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In vitro Susceptibility of *Enterobacter sakazakii* to Natural Products

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Abstract: *Enterobacter sakazakii* is an emerging pathogen that causes food borne illness mainly infecting infants causing nervous system and blood stream infections. In the present study, the prevalence of *E. sakazakii* in buffalo raw milk, milk powders and food products (bread, buns, pizza base etc.) were studied. Out of a total of 25 samples tested *E. sakazakii* was isolated from only 2 buffalo raw milk samples. The antimicrobial activity of crude extracts of 8 natural products including Mulethi, Ajwain, Mousami leaves, Hime, Mango seed kernel, Peepal leaves, Jamun seeds and Choti peepal against *E. sakazakii* using well diffusion method was also studied. All the extracts showed significant antibacterial properties. However, the aqueous extract of Hime and Choti peepal revealed the strongest antimicrobial activity as they indicated low MIC (Minimum Inhibition Concentration) values. It is recommended that these natural products may be used in controlling *E. sakazakii* infections.

Key words: *Enterobacter sakazakii*, antimicrobial activity, natural products, infection, buffalo raw milk, *in vitro*

INTRODUCTION

Enterobacter sakazakii is the member of the family Enterobacteriaceae, genus *Enterobacter* and is recognized as one of the newly emerging pathogens (Son *et al*., 2005). This gram negative and rod shaped bacteria was initially referred to as yellow pigmented *Enterobacter cloacae* upto 1980 but was reclassified (Farmer *et al*., 1980) as *Enterobacter sakazakii*. This bacterium is an emerging opportunistic pathogen predominantly associated with bacterial meningitis (inflammation of the lining of brain), sepsis (bacteria in blood) or necrotizing enterocolitis (severe intestinal infections) (Van Acker *et al*., 2001). *E. sakazakii* infections show signs and symptoms like poor feeding response, jaundice, variation in body temperature, hydrocephalus, developmental delay and death (Muytjens *et al*., 1983) as typically shown by any gram negative bacteria. Although the cases of *E. sakazakii* infections are very less, it has a high mortality rate of about 40-80%.

E. sakazakii can infect individuals of all age groups.The neonates which are immune-compromised, premature, have low birth weight of 2.5 kg or less are likely to be more affected by *E. sakazakii* infections (Lai, 2001). The natural habitat and reservoir of *E. sakazakii* remains unknown but it can be found in surface water, soil, cattle and raw cow's milk (Muytjens and Kollee, 1990), infant milk formulae (Iversen and Forsythe, 2003), milk cartons, fermented bread (Gassem, 1999) etc. *E. sakazakii* is found to be resistant to certain antibiotics like Vancomycin, penicillin, oxacillin, lincosamides (Stock and Wieddemann, 2002) etc. and many forms of *Enterobacter* have developed resistance to the first generation cephalosporins and are also developing resistance to the 2nd and 3rd generation cephalosporins because of the multiple antibiotic resistance operons present in DNA of different strains of *E. sakazakii*.

This drug resistance in bacteria has created additional problems in the treatment of several diseases in patients. As the number of effective antibiotics is decreasing therefore concerted efforts are being made to identify antimicrobial materials from natural products and traditional medicines. India is considered to be the one of the world's richest in flora having about 120 families of medicinal plants comprising 1,30,000 species. Various preparations of these medicinal plants are used in treating infections associated with micro-organisms as these medicinal plants produce certain antimicrobial compounds. Seed extracts of water soluble muscadine were found to be effective against *E. sakazakii* (Kim *et al*., 2009). Certain other natural products like *Terminalia chebula* (Chattopadhyay *et al*., 2007) *Trachyspermum ammi* (Singh *et al*., 2004), *Glycyrhiza glabra* (Gupta *et al*., 2008) etc. were found to be effective against a wide range of micro-organisms. The objectives

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of the present research include isolation of *E. sakazakii* from buffalo raw milk and food products including milk powders, pizza base, bun and bread etc. and also to check the effect of eight different natural products on the standard *E. sakazakii* (MTCC-2958) and its isolates.

MATERIALS AND METHODS

Sample collection: A total number of 25 different milk and food product samples were collected from different areas of Agra which included 16 samples of buffalo raw milk, 3 samples of milk powder and 6 samples of food products (like bread, bun and pizza base).

