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Screening of medicinal plants of Himachal Pradesh for efflux pump inhibitory activity against *Escherichia coli*

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Abstract
Rapid increase of multidrug resistance in bacteria has made the discovery of new antimicrobials necessary. From the time of discovery of bacterial efflux pumps in 1980, many bacteria have been characterized as multi drug resistant. Efflux pumps expel out the antibiotics and other resistance modifying compounds and dyes. Antibiotic resistance developed very quickly due to the changes in expression of efflux pumps. Mechanism of efflux has wildly known as major constituent of resistance in many classes of antibiotics. Multidrug resistance related to efflux is a major factor by which bacteria lowers the effect of antibacterial agents. It is therefore necessary that new antibiotics and efflux pump inhibitor are discovered. The bacterial efflux pump inhibitor helps in the reuse of antibiotics which are therapeutically ineffective. In the present study we study 15 medicinal plants for their efflux pump inhibitory activity against *E. coli* bacteria.

Keywords: medicinal plants, Himachal Pradesh, efflux pump inhibitory activity, *Escherichia coli*

1. Introduction
Constant increase in drug resistant strains of bacteria becomes a big threat to human health [1]. Multidrug efflux pumps unfavorably affects the clinical effectiveness of existing antibiotics and also affects the process of discovery of new antibiotics [2]. The AcrAb system present in *E. coli* is a multidrug efflux system which is related to RND type transporter acrb and also composed of a accessory protein AcrA and it helps in pumping out different types of lipophilic and amphiphilic inhibitors in to the medium [3]. Most of bacteria in gram negative bacteria family are less susceptible to most of the antibiotics, mainly lipophilic and amphiphilic ones, this is due to the presence of outer membrane in gram negative bacteria. The outer membrane layer presents in gram negative bacteria acts as a effective permeability barrier [4]. The AcrAB efflux system present in *E. coli* is responsible for intrinsic resistance of the bacteria against antibiotics, dyes and detergents [5]. Active efflux is established as important factor of bacterial resistance in case of most classes of antibiotics. Mechanism of active efflux is mediated by efflux pumps. Efflux pumps are known as membrane associated active transporters which helps in expelling out toxic compounds, including antibiotics from the cell [6-8].
Antibiotic efflux was first reported in 1980, in case of *E. coli* bacteria as a mechanism for tetracycline resistance. Recently the role of efflux mechanism is reported in bacterial resistance in case of almost all categories of antibiotics [7-11]. In *E. coli* the AcrAB-tolc efflux pump helps in expelling out different types of antibiotics, dyes and detergents [12]. The AcrAB –tolc multidrug efflux pump which is a major efflux system present in *E. coli* Excused a wide range of antimicrobial drugs and hazardous chemicals such as detergents and dyes. Bacterial resistance to most of the classes of antibiotics provided by membrane transporter proteins which are known as drug efflux pumps [13]. These efflux pumps occur as single component system or multi-component systems. Increasing resistance of bacteria to most classes of antibiotics in recent days becomes a big problem [14]. Resistance in antibiotics developed very rapidly because of the changes in the expression of efflux pumps. So it is important to characterized new antibiotics, efflux pump inhibitors and the agents which are resistance modifying [15].
In the present study 15 plants belonging to different families were selected and their EPI activity was checked. Methanolic, extract from these plants were investigated for synergistic activity with ciprofloxacin, gentamycin, erythromycin and tetracycline. Multidrug resistance related to efflux becomes a big complicating factor in the treatment of infections which are related to bacteria [16]. Most of efflux pumps which helps in transporting the drug in gram-negative bacteria are members of the RND family [17]. Overproduction of efflux pump is highly responsible for increasing resistance to many antimicrobial agents such as tetracycline and ciprofloxacin.

