Original Research Article

Plasmid Curing Activity by Seed Extracts of Cuminum cyminum, Coriandrum sativum and Myristica fragrans Houtt. and Fruit Peel Extracts of Orange, Banana and Pineapple Against Gram Negative Bacteria

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ABSTRACT

In this study, previously cultured isolates of Acinetobacter spp., Pseudomonas spp., E.coli, Klebsiella pneumonia and Proteus spp. were used. Methanolic extracts of seeds of Cuminum cyminum, Coriandrum sativum and Myristica fragrans Houtt. as well as fruit peel extracts of orange, banana and pineapple were prepared to study plasmid curing property of all extracts against Acinetobacter spp., Pseudomonas spp., E.coli, Klebsiella pneumoniae and Proteus spp. Plasmid DNA were isolated by Kado and Liu (1981) method from respective isolates. Three plasmid DNA bands and one plasmid DNA band were observed under UV transilluminator by agarose gel electrophoresis from isolates of Acinetobacter spp., Pseudomonas spp., E.coli and Proteus spp. respectively. The susceptibility of isolates to antibiotics such as Tetracycline, Erythromycin, Ampicillin, Neomycin, Levofloxacin, Amoxicillin, Norfloxac, Azithromycin, Amikacin, Cefixime, Doxycillin, Cephalosporin, Trimethoprim, Streptomycin and Ciprofloxacin as well as for heavy metals such as Fe, Cu, Mn, Ca, Al, Mg, Ni, Pb, K, Ba, Co, Mo, Li, Zn, S, Cr, Ag and Hg were tested. It is cleared from this study that the methanolic extracts of Coriandrum sativum showed better plasmid curing activity than the methanolic extracts of Cuminum cyminum and Myristica fragrans Houtt. Coriandrum sativum showed least plasmid curing activity against Pseudomonas spp. Curing percentage for Cuminum cyminum was 33.33-50%, for Coriandrum sativum was 10-75% while for Myristica fragrans Houtt. was 20-72%. Methanolic extracts of orange, banana and pineapple peels showed no curing activity against tested isolates. Rather these extract showed overgrowth of bacteria and fungus other than tested isolates since it supplies nutritional components to organisms. So active biomolecules from methanolic extracts of seeds of Cuminum cyminum, Coriandrum sativum and Myristica fragrans Houtt. may prove to be potential source for dissemination of multiple drug

Keywords
Antibiotic resistance, Cuminum cyminum, Coriandrum sativum and Myristica fragrans Houtt., Fruit peels, Plasmid curing
Introduction

The discovery of antibiotics in 1928 and effective and efficient use of antibiotics to control infectious agents began in 1940 (Barbour et al., 2004). Due to overuse of antibiotics to treat microbial infections, microorganisms are becoming highly resistant to available antibiotics. Bacteria have evolved numerous defenses against the antibiotics, susceptibility to antibiotics get reduced now a days and drug resistant pathogens are on the rise (Kumar et al., 2013). Immense clinical problems arise in immune compromised and cancer patients due to MDR bacteria (Jasmine et al., 2011). So Multiple Drug Resistance (MDR) is a serious, global and future challenge to the chemotherapy or drug discovery programs (Shriram et al., 2010). Medical plants are used in the preparation of herbal medicines since ancient times, as they have antiviral, antifungal and antibacterial activities (Akrayi, 2011). With the development of new technologies, study of plants, isolation of active components, their role and pharmacological effects become easier. Medicinal plants contain wide variety of active compounds such as terpenoids, alkaloids, sulphur compounds, phenols, esters, aldehydes, alcohols, glycosides, thymol are responsible for antibacterial, antifungal and antibacterial activities (Bhattacharya et al., 2013). Phytochemicals have a novel anti-bacterial action like inhibition of MDR efflux pump, anti-antibiotic resistance properties, R plasmid elimination. So, elimination of R plasmid containing genes is responsible for MDR is useful method (Jasmine et al., 2011). Genes responsible for resistance to antibiotics are present in plasmid DNA. R plasmids contains variety of genes encoding resistance to a broad spectrum antimicrobial agents like antibiotics, heavy metals, disinfectant like formaldehyde, mutagenic agents like ethidium bromide (Kumar et al., 2013). Resistance to antibiotics by R plasmid are due to conjugation(Kumar et al., 2013, Shriram et al., 2010, Shriram et al., 2013). Gram Negative microorganisms have developed coping mechanisms in order to survive in environments with high toxic levels of metals. Therefore they can be used as bio-indicators to detect heavy metal pollution in the environment. The survival of these organisms relies on the intrinsic biochemical, structural, and physiological properties and genetic adaptations. There are generally four basic mechanisms used in heavy metal resistance and include: (i)exclusion of toxic heavy metal ions from the cell by the alteration of membrane transport systems involved in initial cellular accumulation, (ii) extra- and intra-cellular sequestration of metal binding components similar to metallothioneins, (iii) cation/anion efflux systems that are encoded by resistance genes, and (iv) enzymatic detoxification of heavy metals from toxic to less toxic forms. Plasmid curing is a process of completely removing plasmids from bacteria by means of chemical agents or now a day by means herbal extracts or phytocompounds. Synthetic plasmid curing agents like acridine orange, ethidium bromide, sodium dodecyl sulfate are toxic and mutagenic in nature. So, they are unsuitable for therapeutic application. But herbal extracts have more effective to use, do not have toxicity and do not have mutagenic effects; so they are safer to use as a plasmid curing agents (Kumar et al., 2013). Resistance genes play important role in acquisition and dissemination by the exchanges between plasmid(s) and the bacterial chromosome and the integration of resistance genes into specialized genetic elements, called integrons (Carattoli, 2003). Plasmids carry resistance genes which are mobile. During plasmid borne antibiotic resistance, the dosage and treatment is also
important. Plasmids which have resistance genes are in competition with other plasmids that do not carry the resistance genes (Svara et al., 2011).