Isolation and identification: A total of 25 samples obtained from different regions of Agra city were tested for the presence of *Enterobacter sakazakii*. About 0.5 g or 0.5 mL of a sample was added to 4.5 mL of *Enterobacter* Enrichment broth (EE broth) and incubated overnight at 37°C. Cultures from EE broth were streaked on VRBGA media (Violet red bile glucose agar) and characteristic pink color colonies from VRBGA media were picked up and examined microscopically. These typical colonies were further streaked on TSA plates (Tryptic Soya Agar) and incubated for 24 h at 37°C. Typical yellow color colonies were picked up and tested biochemically for *E. sakazakii*. Standard strain of *E. sakazakii* MTCC-2958 was used as the positive control.

Natural products: The natural products used in this study. *Glycyrhiza glabra root* (Mulethi), *Carum couticum* seeds (Ajwain), *Citrus sinesis* leaves (Mousami), *Terminalia chebula* (Hime), *Mangifera indica* seed (Mango seed kernel), *Ficus religiosa* leaves (peepal leaves), *Syzygium cumini L.* (Jamun seeds), *Piper chaba* (choti peepal) were obtained from retail shops of Agra.

Preparation of crude extracts: All the natural products were powdered and for making alcoholic and aqueous crude extracts 25 g of each material was soaked in 100 mL of ethyl alcohol (70%) and 25 g of powder soaked in 100 mL of distilled water for 72 h and filtered. Filtrates were concentrated in double boiler at 45°C for 5 days and finally dissolved in DMSO (Dimethyl Sulphoxide) to yield 0.2 g mL^{-1} of the natural product extract.

MIC of natural product: The antimicrobial activity of the crude extracts of natural products was investigated by using agar well diffusion method. MIC (Minimum Inhibitory Concentration) of the extracts against the standard strain MTCC-2958 of *E. sakazakii* and an isolate of *E. sakazakii* was determined. Muller-Hinton Agar was prepared and 100 μL of the test organism (~6×10^7 cells mL^{-1}) were inoculated in the media and wells were bore and in each well 40 μL of crude extracts of different dilutions (such as 2×10^5-0.02 μg mL^{-1}) were added and incubated overnight at 37°C. Inhibition zones were measured and recorded.

Statistical analysis: Statistical analysis was conducted by using the ANOVA test.

RESULTS

Isolation and identification of *E. sakazakii* from samples: About 20% buffalo raw milk samples tested positive for *Enterobacter* species whereas none of the milk powder samples was contaminated with *Enterobacter* species and 8% of the food products indicated positive results on VRBGA media suggestive for *Enterobacter* (Fig. 1). Table 1 shows the number of samples analysed. Results obtained from biochemical tests confirm the presence of *E. sakazakii* only in 2 samples (8%) (Fig. 2).

MIC of natural products: The extent of *In vitro* antimicrobial activity of natural products was considered from MIC values against *E. sakazakii* standard MTCC-2958 and an isolate M-11. Antimicrobial activity was shown by all the natural products used. The results revealed that alcoholic extracts of Mousami leaves, Hime, Mulethi and aqueous extracts of Jamun seeds, Mousami leaves etc. exhibited antibacterial activity against both MTCC-2958 and M-11. Results also show that as the concentration of extracts decreases, the size of the inhibition zone also decreases.

Table 2 shows the MIC of natural products against MTCC-2958 and an isolate M-11. Alcoholic extracts of jamun seeds and choti peepal was found to be most effective against *E. sakazakii* as their MIC values is 2 μg mL^{-1}. MIC for aqueous extracts of Mousami leaves

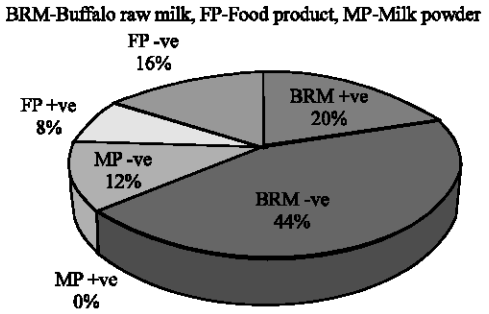

Fig. 1: Relative percentage of positive and negative samples for *Entrobacter* sp.

