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Natural products as inhibitory agents of *Escherichia coli, Staphylococcus aureus* and *Listeria monocytogenes*

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ABSTRACT

Fermented dairy products like curd and cottage cheese are important part of the diet consumed in India prepared by traditional methods. Contamination of indigenous microflora (pathogenic and non-pathogenic) in these milk products is mainly due to processing, handling and unhygienic environment. A potential application of the present research is to search a natural preservative for milk products which could inhibit pathogenic Escherichia coli, Staphylococcus aureus and Listeria monocytogenes. Pathogens were isolated and identified from samples of cottage cheese, biochemically as well as by sequencing of 16S rRNA gene sequence and their control was studied using natural products. Ethanolic extracts were found to be more effective than the aqueous extracts and hime was inhibited E. coli and S. aureus while mausami and peepal leaves inhibited L. monocytogenes most effectively.

Key words: Cottage cheese, *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes* and Natural products.

INTRODUCTION

Milk and milk products represent an ideal growth medium for microorganisms. Most important pathogens found in milk and milk products are *Escherichia coli, Staphylococcus aureus* and *Listeria monocytogenes*. Most strains of *E. coli* are harmless but several strains are extremely pathogenic like *E. coli* 0157:H7 causing complications and death associated with haemorrhagic colitis and acute renal failure, especially in children [Robins-Brown *et al*., 2004]. The infective dose of *E. coli* is estimated to be about 10 cells: in contrast, the infective dose of *S. aureus* is high because the actual cause of the food poisoning is not the organism itself but rather a number of heat stable protein toxins produced by the bacterium and capable of withstanding 100^0C for >30 min [Presscott *et al.*, 2002]. Enterotoxins can be produced at exponential growth phase of the bacterium or stationary growth phase. 20% of the strains of this bacterium produce enterotoxins. The level of enterotoxin associated with triggering food poisoning (in healthy individuals) is estimated to be 1ng/g food (ng=10^-9 g). The infective dose required for *L. monocytogenes* is still unknown but it is believed that it varies with the specific strain of the bacterium and the susceptibility of the individuals. Regular FDA standards include 'zero tolerance' for *L. monocytogenes* in all ready-to-eat products. *L. monocytogenes* is the only species in the genus *Listeria* that has been involved in known food-borne outbreaks of listeriosis, particularly in risk populations including neonates, immunocompromised hosts and pregnant women [Lorber, 1990].

Food borne infections should be cured immediately after being diagnosed because they may lead to serious health problems and some time even death. Drugs used to cure these infections are known as 'antibiotics'. The clinical efficacy of many antibiotics is being threatened by the emergence of multidrug resistant pathogens. Natural products like Neem, Tulsi, and Garlic etc either as pure compounds or as standardized plant extracts provide unlimited opportunities for new drugs due to the unmatched availability of inherent chemical diversity of the natural products. Plant derived antimicrobial compounds may be of value as a novel means for controlling antibiotic resistant zoonotic pathogens which contaminate food animals and their products [Halley and Palamappan 1992]. The urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanism of action for new and re- emerging infectious diseases prompted us to take up the study on the inhibition of *E. coli, S.aureus and L. monocytogenes* applying various natural products. For this purpose we have isolated these microbes from cottage cheese and identified them by using the novel culture dependent methods that involve PCR amplification of bacterial small-subunit rDNA for the three selected microbes using single set of universal primer [Bosshard 1992; Govindaswami 1993; Reyenbach *et al.*, 2000].

MATERIALS AND METHODS

Samples of cottage cheese and curd were collected seasonally from in and around the different market areas of Agra city and examined for the presence of *E. coli, S. aureus* and *L. monocytogenes*. The samples were collected aseptically in sterilized plastic bags and directly transported to the laboratory under cold conditions and were analyzed within 4 hrs. Standard strains of *E. coli* (MTCC-723), *L. monocytogenes* (MTCC-1143) and *S. aureus* (MTCC-3381) were procured from MTCC Chandigarh, India. Isolation and preliminary identification of the 100 isolates of each selected pathogenic bacteria were done by biochemical characterization [Singh and Prakash, 2008] and finally these were identified by 16S rRNA gene sequencing (Vandamme, 1996).