*Cuminum cyminum* belonging to *Apiaceae* family is an annual plant which growing to a height of 15-30cm according to environmental conditions. *Cuminum cyminum* is stomachic, diuretic, carminative, stimulant, astringent, emmenagogic and antispasmodic. It is valuable in dyspepsia diarrhea and hoarseness and many relieve flatulence and colic. It is supported to increase lactation and reduce nausea in pregnancy. It has been shown to be effective in treating Carpel tunnel syndrome as well as indigestion and morning sickness. *Cuminum cyminum* seeds have essential oils containing a high proportion of myrtenal, *p*-cymene, pulgan, cineol, flanderin, Benzaldehyde and small quantities of *c*-Terpinene and 2 J-pinene (Abushama et al., 2013).

*Myristica fragrans* Houtt. is an evergreen tree, native of the *E. Moluccas* and cultivated throughout Malaya. It is found only as a specimen tree in Botanical gardens. The seed of the plant is known as “nutmeg” contains many volatile oils. These oil constituents have a variety of individual pharmacological effects, some of which oppose others (Jellin et al., 2005). The fruit contains ethereal oil-cells often with phenolic and myristicin; the seed and the aril are used for flavouring food. Nutmeg has aromatic, stimulant, narcotic, carminative, astringent, aphrodisiac, hypolipidaemic, antithrombotic, anti-platelet aggregation, antifungal, anticytotoxic, and anti-inflammatory activities. The spice is used as a remedy for stomach ache, rheumatism, and vomiting during pregnancy Sonavanne et al. (2002) explained that the n-hexane extract of nutmeg seeds has anxiogenic, sedative and analgesic activities. Nevertheless, no evidence was found to support the previously published reports of nutmeg’s hallucinogenic or other psychoactive properties besides mild sedation. The major compounds in the oil were sabinene (21.38%), 4-terpineol (13.92%) and myristicin (13.57%). On the other hand, allylbenzene and propylbenzene derivatives (myristicin, safrole, eugenol, and derivatives thereof) were the predominant compounds in nutmeg seeds (Parimala, 2013).

Coriander (*Coriandrum sativum* L.) – an annual of the *Apiaceae* family, is one of valuable medicinal, seasoning and oliferous plants. This species comes from the Mediterranean region and it is grown all over the world. The coriander fruit and the essential oil isolated from it are used for medicinal purposes (Duarte et al., 2012; Mahendra et al., 2011; Sriti et al., 2011; Chung et al., 2012). The coriander fruit shows relaxant activity in the alimentary tract; Coriander is used in the preparation of many household medicines to cure bed cold, seasonal fever, nausea, vomiting, stomach disorders and also used as a drug for indigestion, against worms, rheumatism and pain in the joints. Many of healing properties of coriander can be attributed to its exceptional phytonutrients and hence, it is often referred to as store house for bioactive compounds. Coriander raw material and oil are also used as an aromatising agent in the pharmaceutical, food, cosmetics and perfume industries. Coriander seeds contain petroselinic acid, linoleic acid, oleic acid and palmitic acid. Major components of essential oil are linalool, *α*-pinene, camphor and geraniol (Bhuiyan, 2009).

