulfoxide, PE = Plant extract, (-) = No inhibition zone (n = 3, Values are represented as mean±SD).

Table 3: Preliminary phytochemical screening of the extracts possessing synergistic activity with used antibiotics

S. No.	Name of compounds	*S. aromaticum*	*A. sativum*	*R. cotinus*	*B. aristata*	*P. emblica*
1	Flavonoids (Alkaline reagent test)	+	+	+	+	-
2	Alkaloids (Wagner's reagent)	-	-	-	+	+
3	Reducing sugars (Fehling test)	+	+	+	+	+
4	Saponins (Foam test)	+	-	+	+	+
5	Terpenoids (Salkowki's test)	+	+	+	+	+
6	Phenols	+	-	+	+	+

Legend: (+) = present, (-) = Absent

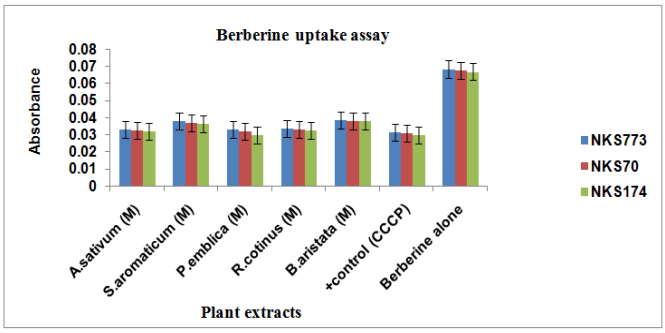

Fig. 1: Absorbance shown by plant extracts at 89 µM berberine

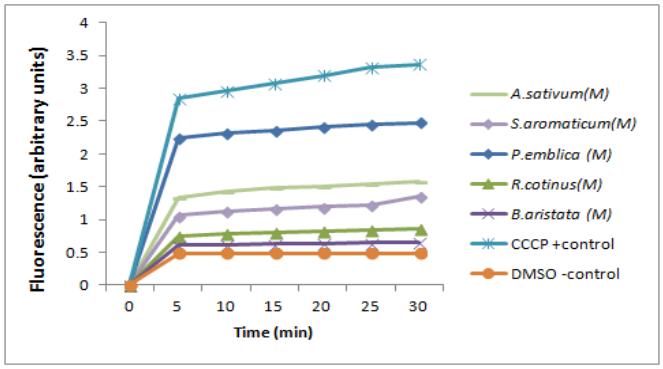

Fig. 2: Effect of plant extracts on the accumulation of 100 µM ethidium bromide by knockout strain (NKS174)

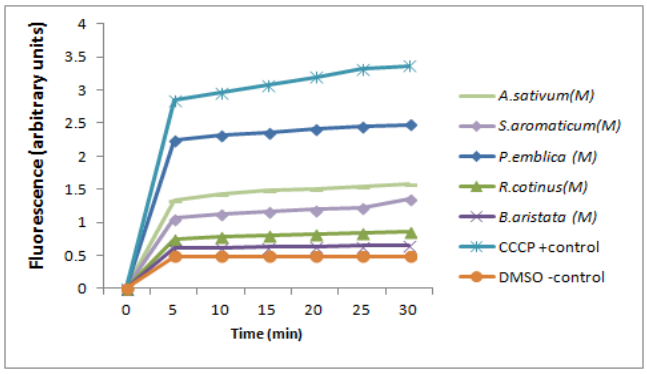

Fig. 3: Effect of plant extracts on the accumulation of 100 µM ethidium bromide by wild type (NKS70) strain

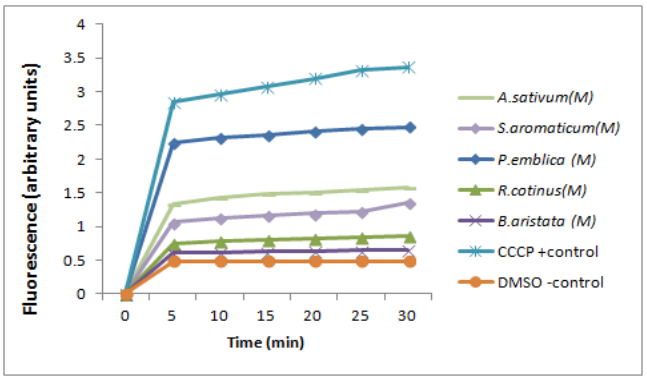

Fig. 4: Effect of plant extracts on the accumulation of 100 µM ethidium bromide by over expressive (NKS773) strain.

Figs 2, 3 and 4 showed the effect of plant extracts on the level of accumulation of ethidium bromide (100 µM) in the NKS174 knockout, NKS70 wild type and NKS773 over expressive strains respectively. Hence, berberine and EtBr at 89 µM and 100 µM respectively, act as a marker for the detection of EPI in plant extracts. Berberine uptake assay and ethidium bromide inhibition assay were performed for all five synergistic plant extracts against *S. Typhimurium* strains. Hence, observations of berberine assay and EtBr assay were found similar and resulted in maximum EPI activity in the methanolic extract of *P. emblica*.