Extraction of DNA

Extraction of the template DNA was done as per the method of Tsai and Olson [1991] with suitable modifications. 100 µl of 24 hrs pure culture was centrifuged in a micro-centrifuge at 4000 rpm for 12 minutes. The recovered pellet was suspended in 100 µl of sterilized DNase and RNase free water, heated in a boiling water bath for 10 mins and then snaps chilled in crushed ice. The obtained lysate was used as the DNA template. 50 µl Master mixture was prepared at a final concentration of 1X of 10 X PCR buffer, 0.2mM dNTP mix, 2mM MgCl2, 5pM of each primer, 1.25U Taq DNA Polymerase, template DNA and MiliQ water. Amplification was done by using primer set 27F (5-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5- TACGGTTACTTGTTACGACTT-3) [Frank *et al.*, 2008; Acedo-Felix and Perez-Martinez, 2003]. The PCR mixture was subjected to thermal cycler (initial incubation 95^0C for 5 min, 34 cycles of 30 s at 95^0C for the denaturation, 30 s at 55^0C for the annealing and 30 s at 72^0C for extension). 8µl of the reaction products were resolved by electrophoresis on a 1% agarose gel containing 0.5µg of ethidium bromide per ml in .5X Tris-borate-EDTA buffer at 7V/cm. A 100 bp DNA ladder (Bangalore genei) was included. The gel was visualized and photographed over the UV transilluminator (Zenith gel documentation system) and analyzed by gel doc software named UN-SCAN-IT gel 6.1.

Inhibition by antibiotics and natural products

Inhibition in the presence of natural products including *Trechyspermum copticum* (Ajwain), *Gycyrrhiza glabar* (Mulethi), *Chebulic myrobalan* (Hime), *Piper chaba* (Choti pepper), *Mangifera indica* (Mango seed), *Ficus religiosa* (Peepal leaves), *Syzygium cumini* (Jamun seed) and *Citrus sinensis* (Mousami) was analyzed by agar well diffusion method. Extracts of natural products were prepared by soaking the product in ethanol and water in a ratio of 1:4 and 1:8 respectively, for 72 hrs, in sterile conical flasks at room temperature with uniform shaking. The extracts were then filtered and concentrated by evaporating to dryness at 45^0C [Mahmood, 2008].

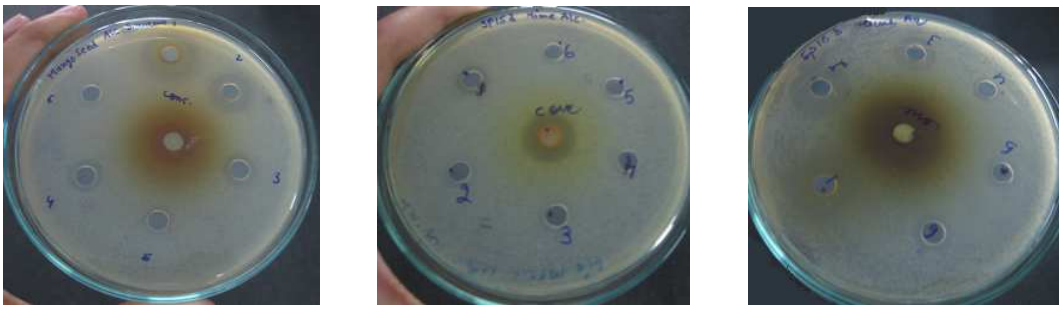
RESULTS AND DISCUSSION

On the basis of Gram's staining and biochemical characterization (MR, VP, Indole, Nitrate, citrate utilization, H_2S production, fermentation of various sugars, haemolysis, and growth on chromogenic medium) 4 isolates were confirmed as pathogenic *E. coli,* 9 isolates as pathogenic *S. aureus* and 6 isolates as pathogenic *L. monocytogenes*. One isolate of *E. coli, S. aureus,* and *L. monocytogenes* each were selected randomly after their biochemical confirmation. They were further confirmed to the species level by the amplification of 16S rDNA coding ~ 1400 bp 16S rRNA gene sequence using universal primer set 27F/1492R. Amplified products were submitted to the Institute of Molecular Medicine, New Delhi for sequencing. Obtained sequences were aligned through National centre for