Fruit peels of Orange, Pineapple and Banana possess antifungal and antibacterial property. Therefore they are used in food
industry cosmetics and as biofertilizers. Being a protective skin of fruit it has property of tolerating harsh environment and is also resistant to microbial population in the environment.

Materials and Methods

Plant materials

Plant material (seeds) of *Cuminum cyminum*, *Corandrum sativum* and *Myristica fragrans* Houtt. were collected from an local shop as well as fruit peels of orange, banana and pineapple were collected from fruit juice shop in Pune, Maharashtra.

Extraction of plant materials

Seeds of *Cuminum cyminum*, *Corandrum sativum* and *Myristica fragrans* Houtt. as well as dried fruit peels of orange, banana and pineapple were finely powdered with auto-mix blender. 20g powder of seeds of *Cuminum cyminum*, *Corandrum sativum* and *Myristica fragrans* Houtt. as well as fruit peel of orange, banana and pineapple were extracted with 200ml methanol using Soxhlet apparatus for a period of 5-6 h. The extracts were concentrated using Vacuum Rota Evaporator (SUPERFIT™) to obtain a semisolid mass and 50°C oven dried to get a dry form of extracts. Dried extracts were stored in a crucible at room temperature.

Bacterial isolates

Previously cultured and identified isolates of *Acinetobacter* spp., *Pseudomonas* spp., *Proteus* spp., *E. coli* and *Klebsiella pneumoniae* were used. Plasmid DNA was isolated by Kado and Liu (1983) method from respective isolates. Plasmid DNA bands were observed under UV Transilluminator by agarose gel electrophoresis from all isolated strains.

Determination of antibiotic and metal resistance

Isolates of *Acinetobacter* spp., *Pseudomonas* spp., *Proteus* spp., *E. coli* and *Klebsiella pneumoniae* were inoculated in sterile Luria broth and kept it on rotary shaker for overnight at 100rpm at 37°C. Dilutions of antibiotics such as Tetracycline, Erythromycin, Ampicillin, Neomycin, Levofloxacin, Amoxicillin, Norflox, Azithromycin, Rifampicin, Ofloxacin, Gentamicin, Cephalosporin, Trimethoprim, Amikacin, Streptomycin, Doxycillin, Cefixime and Ciprofloxacinand metals such as Fe, Cu, Mn, Ca, Al, Mg, Ni, Pb, K, Ba, Co, Mo, Li, Zn, S, Cr, Ag and Hg from 2-1024 µg/ml were prepared and each required dilution were transferred on sterile Muller Hinton Broth. After 24 h. Grid method was performed for spot inoculations of respective isolates on a respective dilution of antibiotic and metal containing Muller Hinton agar plates. The plates were incubated at 37°C for 24–48h. The growth of each isolate on respective dilutions of antibiotic or metal containing Muller Hinton agar plates was monitored. The susceptibility of isolates to antibiotics such as Tetracycline, Erythromycin, Ampicillin, Neomycin, Levofloxacin, Amoxicillin, Norflox and Azithromycin, Rifampicin, Ofloxacin, Gentamicin, Cephalosporin, Trimethoprim, Amikacin, Streptomycin, Doxycillin, Cefixime and Ciprofloxacin as well as to metals such as Fe, Cu, Mn, Ca, Al, Mg, Ni, Pb, K, Ba, Co, Mo, Li, Zn, S, Cr, Ag and Hg were tested.

Curing of plasmid mediated antibiotic resistance

Methanolic extracts of seeds of *Cuminum cyminum*, *Corandrum sativum* and *Myristica fragrans* Houtt. were dissolved in 50ml dimethyl sulfoxide (DMSO) and were used
further. Dilutions of all three methanolic extracts from 2-1024µg/ml were prepared separately and to each dilution 100µl bacterial culture were added having O.D. 0.04-0.05 at 660nm. The tubes were incubated at 37°C for 24 h. and after 24 h. culture were plated on Muller Hinton agar plates to obtain isolated colonies. Isolated colonies were then replica plated to Muller Hinton agar plates with antibiotics. The colonies grew in Muller Hinton agar plates but failed to grow in presence of antibiotics were considered as cure derivatives. The percentage curing efficiency was expressed as number of colonies with cured phenotype per 100 colonies tested (Shriram et al., 2013).