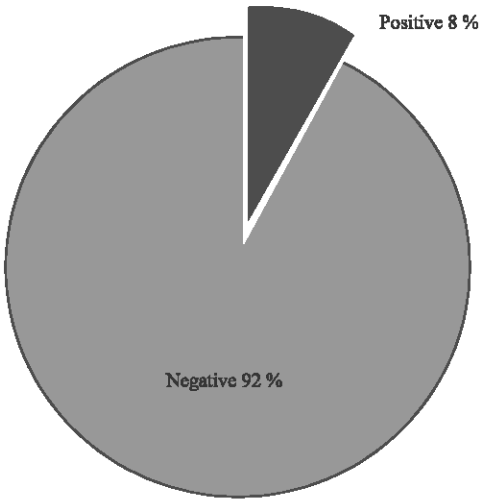

Fig. 2: Percentage of samples contaminated with *E. sakazakii*

Table 1: MIC of natural product against standard MTCC-2958 and an isolate (M-11) of *E. sakazakii*

	MIC (μg mL^{-1}) of natural products	
Natural product	MTCC-2958	M-11
Jamun seeds alcoholic	2	2
Jamun seeds aqueous	2×10^3	2×10^3
Hime alcoholic	2×10^3	2×10^3
Hime aqueous	0.2	20
Choti peepal alcoholic	2	2
Choti peepal aqueous	0.2	20
Ajwain alcoholic	20	20
Ajwain aqueous	2×10^3	2×10^4
Mulethi alcoholic	2	2×10^3
Mulethi aqueous	2×10^3	2×10^2
Mango seeds alcoholic	20	2×10^3
Mango seeds aqueous	2×10^3	2×10^3
Mousami leaves alcoholic	2×10^2	20
Mousami leaves aqueous	2×10^4	2×10^4
Peepal leaves alcoholic	2×10^3	2×10^2
Peepal leaves aqueous	2×10^4	2×10^4

Table 2: MIC of natural product against standard MTCC-2958 and an isolate (M-11) of *E. sakazakii*

	MIC (μg mL^{-1}) of natural products	
Natural product	MTCC-2958	M-11
Jamun seeds alcoholic	2	2
Jamun seeds aqueous	2×10^3	2×10^3
Hime alcoholic	2×10^3	2×10^3
Hime aqueous	0.2	20
Choti peepal alcoholic	2	2
Choti peepal aqueous	0.2	20
Ajwain alcoholic	20	20
Ajwain aqueous	2×10^3	2×10^4
Mulethi alcoholic	2	2×10^3
Mulethi aqueous	2×10^3	2×10^2
Mango seeds alcoholic	20	2×10^3
Mango seeds aqueous	2×10^3	2×10^3
Mousami leaves alcoholic	2×10^2	20
Mousami leaves aqueous	2×10^4	2×10^4
Peepal leaves alcoholic	2×10^3	2×10^2
Peepal leaves aqueous	2×10^4	2×10^4

and peepal leaves is the highest i.e., high dose of these products is required to inhibit *E. sakazakii*. Aqueous extracts of Hime and Choti peepal also show low MIC values and therefore can be used for controlling *E. sakazakii* infections.

Statistical analysis: The results obtained from ANOVA tests show that there exists no significant difference between the responses of standard MTCC-2958 and that of the natural isolate but there exists a significant difference between susceptibility to different natural products ($p<0.05$).

DISCUSSION

Isolation and identification of *E. sakazakii* from samples: As the results show that *E. sakazakii* was not found in any of the milk powder samples and food product samples this may be due to the fact that *E. sakazakii* is not able to tolerate the high manufacturing temperature of these products. It has been also reported that pasteurization is effective in the elimination of *E. sakazakii* (Iversen *et al*., 2004). It has also been found that *E. sakazakii* is more heat sensitive than other pathogenic organisms (Nazarowec-white and Farber, 1997).

MIC of natural products: Even though the natural products used were crude extracts all exhibited antimicrobial activity to a lesser or greater extent. These natural products contain certain active chemical compounds like flavanoids, phenols, glycosides, saponins, oleic acid, linolecic acid and gallic acid etc., which affect the microbial susceptibility. Better results can be obtained if the active components of these products are purified. These products can be easily ingested by individuals in their daily diet. Their side effects are also negligible compared to antibiotics which are used for the control of *E. sakazakii*.

A great care should be exercised to maintain proper hygienic conditions during preparation and handling of raw milk and milk products. Raw milk should be properly pasteurized or boiled before consumption. The study also highlights the use of natural products. The WHO (1993) reported that about 80% of the world's population depends mainly on traditional medicine which involves the use of plant extracts.

CONCLUSION

In the present study, the alcoholic and aqueous crude extracts of some natural products were found to be effective against *E. sakazakii*. To identify the active

principle of these chemical compounds present in the natural products a detailed chemical investigation is needed which would uncover the real therapeutic value of these extracts in controlling *E. sakazakii* infections.

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