biotechnology Information (NCBI) database by using the Basic Local Alignment Tool (BLAST, 2.0 search program) to determine their approximate phylogenetic affiliations. Sequence alignment with BLAST database shows the best match of the three pathogenic bacteria as *Escherichia coli* F11 gcontig_1112495919726, whole genome shotgun, *Staphylococcus aureus subsp. aureus* ATCC BAA-39 contig00105, whole genome shotgun sequence and *Listeria monocytogenes* FSL N1-017 cont5.59, whole genome shotgun sequence.

Inhibition of two of the confirmed isolates of each pathogen, along with standard strain was studied in the presence of natural products (*Trechyspermum copticum (Ajwain), Gycyrrhiza glabar (Mulathi), Chebulic myrobalan* (Hime), *Piper chaba* (Choti pepper), *Mangifera indica* (Mango seed), *Ficus religiosa* (Peepal leaves), *Syzygium cumini* (Jamun seed) and *Citrus sinensis* (Mausami)).

There are many reports available that prove antiviral, antibacterial, antifungal, antihelmintic, antimolluscal and anti-inflamatory properties of plants [Stepanovic, 2008; Behera and Misra 2005]. For determining the MIC of the natural products the agar well diffusion method was employed (on Muller Hinton Agar, MHA) against all the pathogenic microbes (Fig. 1).



(a) **(b)** **(c)**

Fig. 1. Plate showing MIC (a) *E.coli* (b) *S. aureus* (c) *L. monocytogenes*.

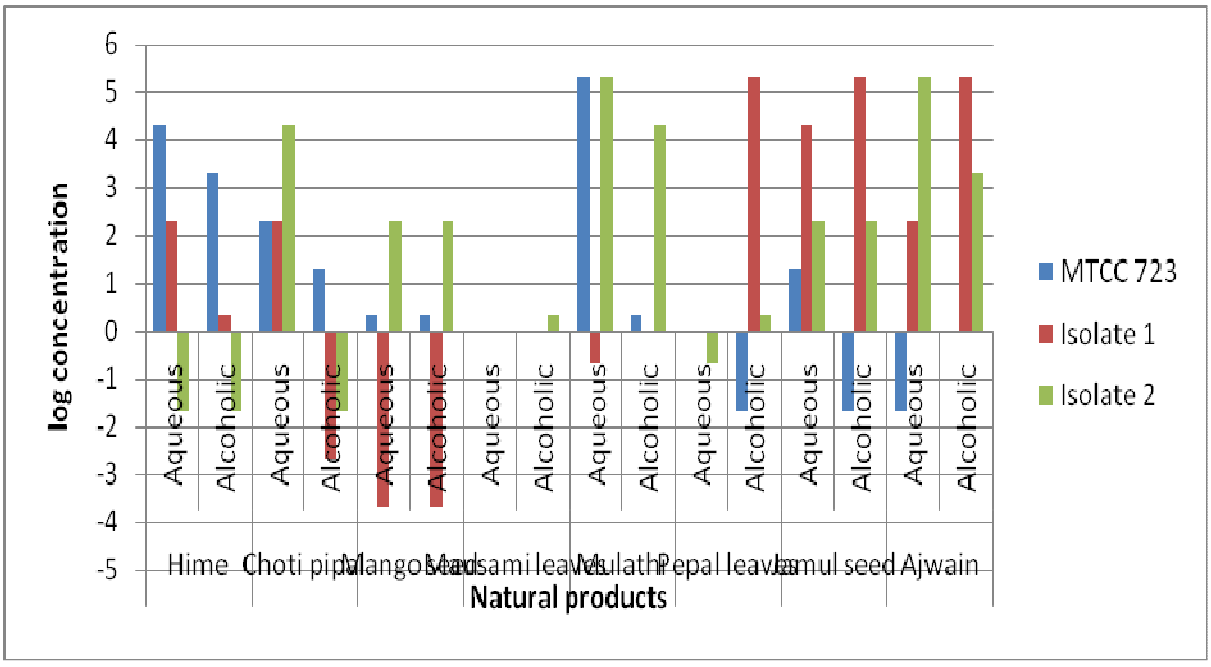


Fig-2: Minimum Inhibitory Concentration of all the natural products against *E. coli*