**Results and Discussion**

The extraction of all three plant materials such as *Cuminum cyminum*, *Coriandrum sativum* and *Myristica fragrans* Houtt. were done successful by Soxhlet Extraction method. Plasmid DNA was isolated by Kado and Liu (1981) method from respective isolates. Three plasmid DNA bands having molecular weights 4000bp, 5000bp and 7000bp and one plasmid DNA band having molecular weight 7000bp were observed under UV transilluminator by agarose gel electrophoresis from isolates of *Acinetobacter spp.*, *Pseudomonas spp.*, *Klebsiella pneumoniae*, *E. coli* and *Proteus spp.* respectively. In our study the strain of *E.coli* was isolated from sewage sample, strain of *Klebsiella pneumonia* and *Proteus spp.* were isolated from the hospital samples. The isolated strain of *Acinetobacter spp.* showed highest resistance to almost all antibiotics except Rifampicin, Oflaxacin, Ampicillin, Amoxicillin, Levofloxacin, Norflox and Azithromycin. The isolated strain of *Pseudomonas spp.* showed highest resistance to almost all antibiotics except Oflaxacin, Levofloxacin, Norflox and Streptomycin. The isolated strain of *Klebsiella pneumonia* and *Proteus spp.* showed highest resistance to almost all antibiotics except Oflaxacin, Gentamicin, Ampicillin, Tetracyclin and Levofloxacin. All isolated strains were resistant to all metals such as Fe, Cu, Mn, Ca, Al, Mg, Ni, Pb, K, Ba, Co, Mo, Li, Zn, S, Cr, Ag and Hg. In our study, the methanolic extracts of *Coriandrum sativum* showed better plasmid curing activity than the methanolic extracts of *Cuminum cyminum* and *Myristica fragrans* Houtt. *Coriandrum sativum* showed least plasmid curing activity against *Pseudomonas spp.* Curing percentage for *Cuminum cyminum* was 33.33-50%, for *Coriandrum sativum* was 10-75% while for *Myristica fragrans* Houtt. was 20–72% (Table 2).

Using methanolic extracts of seeds of *Cuminum cyminum*, *Coriandrum sativum* and *Myristica fragrans* Houtt., all plasmid DNA present in the *Acinetobacter spp.*, *E.coli* and *Proteus spp.* were cured completely. But, only one plasmid DNA having molecular weight 7000 bp present in *Klebsiella pneumonia* and *Pseudomonas spp.* was cured. So, to get complete curing, it is necessary to increase the concentration of methanolic extracts of seeds of *Cuminum cyminum*, *Coriandrum sativum* and *Myristica fragrans* Houtt. Methanolic extracts of orange, banana and pineapple peels showed no curing activity against tested isolates. Rather these extract showed overgrowth of bacteria and fungus other than tested isolates since it supplies nutritional components to organisms.
Table 1: Metal resistance of isolated strains

<table>
<thead>
<tr>
<th>Name of Metal</th>
<th>Concentration of metals in μg per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Fe</td>
<td>+</td>
</tr>
<tr>
<td>Cu</td>
<td>+</td>
</tr>
<tr>
<td>Mn</td>
<td>+</td>
</tr>
<tr>
<td>Ca</td>
<td>+</td>
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<tr>
<td>Al</td>
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<tr>
<td>Mg</td>
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<tr>
<td>Ni</td>
<td>+</td>
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<td>Pb</td>
<td>+</td>
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<tr>
<td>K</td>
<td>+</td>
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<td>Ba</td>
<td>+</td>
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<td>Co</td>
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<td>Mo</td>
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<td>Li</td>
<td>+</td>
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<td>Zn</td>
<td>+</td>
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<tr>
<td>S</td>
<td>+</td>
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<tr>
<td>Cr</td>
<td>+</td>
</tr>
<tr>
<td>Ag</td>
<td>+</td>
</tr>
<tr>
<td>Hg</td>
<td>+</td>
</tr>
</tbody>
</table>

Legends:
C: *E. coli*                      D: *Klebsiella pneumoniae*
E: *Proteus* spp.                #: Antibiotic Sensitive
+: Antibiotic Resistance
**Table 2** Percent plasmid curing for all tested strains

