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Research Article

Experimental Assessment of Combined Antimicrobial Effect of Lactic Acid Bacteria and Clove Oil against *Escherichia coli*

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ABSTRACT

There has been an increasing threat to the efficacy of antibiotics as more and more pathogens are developing anti-microbial drug resistance. Alternatives are required to be developed for therapeutic purposes so that use of antibiotics can be restricted and their activity can be conserved. The present study is based on the use of combination of Lactic acid bacteria and clove oil against one of the commonest entero- and uro-pathogen, *E.coli* in order to check the anti-microbial activity of the two in a synergistic way. This study is an attempt to look for the possibility of increased anti-microbial activity of lactic acid bacteria and clove oil against *E.coli*, when they are used in combination.

Keyword: Lactic acid bacteria; *E.coli*; Clove oil; antimicrobial activity; anti-microbial resistance

INTRODUCTION

Escherichia coli is a well-known ubiquitous entero- and uro-pathogen that is constantly evolving as antibiotic resistant species. According to a recent cross-sectional study done by Purwar et. al. (2016), enter-pathogenic *E.coli* is one of the two most frequently isolated pathogens among diarrheal patients in India [1]. *E.coli* has also been recently identified as the most common microbe causing Urinary Tract Infection (UTI) and Laboratory Confirmed Blood-Stream Infection (LCBI) in a recently done active surveillance of health care associated infections among neuro-surgical patients at AIIMS, New Delhi [2]. An even more serious observation was that all the isolates were multi-drug resistant [2]. *E.coli* has also been found to be one of the most common pathogenic agents isolated from device associated-hospital associated infections in a study done in a tertiary care multi-disciplinary intensive care unit of a teaching hospital in eastern India [3]. The gravity of the problem is continuously increasing as a decline in sensitivity towards antibiotics has been observed. A recent example is a report from a tertiary care hospital of North-Eastern Karnataka which shows that a significant number of UTIs had been there due to multi-drug resistant *E.coli* along with continuous decrease in its sensitivity pattern towards different antibiotics [4]. Not only human, but animals have also been affected by the antibiotic resistant strains of *E.coli* [5,6]. Considering the global status, there is dissemination of extended spectrum ß-lactamases (ESBLs), broad spectrum

ß-lactamases, plasmid mediated AmpC and carbapenemases hosted by Enterbacteriaceae clones and *P.aeruginosa* with fewer therapeutic options [7,8].

Need for alternative of antibiotics was felt quite many years ago to decrease their usage and to preserve their activity. Few approaches have been successful also, though partially. Prophylaxis with PGG glucan (an immunomodulator) in combination with antibiotics has been shown to exhibit increased protection against *E.coli* as compared to antibiotics alone [9]. Antimicrobial peptides like Ib-AMP1 have also been promising alternatives [10]. Restrictive policy on use of antibiotics has also been a fair strategy so that inappropriate use of antibiotics can be avoided [11]. Natural product like cranberry (*Vaccinium macrocarpon*) is reported to decrease recurrent urinary tract infections and hence help reducing antibiotic usage [12]. Live attenuated vaccines can be developed using non-pathogenic *E.coli* [12]. Many *lactobacilli* species are used since long as probiotics and antagonistism of some *lactobacilli* is known to be quite pronounced towards some pathogenic and opportunistic bacteria like enterpathogenic *Escherichia, shigellae, proteus, staphylococci* [13].A combination of an antibiotic with Lactobacilli (Lactobacillus acidophilus-LA-5) and Bifidobacterium is reported to block *in vitro* attachment of uro-pathogenic bacteria to uro-epithelial cells and found to reduce the incidence of febrile urinary tract infections [12]. Ouwehand et. al. (2010) had also reported that some essential oils exhibit the advantage of inhibiting the growth of potential pathogens of the intestine while only moderately influencing the beneficial members of intestinal microflora [14]. Hawrelak et. al. (2009) suggested that the minimum inhibitory concentrations of some essential oils against pathogenic bacteria are lesser in comparison to those for probiotic bacteria [15]. Moritz et. al. (2012) reported that clove essential oil exhibits only sub-lethal effect on *Lactobacillus rhamnosus* [16]. A strategy based on these observations can be applied against *E.coli* using a combination of probiotic bacteria with other anti-microbial agents like essential oils. Moreover, there have been evidences for antagonistic activities of lactic acid bacteria against *E.coli*. For instance, Genis et. al. (2016) reported that few species of lactic acid bacteria could modulate *E.coli* and the bovine endometrial cells' inflammation [17]. Another evidence is the inhibitory activity of Lactic acid bacilli (*Lactobacillus lactis*) against shiga toxin producing *E.coli* [18, 19] that is thought to result mainly due production of organic acids by lactic acid bacteria [19]. Bacteriocins produced by the probiotic bacteria are also known to act against pathogenic agents. Shipradeep et. al. (2012) have also proposed the possiblity of successful use of combinations of probiotic bacteria and essential oil based on the facts that MICs of many essential oils are higher for probiotic bacteria than those of pathogenic agents and secondly, pathogens do not have any enzymatic activity to inactivate essential oils [20]. Thus, a well optimized combination of an essential oil and probiotic bacteria can be effective against pathogenic *E.coli* [21].