MIC of the natural products observed for *E. coli* shows that the least effective natural products were the extract of mausami leaves, jamun seed and ajwain while the most effective were the extracts of mango seed, followed by hime, choti peepal, and mulathi. (Fig. 2)

The results show that all the natural products except mausami leaves form a zone of inhibition against *S.aureus*. However, it was observed that hime was the most effective in inhibiting *S. aureus* while peepal leaves were the least effective both for the standard and the isolate. Aqueous form of mulathi was also found to be more effective against the standard *S. aureus* to a greater extent than the alcoholic form. Aqueous extract of ajwain was effective, but to a much lesser extent than the alcoholic extract of ajwain which depicted a very high MIC ($2x10^{-5}$ µg/ml) as observed for the standard *S. aureus* (MTCC 3381). The *S.aureus* isolates show that ajwain extracts are not able to inhibit them effectively as the MIC required is $2x10^{5}$ µg/ml.

Extracts of all the natural products were effective against the isolates but inhibited the isolates to a lesser extent as compared to the standard *S. aureus* (MTCC 3381). The genetic make up of the isolates may be different from the standard strain of *S. aureus*, probably the former being more virulent. From the present study it is obvious that pathogenic *S. aureus* could be effectively treated by mulathi, hime and mango seed extracts, as these extracts were very effective in inhibiting even at low concentrations (lower MIC value) (Fig. 3)

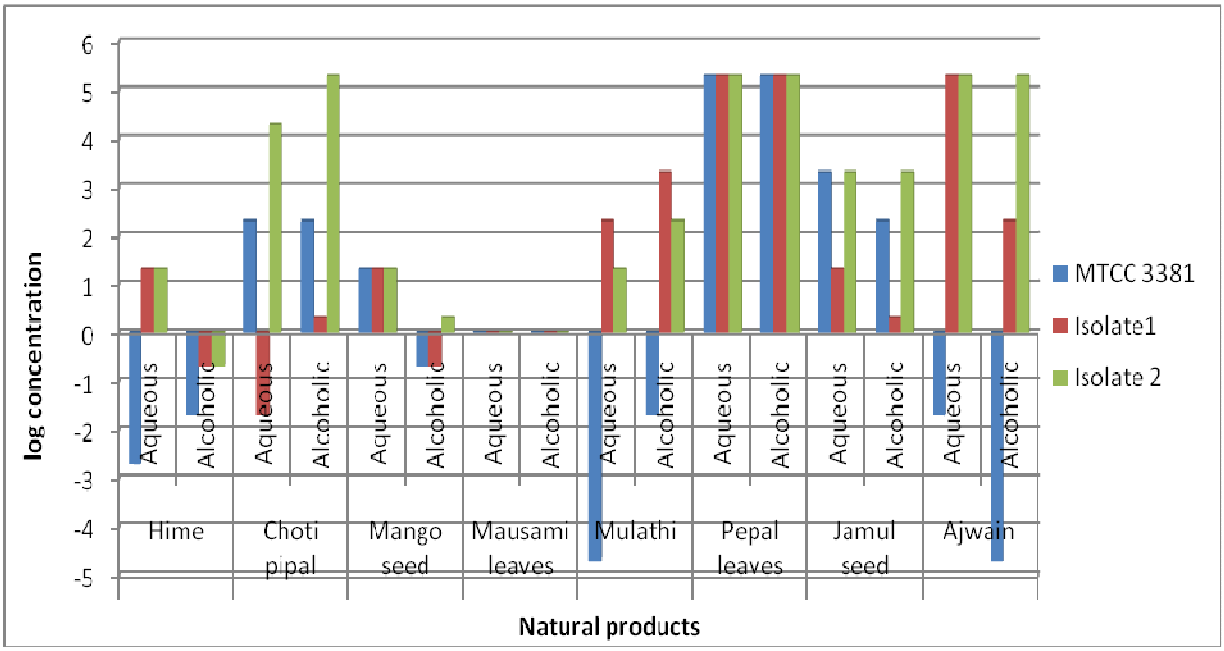


Fig-3: Minimum Inhibitory Concentration of all the natural products against *S. aureus*