2. Material and Methods

2.1 Materials

Table 1: List of plants selected for screening

S.no	Common name	Botanical name	Family	Part
1	Clove	*Syzygium aromaticum*	Myrtaceae	Buds
2	Ginger	*Zingiber officinale*	Zingiberaceae	Rhizome
3	Thyme	*Thymus vulgaris*	Labiaceae	Leaves
4	Cinnamon	*Cinnamomum zeylanium*	Lauraceae	Bark
5	Rosemary	*Rosmarinus officinalis*	Lamiaceae	Leaf
6	Garlic	*Allium sativum*	Liliaceae	Bulb
7	Coriandrum	*Coriandrum sativum*	Umbelliferae	Leaf
8	Black cumin	*Nigella sativa*	Umbelliferae	Seeds
9	Mint	*Mentha longifolia*	Lamiaceae	Leaves
10	Black pepper	*Piper nigrum*	Piperaceae	Seeds
11	Sage	*Salvia officinalis*	Lamiaceae	Leaves
12	Eucalyptus	*Eucalyptus globules*	Myrtaceae	Leaves
13	Neem	*Azadirachta indica*	Meliaceae	Leaves
14	Babul	*Acacia arabica*	Fabaceae	Roots, bark
15	Turmeric	*Curcuma longa*	Zingiberaceae	rhizome

2.2 Microbial strains: The strains of *Escherichia coli* used in the present study were procured from Sabine Schuster and Winfried V. Kern, Center for Infectious Diseases and Travel Medicine, University Hospital, and Department of Medicine, Albert-Ludwigs-University, Freiburg, Germany and from Mtcc and Nctc. The *E. coli* strains used were one knock out strain 1- DC14, wild type strain K-12 AG100 and two standard strains with NCTC number 12923 and MTCC number 1302.

2.3 Chemicals and reagents: Nutrient agar, Nutrient broth, DMSO, Methanol, CCCp, Ethidium bromide, Berberine and Mercuric chloride were purchased from Hi- media Pvt. Ltd.

2.4 Methods

2.5 Collection of plant material: Plants from different families were selected for screening. Plants were collected from botanical garden of Shoolini university, Solan and from the local markets of Himachal Pradesh. A total of 15 plants were collected belonging to different families and their synergistic activity was checked with different antibiotics against *E. coli*. Only the required plant parts were collected either in dry or in fresh form and stored in sterile containers at 4 ^{0}C (Table -1).

2.6 Preparation of plant extracts: The parts of plants used for extract preparation were first washed with tap water and then washed with 0.1% $HgCl_2$ to remove the contamination and after that washed with distilled water. The plant parts washed were then dried for 4 to 5 days in shade. Then the dried plant parts were grinded in to fine powder with the help of mortar and pestle. Plants powder were stored at 4 ^{0}C until use.

2.7 Soxhlet extraction: Powdered plants were subjected to soxhlet extraction with methanol as a solvent. Methanol is widely used as a solvent, because many of the compounds dissolve in it easily, which is important for the plant material, moreover methanol easily evaporates. So, it can be separated from the extract and it is also easily available at low cost. The concentration of methanol used for preparation of extract was 1gm of plant extract dissolved in 10 ml methanol. Apparatus was run for 18-24 hours to get final concentrated slurry. Then extract was poured in china dish. Methanol was evaporated from the extract by incubation at 35-38 ^{0}C. Powder obtained were weighed and stored in a sterile vials at 4 ^{0}C till use (Stefanovic & Comic, 2012).

2.8 Study of combined effect of extract and antibiotics using well diffusion method (NCCLS, 1993)

Antibacterial activity was measured using well diffusion method according to National Committee for Clinical Laboratory Standard 1993. The bacterial culture was inoculated and incubated at 37 ^{0}C for overnight. Inoculums was adjusted according to 0.5 McFarland standard (McFarland solution was prepared by dissolving 99.5 mL of 1% H_2SO_4 and 0.5 mL of 1.175% $BaCl_2$ and stored in dark at room temperature). Nutrient agar plates were prepared. Sterilized swabs were dipped in standardized bacterial suspension with inoculum size of 1.5 x 108 cfu/mL and excess of culture was removed by turning the swab against the side of the tube. Inoculum was spread over the entire surface of Nutrient Agar plates. These plates were allowed to dry for at least 15 min. Wells of 7 mm in diameter were punched in agar and were filled with 40 µL of plant extract (2 concentrations 100 µg/mL and 1000 µg/mL of plant extract were used) and antibiotic alone as well as 30 µL of both plant extract and drug were added. The three replicates of each plate have been performed. The plates were incubated at 37 ^{0}C for 24h.