The present work aims to experimentally analyze whether it is feasible to use a combination of clove essential oil and lactic acid bacteria *(Lactobacillus sporogenes)* against *E.coli*, along with assessing an enhancement (if any) of the antimicrobial property of the combination. For this, two approaches have been used-

Low concentration approach: Lactic acid bacteria were allowed to grow in presence of such a concentration of clove oil in which it can survive for defined time (1 hr) and then use the conditioned medium 1 (CM1) against *E.coli* to check its antimicrobial effect;

High concentration approach: Since lactic acid bacteria cannot survive high concentrations of clove oil, they were first allowed to grow in nutrient broth for 24 hrs and then after separation from broth by centrifugation and filtration, clove essential oil at definite higher concentrations were added in it (conditioned medium 2 = CM2) for use against *E.coli*.

MATERIALS AND METHOD

Media for culture of *E.coli* and Lactic acid bacteria were purchased from HiMedia. Stains and chemicals were obtained from Spectrachem and HiMedia. The clove oil used in the study was the commercially available itra. All glassware used in the study was sterilized and all procedures were performed aseptically.

The *E.coli* was isolated from sewage water collected from areas surrounding College of Life

Science, Gwalior and Cancer Hospital, Gwalior. Confirmation of *E.coli* was done by its Gram's staining, metallic green sheen bearing colonies on EMB medium and standard biochemical tests like IMViC, motility and catalase. Well isolated pure colonies were preserved as slants.

For obtaining pure culture of the Lactic acid bacilli, commercially available tablets were purchased (Sanzyme (P) Ltd). A single tablet was aseptically dissolved in 5 ml of autoclaved distilled water. The solution (300 µl) was immediately inoculated in eugonic medium by spread plate method. After 24 hrs of incubation, growth of the Lactic acid bacilli was observed in the form of small white circular colonies. The obtained colonies were Gram stained and tested for catalase activity and motility. Slants were prepared for preservation.

Determination of %age concentration of clove oil in which Lactic acid bacilli can survive for 1 hour: Solutions with various %age concentration of clove oil (5%, 2.5%, 1%, 0.5%, 0.25% and 0.1%) were prepared in sterile nutrient broth by dissolving the stock solution (prepared in DMSO) in it. Lactic acid bacilli ($1X10^5$ CFU/ml) were inoculated in each clove oil-broth solution in separate culture tubes and incubated for 1 hour at 37 ˚C, in aerobic conditions. The solutions were then spread plated in duplicate (300 µl per petridish) on nutrient agar and incubated for 24 hrs at 37 ˚C, in aerobic conditions. Broth with equivalent DMSO content corresponding to each solution of clove oil were also inoculated with lactic acid bacilli and were incubated and plated in duplicate in the same way as the clove oil-broth solutions. These broth cultures of lactic acid bacilli with only DMSO (no clove oil) acted as controls for respective test solutions. In 0.1%, 0.25% and 0.5% concentrations, no adverse effect on the growth of lactic acid bacteria was observed while the rest three sets did not show any growth of lactic acid bacteria. This led to the conclusion that lactic acid bacteria were unaffected by low concentration of the clove oil (1 hr incubation) but under increased clove oil concentrations, they could not survive even for 1 hour.