Results obtained for the MIC of natural products against *L. monocytogenes* show that all the natural products were effective in inhibiting both the standard *L. monocytogenes* (MTCC 1143) and the two isolates, though the former was inhibited to a greater extent than the latter. The difference may be due to the differences in the virulent genes present in the standard *L. monocytogenes* (MTCC 1143) and its isolates from milk products. Of all the natural products the extracts of mausami leaves and peepal leaves were found to be most effective in inhibiting *L. monocytogenes* (Fig. 4).

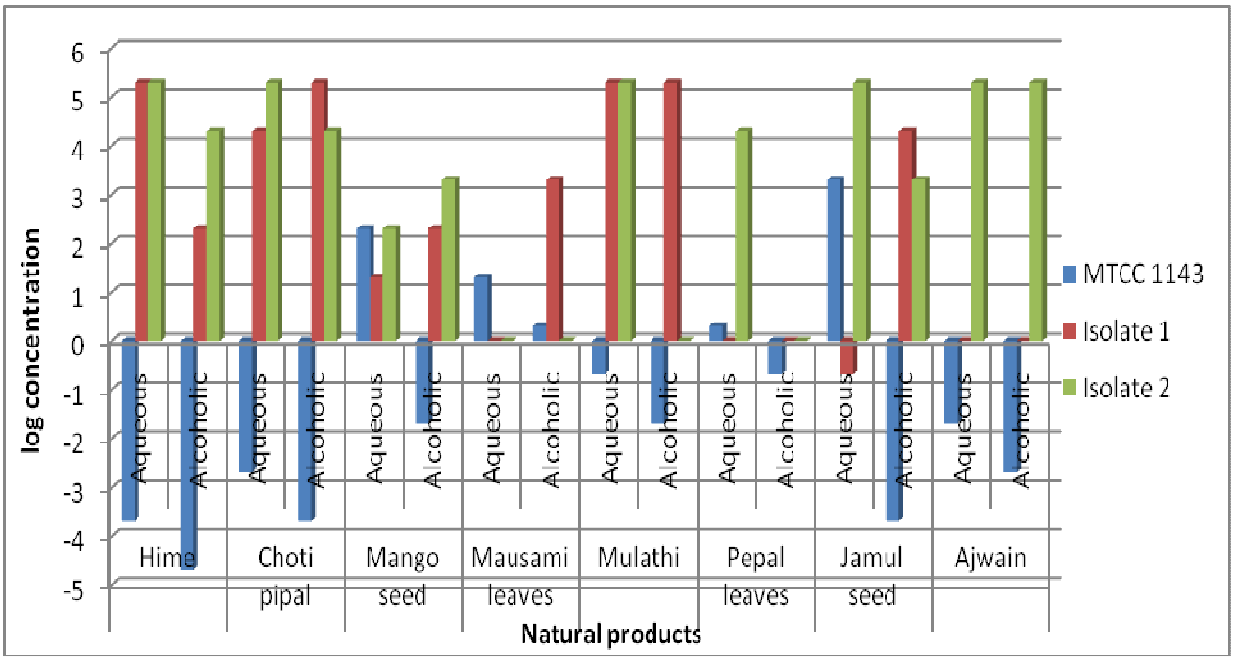


Fig-4: Minimum Inhibitory Concentration of all the natural products against *L. monocytogenes*.

CONCLUSION

Ethanolic extracts of the products used were found to be more effective than aqueous extracts. This difference may be due to the better extraction of biologically active compounds (Alkaloids, flavonoides, essential oils, tennins etc.) in the presence of ethanol [Ghosh, 2008; Falodun, 2006]. The present work suggests that the active molecules isolated from the crude extracts of the natural products may prove to be more effective and may substitute the well known antibiotics currently in use for the control of these pathogens. These products can be easily incorporated in one's diet without producing any side effect in contrast to the antibiotics. The antibiotic resistance, which poses an insurmountable problem in the cure of these bacterial infections, could be taken care of by an increased use of natural products which possess a magical potential against these pathogens.

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