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Natural Products as Inhibitory Agents of *Escherichia coli* and *Listeria monocytogenes*

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Abstract

The potential application of the present research findings is of particular interest to search a natural preservative for milk products which could inhibit pathogenic Escherichia coli and Listeria monocytogenes. To achieve the aim pathogens were isolated and identified from cottage cheese samples, produced by the traditional methods using biochemical tests as well as sequencing of 16S rRNA gene sequence and their control was studied using natural products extracted in ethanol and water. In vitro studies shows that Mango seed and hime are more effective against E. coli whereas mausami leaves and peepal leaves inhibit L. monocytogenes more effectively.

Introduction

Milk and milk products represent an ideal growth medium for microorganisms. *Escherichia coli,* and *Listeria monocytogenes* are the most important pathogens which indicate the unhygienic conditions in milk and milk products. Most strains of *E. coli* are harmless but several strains are extremely pathogenic like *E. coli* 0157:H7 causing complications and death associated with hemorrhagic colitis and acute renal failure, especially in children. The infective dose of *E. coli* is estimated to be very low, about 10 cells: in contrast, the infective dose required for the *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, immune-compromised hosts and pregnant women.

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 [1]. 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 and L. monocytogenes* by using various antibiotics and 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 of all the two selected microbes using single set of universal primer.

Materials and Methods

Samples of cottage cheese were collected seasonally from in and around the different market areas of Agra city and examined for the presence of *E. coli and L. monocytogenes*. Standard strains of *E. coli* (MTCC-723) and *L. monocytogenes* (MTCC-1143) were procured from MTCC Chandigarh, India. Isolation and

M.M. Srivastava, L. D. Khemani, S. Srivastava, *Chemistry of Phytopotentials: Health, Energy and Environmental Perspectives*, DOI:10.1007/978–3-642–23394-4_12,

preliminary identification of the 100 isolates of each selected pathogenic bacteria were done using biochemical characterization (Singh and Prakash, 2008) and finally these were identified by 16S rRNA gene sequencing [2].

Extraction of DNA

Extraction of the template DNA was done as per the method of Tsai and Olson (3) 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 min and then snap 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 (10 X PCR buffer), 0.2mM (2mM dNTP mix), 2mM (25mM $MgCl_2$), 5pM (22pM 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-TACGGTTACCTTGTTACGACTT-3) [4, 5]. The PCR mixture was subjected to thermal cycler (initial incubation 95°C for 5 min, 34 cycles of 30 s at 95°C for denaturation step, 30 s at 55°C for annealing step and 30 s at 72°C for extension step). 8µl of the reaction products were resolved by electrophoresis on a 1 % agarose gel containing 0.5 µg 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 under the influence 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°C [6].

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, hemolysis, and growth on chromogenic medium) 4 isolates have been confirmed as pathogenic *E. coli,* and 6 isolates as pathogenic *L. monocytogenes.* 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 programs) to determine their approximate phylogenetic affiliations. Sequence alignment with BLAST database Sequence determination by 16S rRNA gene confirmed the isolates as *E. coli* and *L. monocytogenes.*

Inhibition of two of the confirmed isolates, 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)) (Fig 1).

There are many reports available that prove antiviral, antibacterial, antifungal, antihelmintic, antimolluscal and anti-inflamatory properties of plants [7, 8]. For determining the MIC of the natural products the agar well diffusion method was employed (on Muller Hinton Agar, MHA). 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).

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,

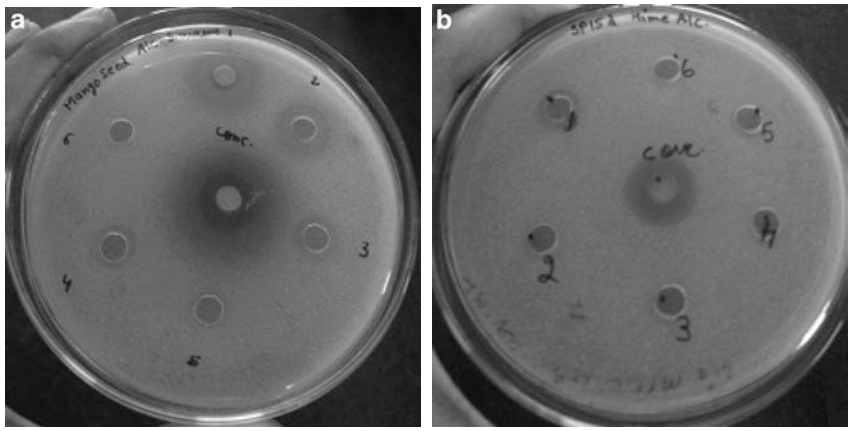

Fig. 1: Plate showing MIC **(a)** *E. coli* **(b)** *L. monocytogenes*

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 3).

Ethanolic extracts were found to be more effective then aqueous extracts, this variation in the activity may be due to the better extraction of biologically active compounds (Alkaloids, flavonoids, essential oils, tennins etc.) which were enhanced in the presence of ethanol [9,10].