2.9 Screening of methanolic plant extracts for efflux pump inhibitory activity

Berberine potentiating assay (Belofsky *et al*., 2006): This assay was used to detect the presence of efflux pump inhibitory activity in the plant extracts. Berberine is a well classified antimicrobial agent and it also acts as substrate for efflux pumps and it can be effluxed out by resistant bacteria with the help of efflux pumps. When berberine is combined with plant extracts in which efflux pump inhibitors are present, the efflux pump inhibitor presents in the extract inhibits the activity of multidrug resistance bacteria and highly increases the concentration of berberine inside which leads to the death of bacterial cells because of the antimicrobial activity of berberine.

a) **Culture preparation:** Cultures of *E. coli* were incubated at 37 ^{0}C for 24 hours. Then culture was centrifuged for 2 min at 12,000 rpm, further 0.5 ml, glucose was added in to the culture and was used for the assay.

b) Berberine Assay: This assay was performed in 96 well micro-titre plate and two different concentrations of plant extracts i.e. 100µg/ml and 1000µg/ml, was taken to perform the assay. 170 µl of nutrient broth and 5 µl of *E. coli* culture was loaded in to each well followed by addition of 20µl berberine (30 µg berberine dissolved in 1 ml Dmso) and 5µl of plant extract(15 µg plant extract dissolved in one ml DMSO). DMSO (5µl) was used as negative control along with the addition of berberine and culture. CCCP was used as a positive control along with berberine. Plates were incubated at 37 ^{0}C for 24 hours. Then OD was taken on 595nm in Elisa plate reader (BioTek). An OD less than 0.04 considered to reveal no bacterial growth. No bacterial growth in the presence of berberine indicates the presence of an MDR inhibitor in plant extract.

c) Ethidium Bromide Efflux Inhibition Assay (Kamicker *et al*., 2008): Ethidium bromide is easily effluxed out by the resistant bacteria and will only accumulate in cells in the presence of an efflux pump inhibitor and emits strong fluorescence. Assay was performed in 96 well ELISA plate in duplicate. Two different concentrations of plant extracts were taken i.e, 100µg/ml and 1000µg/ml. In each well of the 96 well plate, 170µl nutrient broth, 5µl of inoculums, 20µl etbr and 5µl plant extract was added. CCCP (5µl) which is a efflux pump inhibitor was used as a positive control. 5µl of DMSO was used as negative control. Then fluorescence was measured for 30 minutes with 5 minute interval at excitation wavelength of 530 nm and emission wavelength of 600 nm. Readings were taken in the fluorescent ELISA reader.

3 Results

3.1 Synergistic activity of plants

Table 2: Combined effect of plants and antibiotics (Mean ± S.D)

	Zone of inhibition in mm		
Plant extract/antibiotic	***E. coli***		
tetracycline	A alone	E alone	A+e
Tet+ Garlic	23±2	13±1	31±2
Tet+ Ginger	14±1	9±2	28±0
Tet+ Clove	24±0	10±0	35±0
Tet+ Thyme	13±1	7±0	20±1
Tet+ Cinnamon	18±1	12±0	27±1
Tet+ Rosemary	16±1	11±1	22±1
Tet+ Coriandrum	16±0	9±0	20±0
Tet+ Black cumin	23±1	8±1	27±1
Tet+ Mint	17±0	9±1	27±1
Tet+ Black pepper	21±1	11±1	33±2
Tet+ Sage	15±1	10±2	24±0
Tet+ Eucalyptus	22±1	13±1	33±1
Tet+ Neem	22±1	11±1	29±1
Tet+ Babul	20±1	11±1	27±0
Tet+ Turmeric	21±1	11±1	29±0
erythromycin			
Ery+ Garlic	18±1	11±1	34±1
Ery+ Ginger	13±1	7±0	24±1
Ery+ Clove	19±1	10±1	27±1
Ery+ Thyme	21±0	9±0	24±1
Ery+ Cinnamon	23±1	14±1	29±1
Ery+ Rosemary	18±1	12±1	31±1
Ery+ Coriandrum	22±1	10±1	30±0
Ery+ Black cumin	19±1	9±0	27±1
Ery+ Mint	21±1	15±	32±1
Ery+ Black pepper	24±1	11±0	29±1
Ery+ Sage	16±1	7±1	25±0
Ery+ Eucalyptus	20±0	13±1	31±1
Ery+ Neem	23±1	14±1	33±1
Ery+ Babul	16±1	12±1	29±1
Ery+ Turmeric	21±1	15±1	33±1
ciprofloxacin			
Cip + Garlic	15±1	8±0	24±1
Cip +ginger	20±1	14±1	26±1
Cip +clove	18±1	11±0	29±1
Cip + Thyme	17±1	6±0	31±1
Cip +cinamon	15±1	14±1	28±1
Cip +rosemary	15±1	9±1	31±1
Cip + Coriandrum	15±1	7±1	26±1
Cip +black cumin	14±1	10±0	22±1
Cip +mint	18±1	13±1	35±1
Cip + Black pepper	15±1	11±1	32±1
Cip +sage	13±1	7±1	22±1
Cip +eucalyptus	20±1	9±1	28±1
Cip +neem	15±1	7±1	26±1
Cip + babul	15±1	6±0	22±1
Cip + termeric	18±1	15±0	35±1

