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Evaluation of carvacrol as an antibacterial agent against *Escherichia coli* isolated from different animal species

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Abstract

Excessive and indiscriminate use of antibiotics as therapeutic agent against many infectious diseases or as growth promoters lead to antimicrobial resistance to a greater extent. Antibiotic resistant bacteria are of public health importance and there is need to identify new compounds with antibacterial properties without any side effect. Herbal drugs offer an alternative approach to prevent the infectious diseases and hence can help in minimizing the antimicrobial resistance. In the present investigation, we have evaluated the *in vitro* antibacterial activity of carvacrol and its synergistic effects with antibiotics (Imipenem and colistin) against *Escherichia coli* isolates from faecal samples of various animal species by disc diffusion method. Total 430 *E. coli* isolates were screened for the carvacrol sensitivity and it was observed that all *E. coli* isolates were sensitive to carvacrol. The same isolates were screened for synergy between carvacrol and antibiotics (Imipenem and colistin). Total 14 (3.25%) *E. coli* isolates have shown synergy With Imipenem whereas none was synergistic with colistin. Among these, 6 (5.45%), 3 (2.6%), 2 (1.9%), (2.5%) and (4.1%) isolates were of pig, cattle, poultry, sheep and goat respectively. In conclusion, the carvacrol can be used as therapeutic agent against *E. coli* as alone or in combination with other antibiotics. Further work is needed to explore the antibacterial potential of carvacrol against another bacterial species as well as synergy with other antibiotics.

Keywords: Carvacrol, antibacterial, *Escherichia coli*

Introduction

Herbal drugs offers as an alternative to antibiotics as therapeutic agent due to increase in antibiotic resistant bacteria in last few years [2]. Herbal drugs possess various properties which includes antimicrobial, antibacterial and antifungal properties and are frequently used as preservatives in the food industry [3]. Also, the herbal drugs show increased antimicrobial activity due to synergistic effect with some antibiotics [4].

Carvacrol (2-Methyl-5-(1-methylethyl) phenol] is a hydrophobic monoterpene which has the ability to penetrates bacterial cell membrane, disrupts its integrity and cause release of bacterial cell contents [5, 6]. Carvacrol can inhibit growth of gram-positive and gram-negative bacteria [7] Many essential oils i.e. *Thymus vulgaris*, *Origanum vulgare*, *Trachyspermum ammi*, *Lepidium africanum* and *Citrus bergamia* has carvacrol in them and it is reported that these essential oils has an antimicrobial properties [7, 8, 9]. The carvacrol has been tested against number of bacteria like *Escherichia coli* O157:H7, *Bacillus cereus*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Pseudomonas fluorescens*, *Salmonella typhimurium*, *Vibrio cholera* etc. and proved as bacteriostatic as well as bactericidal [10, 11, 12, 13, 14]. In case of planktonic staphylococci, *Staphylococcus aureus* was resistance to higher concentrations of carvacrol (20 mM) [15]. The synergistic effects of carvacrol in combination with macrolides have also been reported [4].

As the antimicrobial resistance scenario is continuously changing, the present study was conducted to determine the antibacterial activity of carvacrol and synergistic activity with selected antibiotics in the *E. coli* isolates of animal origin.

Material and Methods**Isolation of *E. coli***

A total of 430 *E. coli* isolates from faecal samples of different animal species (cattle, goat, pig, poultry and sheep) were cultured by following the standard protocol. The isolates were stored in glycerol stocks at -20°C.

Sample processing

The rectal swab was transferred to 5 ml of Mac Conkey broth (MLB) and incubated for 18-24 h at 37°C. The broth culture was streaked on to Mac Conkey agar (MLA) plates and Eosin Methylene Blue Agar (EMB) plates and incubated at 37°C overnight. All the 430 isolates were identified based on colony characteristics as putative *E. coli* spp. for the further confirmation. A series of biochemical tests were carried out to confirm the *E. coli*.

Isolation and Identification of *E. coli*

The colonies showing metallic sheen or dark purple colonies on EMB agar plates were picked individually and inoculated into LB agar to get isolated pure colony. The presumptive isolates were tested for *E. coli*, specific tests as production of indole, lysine decarboxylase, Utilization of citrate [16], malonate, sucrose, lactose and glucose [17].