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Name of Extracts</th>
<th>Name of Micro-organisms</th>
<th>Number of Colonies with Cured Phenotype</th>
<th>Total Number of Colonies</th>
<th>Percent Plasmid Curing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Cuminum cyminum</em></td>
<td>Acinetobacter <em>spp.</em></td>
<td>4</td>
<td>12</td>
<td>33.33%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Pseudomonas spp.</em></td>
<td>3</td>
<td>7</td>
<td>42.85%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>E.coli</em></td>
<td>1</td>
<td>2</td>
<td>50%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Klebsiella pneumonia</em></td>
<td>2</td>
<td>6</td>
<td>33.33%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Proteus spp.</em></td>
<td>4</td>
<td>8</td>
<td>50%</td>
</tr>
<tr>
<td>2.</td>
<td><em>Coriandrum sativum</em></td>
<td>Acinetobacter <em>spp.</em></td>
<td>6</td>
<td>8</td>
<td>75%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Pseudomonas spp.</em></td>
<td>2</td>
<td>20</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>E.coli</em></td>
<td>1</td>
<td>2</td>
<td>50%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Klebsiella pneumonia</em></td>
<td>1</td>
<td>2</td>
<td>50%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Proteus spp.</em></td>
<td>4</td>
<td>8</td>
<td>50%</td>
</tr>
<tr>
<td>3.</td>
<td><em>Myristica fragrans Houtt.</em></td>
<td>Acinetobacter <em>spp.</em></td>
<td>5</td>
<td>9</td>
<td>55.55%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Pseudomonas spp.</em></td>
<td>8</td>
<td>11</td>
<td>72%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>E.coli</em></td>
<td>1</td>
<td>3</td>
<td>33.33%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Klebsiella pneumonia</em></td>
<td>3</td>
<td>7</td>
<td>42.85%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Proteus spp.</em></td>
<td>3</td>
<td>14</td>
<td>20%</td>
</tr>
</tbody>
</table>
Figure 1. Plasmid curing mediated antibiotic resistance reversal for *Pseudomonas spp.* by methanolic extracts of seeds of *Coriandrum sativum* (a) Control plate, (b) Absence of colonies on antibiotic Muller Hinton agar plates indicites putative cured derivatives.
Figure.2 Plasmid curing mediated antibiotic resistance reversal for *Proteus spp.* by methanolic extracts of seeds of *Myristica fragrans* Houtt. (a) Control plate, (b) Absence of colonies on antibiotic Muller Hinton agar plates indicates putative cured derivatives

The extraction of *Richardia brasiliensis* was done by Soxhlet Extraction method (Morias, 2013). The ethanolic extract of *Trachyspermum ammi* was also prepared by Soxhlet Extraction to separate the secondary metabolites (Mobashshera, 2014) which is similar to our study. The strain of *Proteus spp.* was collected from Agharkar Research Institute, Pune and Microbial Type Culture Collection (MTCC), they have got only one plasmid DNA band (Shriram, 2013) as well as the strain of *Proteus spp.*(ATCC 25938) is also contain only one plasmid DNA (Bhattacharya, 2013) which are similar to our study. The resistance of micro-organisms occurs by several mechanisms like efflux pump, production of enzymes that inactivate the drug and change the target of action of antibiotic. The resistance mechanisms are transmitted through exchange of genetic materials between micro-organisms of the same species (Oliveira, 2006). In the study carried out by Ronald *et al.* (2002), over 40% of the bacteria resistant to more than one antibiotic had atleast one plasmid which is comparable to our study. From Fever Hospital and Chest Department, Zagzig University, Egypt, the isolated strains of *Klebsiella pneumoniae* as well as *Pseudomonas aeruginosa* showed resistance to Amikacin, Trimethoprim and to Gentamicin, Azithromycin respectively (Azza *et al.*, 2010) which are similar to our study. In the study of plasmid profiling and curing analysis, metal such as nickel shows resistance against *Pseudomonas aeruginosa* (Raja *et al.*, 2009) which is similar to our study. It is well known that there are no currently acceptable concentrations of metal ions which can be used to distinguish metal resistant and metal sensitive bacteria (Malik and Jaiswal, 2000). The curing efficiency of methanolic extract of *Elettaria cardamomum* is in the range of 3-33% (Akrayi, 2011) which is comparable with the curing efficiency of *Cuminum cyminum* as well as the curing efficiency of methanolic extract of *Piper longum* fruitshows 64% of plasmid curing efficiency (Kumar *et al.*, 2013) which is comparable with the curing efficiency of *Coriandrum sativum* and *Myristica fragrans* Houtt. It can be
concluded from the present results that the methanolic extracts of seeds of *Cuminum cyminum*, *Coriandrum sativum* and *Myristica fragrans* Houtt. successfully reversed the multiple drug resistance in cured derivatives and making them sensitive to antibiotics. This antibiotic resistance reversal may be attributed to the curing of R-plasmids harbored by these MDR bacterial strains. These active biomolecules from methanolic extracts of seeds of *Cuminum cyminum*, *Coriandrum sativum* and *Myristica fragrans* Houtt. proved to be potential source for dissemination of multiple drug resistant organisms.

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