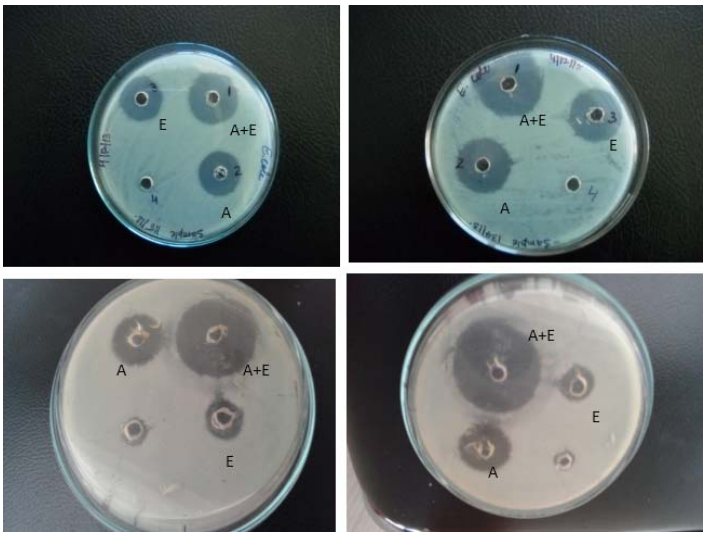

Fig 1: A- Antibiotic alone, A+E- Antibiotic + Extract and E- Extract alone. Synergistic activity expressed in terms of clear zone (mm dia) produced around the well (6 mm) by combination of different plant extracts and antibiotics after incubation at 37°C for 24 hrs of incubation.

3.2 Berberine potentiation assay

Out of 15 methanolic Plant extracts of only 8 plant extracts Shows efflux pump inhibitory activity while 7 methanolic plant extracts have not shown any efflux pump inhibitory activity as shown in table (3). The 1000 µg/ml concentration shows effective efflux pump inhibitory activity while 100µg/ml concentration of all plant extracts have shown less EPI activity for *Escherichia coli* as shown in (Fig 2). An OD less than 0.04 considered to reveal no bacterial growth. No bacterial growth in the presence of berberine indicates the presence of an MDR inhibitor in plant extract.

Table 3: Detection of efflux pump inhibitory activity in methanolic plant extracts by using berberine as a marker for different strains of *E. coli*

S. No	English name	Botanical name	Parts used	EPI activity
1	Garlic	*Allium sativum*	Bulbs	Detected
2	Ginger	*Zingiber officinale*	Rhizome	Detected
3	Turmeric	*Curcuma longa*	Rhizome	Detected
4	Clove	*Syzygium aromaticum*	Bud	Detected
5	Rosemary	*Rosmarinus officinalis*	Leaf	Detected
6	Neem	*Azadirachta indica*	Leaf	Detected
7	Mint	*Mentha longifolia*	Leaf	Detected
8	Coriandrum	*Coriandrum sativum*	Leaf	Detected