Antibacterial agents used in the study: Discs of Carvacrol were prepared by soaking 4 mm diameter discs of Whatman filter paper no. 3 in 4% Carvacrol (Sigma) in methanol. Imipenem (IPM-10µg) and colistin (CL-10µg) discs were obtained from Becton, Dickinson and Company (BD, Difco).

Preparation of *E. coli* culture for sensitivity testing: The *E. coli* culture was grown overnight in Luria Bertani (LB) broth at 37°C. The loopful culture was inoculated on Muller Hinton agar (MHA) and incubated for 24 hrs. Later on, colony was inoculated into Muller Hinton broth (MHB) and the concentration was adjusted to 0.5 McFarland standard to contain approximately count of 1×10^4 cfu/ml.

Screening of *E. coli* for carvacrol resistance by disc diffusion assay

The isolate was streaked on a single plate providing a distance of 10 -15 mm apart from other discs. The plates were incubated at 37 °C overnight in a bacteriological incubator. Each plate was observed for growth or zone of inhibition around or near the carvacrol disc. The isolates that did not grow near the carvacrol disc were considered as sensitive to carvacrol where as those isolates which grow near the discs were considered as carvacrol resistant isolates.

Screening of *E. coli* isolates for synergy by disc diffusion assay

The culture was swab inoculated on to MHA. Thereafter, the carvacrol disc was placed in the centre of the agar plate and both antibiotic discs at 15 mm apart on the periphery. The plates were incubated overnight to check the zone of inhibition around antibiotic discs. The key-hole formation between growth inhibition zones of carvacrol and any of the antibiotics tested was considered as a positive indicator of synergy between the two. Absence of keyhole is considered as control against positive result.

Results

Total 430 *E. coli* isolates were screened for carvacrol resistance. None of isolates were resistant to carvacrol. Furthermore, 14/430 (3.25%). *E. coli* isolates showed synergy between carvacrol and imipenem by disc diffusion method (Fig. 1). None of the isolates have shown synergy with CL (Fig. 2). Out of 14 *E. coli* isolates, 6 (5.45%) isolates were of pig, 3 (2.6%) of cattle, 2 (1.9%) of poultry and one isolate each from sheep (2.5%) and goat (4.1%) (Table 1).

Table 1: *E. coli* isolates of different origin showing synergy between carvacrol and imipenem

Sources of isolates	<i>E. coli</i> tested	Number of <i>E. coli</i> isolates showing synergy (%)	
		IPM	CL
Cattle	114	3 (2.6)	Nil
Sheep	39	1 (2.5)	Nil
Goats	24	1 (4.1)	Nil
Pigs	110	6 (5.45)	Nil
Poultry	105	2 (1.90)	Nil
Drainages	38	Nil	Nil
Total	430	14 (3.25)	Nil

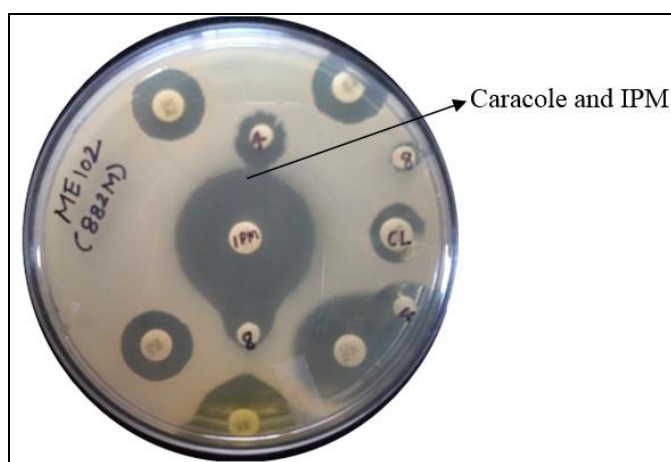


Fig 1: Synergy between Carvacrol and Imipenem (IPM)