Determination of combined antimicrobial activity of clove oil and Lactic acid bacteria against *E.coli*

Low Concentration Approach: Lactic acid bacilli ($1X10^5$ CFU/ml) were inoculated in 0.5%, 0.25% and 0.1% clove oil containing nutrient broth tubes in duplicates and were incubated for 1 hour at 37 ˚C, in aerobic conditions. All the tubes were centrifuged at 2000 rpm for 5 minutes. Supernatant from each tube was collected separately leaving behind the pellet. The supernatants were passed twice with bacterial filter to remove any bacteria resulting in CM1 with three different concentrations (0.5%, 0.25% and 0.1%) of clove oil. All the supernatants were checked for their antibacterial activity against *E.coli* by well diffusion method [22, 23]. Broth with only Lactic acid bacteria incubated for 1 hr (with no clove oil) acted as negative control after centrifugation and filtration.

High Concentration Approach: This approach is based on the method used by Lee et. al (2013) to obtain cell free supernatants however, with required modifications as per the need of the experiment [24]. Lactic acid bacilli ($1X10^5$ CFU/ml) were inoculated in nutrient broth tubes in duplicates and were incubated for 24 hours at 37 ˚C, in aerobic conditions. All the tubes were centrifuged at 2000 rpm for 5 minutes after incubation. Supernatant from each tube was collected separately leaving behind the pellet. The supernatants were passed twice with bacterial filter to remove any bacteria. Clove oil in three different concentrations (10%, 5% and 2.5%) was added in the supernatant in well marked separate tubes (in duplicate). All the supernatants were checked for their antibacterial activity against *E.coli* by well diffusion method. Broth with only respective clove oil concentrations (10%, 5% and 2.5%) acted as controls after centrifugation and filtration.

RESULTS AND DISCUSSION

Lactic acid bacteria cannot survive high range concentrations but remain unaffected in lower range concentrations of clove essential oil for 1 hour: Lactic acid bacteria were incubated with both high (10%, 5%, 2.5% and 1%) and low (0.5%, 0.25% and 0.1%) concentrations of clove oil for 1 hr. Their growth was totally inhibited in

the higher concentration range showing that these concentrations caused killing of the treated bacteria. Those incubated with lower range concentrations were not significantly inhibited showing that they could survive low concentration clove oil for at least one hour (Table 1).

Table 1: Determination of optimum concentration of clove oil in broth which does not affect Lactic acid bacteria during 1hr incubation

S.No.	Concentration of clove oil	Avg. No. of CFUs of Lactic acid bacteria obtained
1	10%	None
2	5%	None
3	2.5%	None
4	1%	None
5	0.5%	$1.81X10^5$
6	0.25%	$3.10X10^5$
7	0.1%	$3.30X10^5$
8	Control (No clove oil)	$3.32X10^5$

CM1 did not inhibit E.coli growth: Lactobacilli produce many anti-microbial substances such as hydrogen peroxide, organic acids and bacteriocins [25]. Following the low concentration approach it was expected that the CM1 could have some secreted products of the lactic acid bacteria grown in it for 1 hour. Combination of the secreted products and clove oil (0.5%, 0.25% and 0.1%) were expected to have enhanced antimicrobial activity towards *E.coli*. Such speculation was based on the study done by Palmer et. al. (1998) according to which essential oils including that of clove were found to be bacteriostatic for 5 major food pathogens including *E.coli* at concentration as low as 0.075% or less [26]. However, in the present study, no such effect was observed as indicated by no zones of inhibition obtained in any of the three low concentrations (Figure 1).