DISCUSSION

Multidrug efflux pumps are the major mechanism of drug resistance in *Salmonella*. Most effective multidrug efflux pump is AcrAB, exporting various drugs, dyes, and detergents. The EPI evaluation assay studies suggest that some moderate or strong polar components play a role in inhibiting bacteria. The approach to the detection of molecules that can interfere with the process of efflux to enhance the activity of existing antibiotics helps in tackling with antibiotic resistance and explore genomic-driven new lead molecules [32].

The present study was done with an aim to find plant extracts that may be combined with antibiotics to produce effects greater than their individual effect and act as efflux pump inhibitors. Synergism from natural sources has received much attention and efforts have been put in to identify compounds by phytochemical screening that can act as efflux pump inhibitors. The ability of the compounds to reverse antibiotic resistance conferred by the expression of the AcrAB-TolC efflux pump was determined. *Salmonella enterica* serovar *Typhimurium* is resistant to fluoroquinolones, chloramphenicol-florfenicol, and tetracycline due to the presence of the AcrAB-TolC efflux pump [33]. Drug synergism between known antibiotics and bioactive plant extracts is a novel concept and could be beneficial (synergistic or additive interaction) or deleterious (antagonistic).

Our potentiation study revealed that plants, namely, *Allium sativum*, *Syzygium aromaticum*, *Berberis aristata*, *Rhus cotinus* and *Phyllanthus emblica* synergistically increased the antimicrobial activity of ciprofloxacin and tetracycline. Our results were consistent with previous *in vitro* studies reported that crude extracts of Indian medicinal plants, *Acorus calamus, Hemidesmus indicus, Holarrhena antidysenterica* and *Plumbago zeylanica* showed their synergism with tetracycline and ciprofloxacin against an extended spectrum of beta-lactamase, producing multidrug-resistant enteric bacteria in which ciprofloxacin showed more synergy with the extracts than tetracycline [34-40]. Plants either contain antimicrobials that act synergistically with antibiotics or possess compounds that have no intrinsic antibacterial activity, but are able to sensitize the pathogen to a previously ineffective antibiotic [37].

Our secondary metabolite studies of above five medicinal plants have shown the presence of flavonoids, saponins, alkaloid, terpenoid, reducing sugars and phenols which are of great importance in the field of drug research. All the five medicinal plants selected for screening were found to possess terpenoids and reducing sugars. Phenols are also present in all plant extracts except *A. sativum.* The phenolic compounds are one of the largest and most ubiquitous groups of plant metabolites [41]. A literature search revealed that they possess biological properties such as antiapoptosis, anti-aging, anticarcinogen, antiinflammation and improvement of endothelial function, as well as inhibition of angiogenesis and cell proliferation activities [42]. Alcoholic and aqueous Heartwood extracts of medicinal plant *Berberis aristata,* containing berberine alkaloid also known to be a potent anti-inflammatory agent [43]. The plant extracts revealed the presence of saponins, which are known to produce an inhibitory effect on inflammation [44]. Moreover, alkaloids have been associated with medicinal uses for centuries and are one of the diverse groups of secondary metabolites found to have antimicrobial activity by inhibiting DNA topoisomerase. Terpenoids are also known to possess antimicrobial, antifungal, antiparasitic, antiviral,

antihyperglycemic, anti-inflammatory and immune-modulatory properties [45, 46].

A fluorimetric study was employed to assess the fluorescence or accumulation of berberine and ethidium bromide, a substrate of the efflux pump. The fluorescence of ethidium bromide is maximal inside cells; thereby the accumulation was evidenced by an increase in the fluorescence units. The addition of the methanolic *P. emblica* showed slightly lesser accumulation of ethidium bromide in the cells than in the presence of CCCP. However, the accumulation was significantly higher than that of the control without any EPI, in all three strains. However, it was evident that *P. emblica* play a major role in AcrAB efflux pump inhibition, therefore, act as an efflux pump inhibitor. One of the previous study of Piddock *et al.* [47] observed the efflux pump inhibitors of the AcrAB efflux pump from *Theobroma cacao* and *Catha edulis* sources. Therefore, efflux pump inhibitors (EPIs) are promising therapeutic agents, as they restore the standard antibiotic activity. Co-administration of EPIs with antibiotics that act as pump substrates could increase drug levels intracellularly and therefore renew the efficacy of existing antibacterial agents.

CONCLUSION

In conclusion, the present study probably suggests the possibility of concurrent use of these antimicrobial agents and extracts in combination in treating infections caused by *S. Typhimurium* strains and helps in the reuse of these antibiotics into clinical. Methanolic extract of *P. emblica* found to be a potent inhibitor of the AcrAB-mediated efflux of ethidium bromide and berberine. Though, *P. emblica* has shown EPI activity in bacteria, as per our knowledge, there is no report on EPI activity of *P. emblica* in AcrAB-efflux systems. As the incidence of drug-resistant *S. Typhimurium* is increasing at an alarming rate, a combination therapy of antibiotics with these phytochemicals would be a better approach to combating the multidrug resistance. This will eventually help in better treatment outcome and help in decreasing the mortality rate of patients with infections caused by the MDR pathogens. A better understanding of plant-derived bioactive compounds, EPI of substantial medicinal merit may be fundamental to the development of pharmacological agents and may lead to the improved application of existing plant-derived CAM therapies.

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CONFLICT OF INTERESTS

The authors have no conflict of interest to report

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