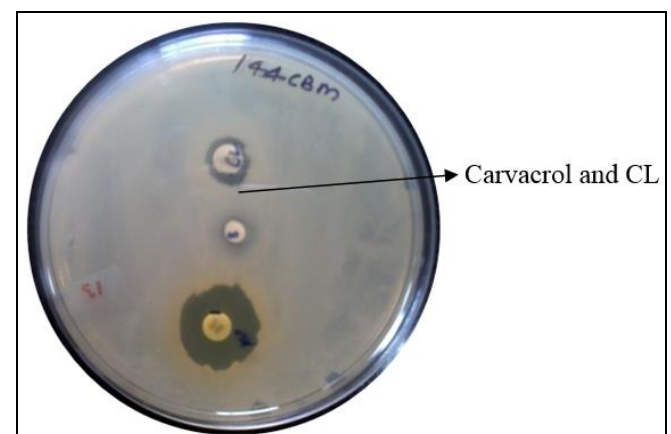


Fig 2: Absence of Synergy between Carvacrol and Colistin

Discussion

In past few years, the emergence of MDR organisms is making difficult to control bacterial infections [18]. Therefore, the emphasis is given by researchers on alternative therapeutic strategies to combat infections without aggravating drug resistance further [19]. Herbal drugs have potential to be used as alternative to growth promoting antibiotics [20]. Among these, carvacrol is the main component of commonly used oregano and thyme oil [21].

In the present study, strong antibacterial effect of carvacrol was observed *in vitro* against *E. coli*. None of isolates were resistant to carvacrol. Furthermore, 14/ 430 (3.25%). *E. coli* isolates showed synergy between carvacrol and imipenem by disc diffusion method (Fig. 1). None of the isolates have shown synergy with CL (Fig. 2). Out of 14 *E. coli* isolates, 6 (5.45%) isolates were of pig, 3 (2.6%) of cattle, 2 (1.9%) of poultry and one isolate each from sheep (2.5%) and goat (4.1%). The *E. coli* isolates were of different animal origin, so

they were compared to access the effect of synergy on each other. On comparing *E. coli* isolates from different sources revealed that isolates from cattle showed significantly higher levels of synergy than in pig ($p < 0.01$), poultry ($p < 0.05$) and goat ($p < 0.01$) isolates while there is no significant difference with sheep isolates. Likewise, isolates from goats showed significantly ($p < 0.01$) higher synergy than isolates of poultry origin. The isolates from pigs has significant ($p < 0.01$) higher synergistic activity than isolates from poultry and sheep. Isolates from poultry had no significant difference for synergy than isolates from sheep origin.

The study is supported by few researchers who suggested that carvacrol has potent antimicrobial activity against ESBL *E. coli* isolated from ascetic fluid of UTI patients [22]. Also in previous studies, same antimicrobial effects of carvacrol have been reported against *Salmonella enteritidis* and *Campylobacter jejuni* [23]. Many studies included determination of MIC for carvacrol against many bacteria including *E. coli*. The MIC of carvacrol against *E. coli* was 400 µg/mL [11]; 160 and 320 µg/mL against *E. coli* and *S. enteritidis* for thymol, respectively [24] and MICs of 240 and 300 µg/mL, for thymol and carvacrol respectively against *C. perfringens* [25]. Carvacrol in combination with antibiotics (azithromycin, clarithromycin, tigecycline and minocycline) showed synergism using checkerboard analysis [26]. Carvacrol has increased susceptibility of *S. typhimurium* to antibiotics (ampicillin, tetracycline, penicillin, bacitracin, erythromycin and novobiocin) [7]. Carvacrol permeabilize and depolarize the bacterial membrane and inhibit growth of *E. coli*. [11].

The carvacrol was synergistic with imipenem against few *E. coli* isolates and has not shown any synergistic activity with colistin in disc diffusion method. Although the antibacterial mechanism of carvacrol is not fully understood, studies have shown the drugs with phenol group like eugenol, carvacrol and thymol have the greatest bactericidal activities, followed by aldehydes, ketones, alcohols, ethers and hydrocarbons [27]. Carvacrol may induce inhibition of growth of both gram positive and gram negative bacteria via membrane damage resulting in an increase in membrane permeability and disruption of cell wall [28]. So, it is possible that carvacrol is acting synergistically with Imipenem. On other hand, colistin also cause damage to cell membrane but even then it has not shown synergy with carvacrol, might be due to some other reason.

So, if we compare herbal drugs and antibiotics in terms of activity against bacteria, one may neither be effective nor ineffective against all bacteria [29]. In previous studies, carvacrol was reported as effective herbal drug against *E. coli* isolates from different sources [30]. The strong antibacterial activity of carvacrol makes it a potent antimicrobial agent whereas it can be used as future alternative to many life threatening infections on combination with imipenem.

Conclusion

The present study showed that carvacrol possessed strong antibacterial activity against *E. coli* *in vitro*. In addition, an additive effect was found when carvacrol and imipenem were applied in combination. More specific studies are required to prove the actual reason behind the synergistic activity between carvacrol and imipenem.

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