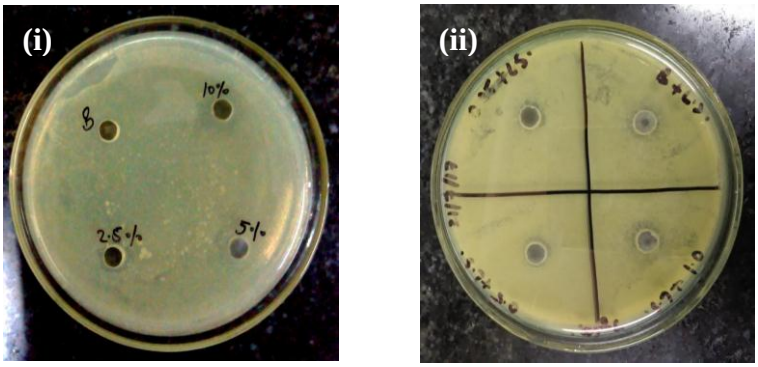

Fig.1: Representative nutrient agar plates showing no effect on growth of *E.coli* treated with CM1 (Low concentration approach) using well diffusion method: Control plate (i) and treated plate (ii).

CM2 showed slightly enhanced anti-microbial effect against E.coli: It was already experimentally proved that lactic acid bacteria could not survive high range concentrations of clove oil and hence in the high concentration approach, clove oil was added after incubating the lactic acid bacteria in the broth for 24 hrs. This approach was expected to yield better results as the concentrations of clove oil used were higher (10%, 5% and 2.5%) and the lactic acid bacteria was incubated for longer time in broth allowing them to produce more secretions. The antimicrobial activity of CM2 was higher (slightly though) than that of respective "Only-clove oil containing" counterparts (Figure 2, Table 2). Observation of only slightly increased activity may be due to use of cell free supernatant without concentrating it, as per the method followed by Lee et. al. (2013) [24].

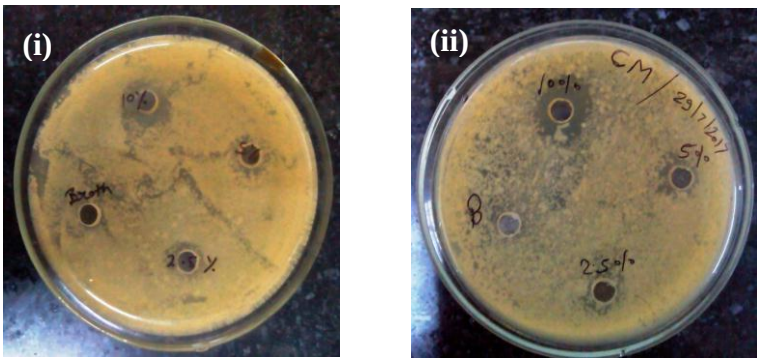

Fig.2: Demonstration of combined antimicrobial activity of clove oil and lactic acid bacteria against *E.coli* using high concentration approach by well diffusion method: Broth with only-clove oil (i) and CM2 (ii)

Table 2: Antimicrobial effect of combined treatment of high range concentrations of clove oil with broth in which lactic acid bacteria were incubated for 24 hrs against *E.coli*

Set	Concentration of Clove oil used	Zone of Inhibition observed		Average
		1st Attempt	2nd Attempt	
Control: Only clove oil	10%	15mm	16mm	15.5mm
	5%	12mm	11mm	11.5mm
	2.5%	Diffused	Diffused	-
	B (Only broth)	No zone	No zone	-
Treated: Broth in which Lactic acid bacteria were grown for 24 hrs + Clove oil	10%	18mm	17mm	17.5mm
	5%	13mm	11mm	12mm
	2.5%	Diffused	Diffused	-
	B (Only broth)	No zone	No zone	-

CONCLUSION

It can be concluded from the present preliminary work that no antimicrobial activity is exhibited by low range clove oil combined with lactic acid bacteria against *E.coli*. Visible enhancement of antimicrobial effect is observed only in high range concentration of clove oil combined with broth incubated with lactic acid bacteria. Further experimental studies involving higher range concentrations of the clove oil combined with broth incubated with lactic acid bacteria for longer time (more than 24 hrs) are expected to yield significantly better results. Concentrating the cell free supernatants can also enhance the antibacterial activity of the combinational treatment.

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CONFLICT OF INTEREST

The author confirmed that there is no conflict of interest for this research paper.

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