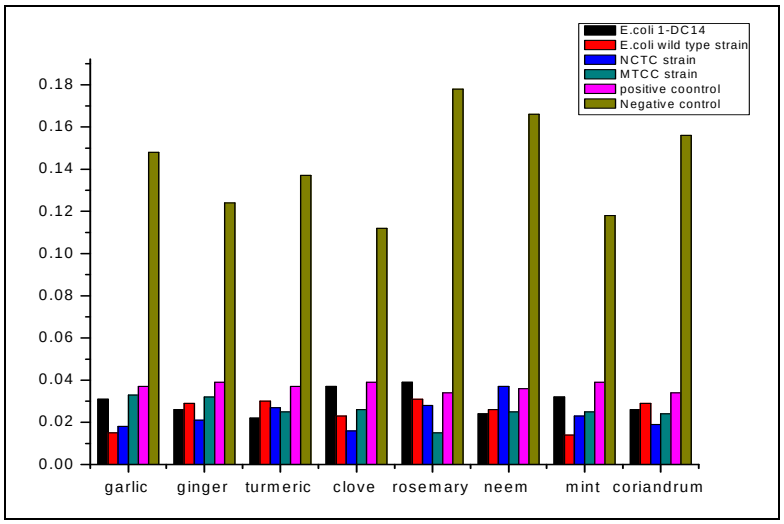

Fig 2: Absorbance shown by garlic, ginger, turmeric, clove, rosemary, neem, mint, coriandrum at concentration of 1000 µg/ml by *E. coli* strains.

3.3 Ethidium bromide assay:

Out of 15 methanolic plant extracts of only 8 plants shows efflux pump inhibitory activity while other 7 plants did not show any efflux pump inhibitory activity. At 1000µg/ml concentration the plants were more effective in accumulating ethidium bromide while 100µg/ml concentration shows less effective results as explained in (Fig 3).

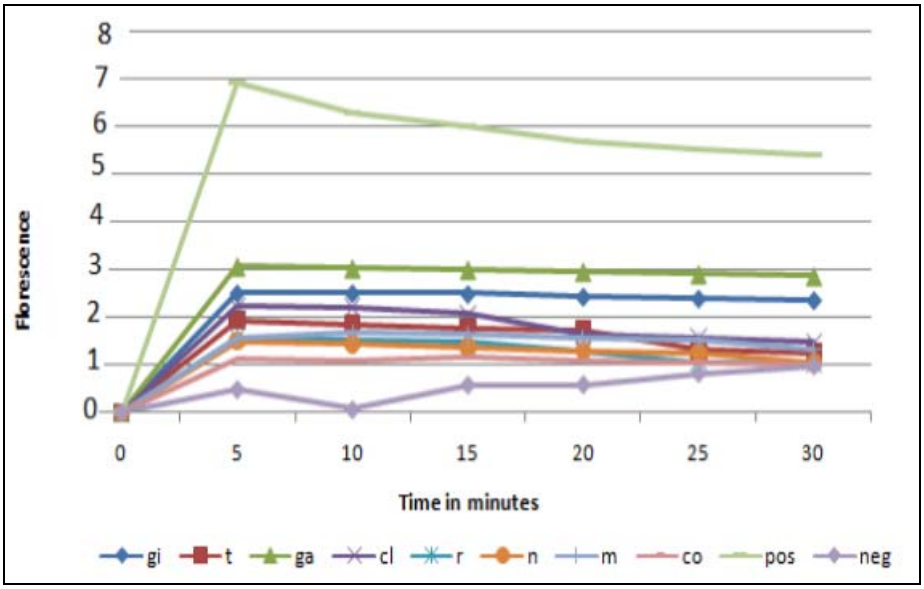

Fig 3: Effect of plants on accumulation of Ethidium bromide at a concentration of 1000ug/ml by *E. coli* strains.

4 Discussion

The study of efflux pump inhibitor assays shows that there are some chemical components present in plants which plays an important role in inhibition of bacteria. One important property that a putative efflux pump inhibitor should possess is that it should show synergistic activity with antibiotics against bacteria. Ciprofloxacin is used as a substrate in case of numerous bacterial efflux pumps [18]. The degree of sensitivity varies in bacteria because of the presence of different efflux pumps in different strains of bacteria and nature of the phytocompounds which are present in the crude plant extract. Out of the 20 methanolic plant extracts, in 8 methanolic plant extracts the efflux pump inhibitory activity was detected. The present study supports Starvi *et al*. [19], as most of the plant extracts shows efflux pump inhibitory activity against *E. coli* [20] This is because of the availability of different chemical compounds which are present in the crude plant extracts having efflux pump inhibitory activity. So, the methanolic extract of these 8 plants have a great future ahead in the development of effective efflux pump inhibitor which helps in improving the antibacterial activities of standard antibiotics. The determination of plants which shows efflux pump inhibitory activity is important for the future use of these plant extracts with existing antibiotics which are in effective because of the presence of MDR pumps in gram- negative bacteria.

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