However, the present work suggests that if the active molecules are isolated from the crude extracts of the natural products they may prove to be more effective than the well known antibiotics currently in use for the control of these pathogens and can be easily incorporated in one's diet and exert no side effect as compared to antibiotics.

Besides 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.

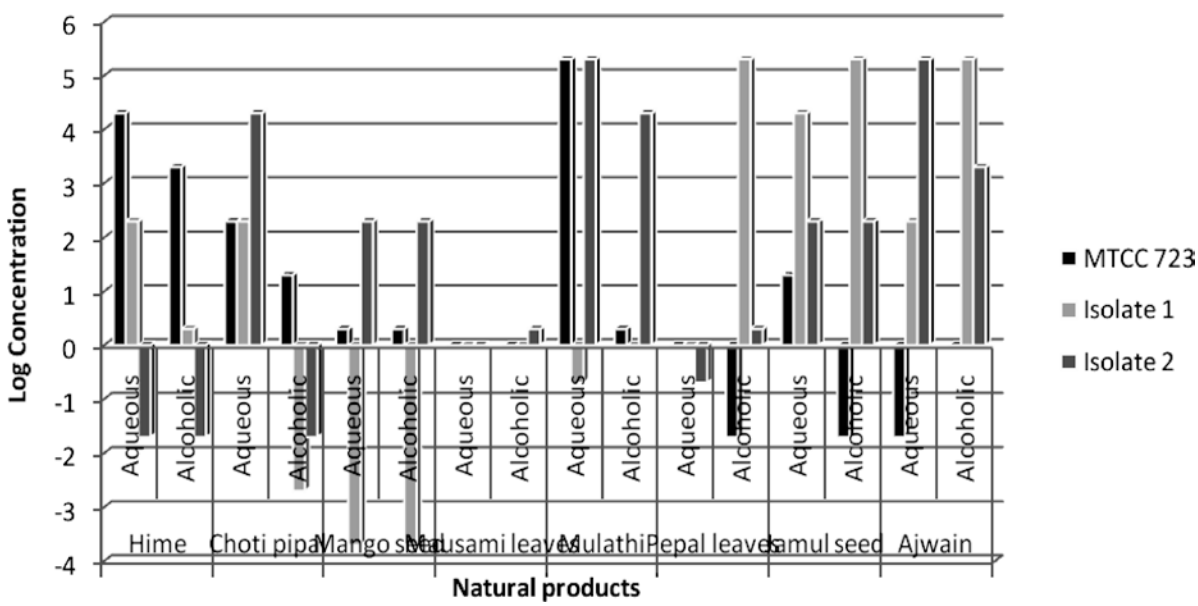

Fig. 2: Minimum Inhibitory Concentration of all the natural products against *E. coli*

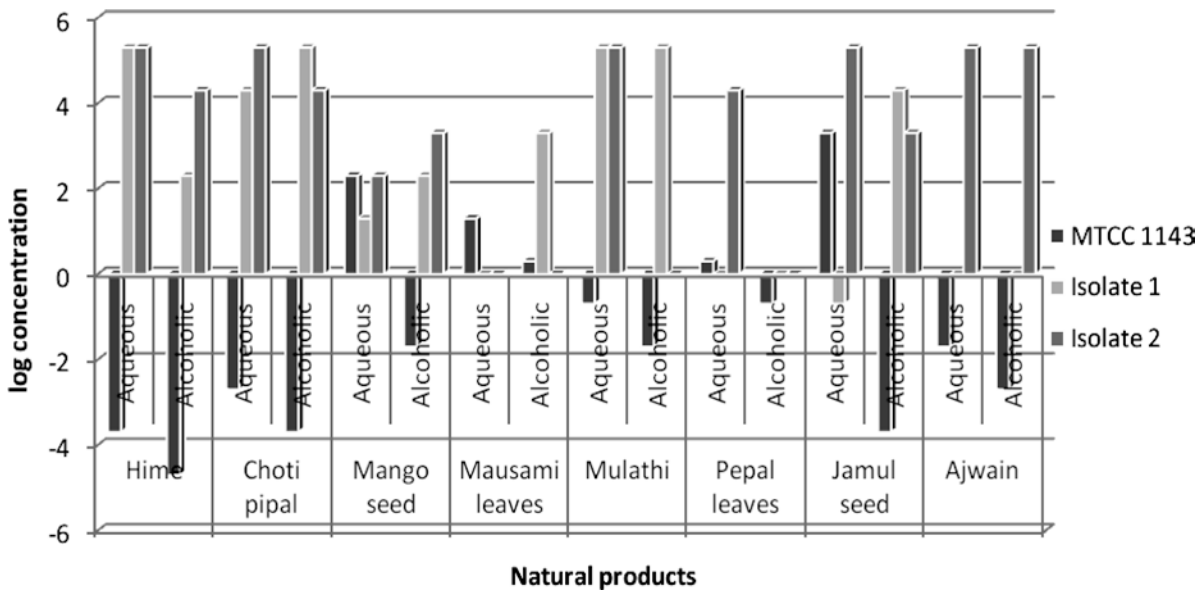

Fig. 3: Minimum Inhibitory Concentration of all the natural products against *L. monocytogenes*

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