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ANTIMICROBIAL ACTIVITY OF SPICE EXTRACTS AGAINST *CRONOBACTER SAKAZAKII*

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ABSTRACT

Cronobacter species, isolated from a wide variety of foods have been described as an ubiquitous opportunistic pathogen causing neonatal bacteremia and meningitis. This organism is found in water and the soil environment and also reported to be prevalent in infant milk formula, milk cartons & fermented breads. Dried coconut is a condiment used widely in India as a base for several food preparations. *C. sakazakii* has been recently isolated from dried coconut in our laboratory. The present study aimed to investigate the effects of spices on *C. sakazakii* in order to prevent the contamination of foods using dried coconut. In this study, different extracts of spices red chilli, coriander, turmeric, fenugreek, fennel, mint leaves, asafoetida, star anise, bay leaf and poppy seeds were made using methanol, ethanol, acetone, chloroform including aqueous extracts of spices. Antimicrobial activity of spice extracts was assessed by using agar well diffusion technique both singly and synergistically. Ethanolic extract of asafoetida was found to have highest activity against *C. sakazakii*. Fennel, fenugreek, coriander seeds and star anise did not show any activity against the test culture. The combination of turmeric- poppy seeds showed effective synergism against the test culture. Minimum inhibitory concentration of selected spices was also determined against both isolated and pure culture of *C. sakazakii*. Minimum inhibitory concentration of curcumin was determined and found to be 0.04mg and 0.055mg against pure and isolated culture of *C. sakazakii* respectively. The results showed that the antimicrobial activity of these spices against the opportunistic pathogen *C. sakazakii* suggesting their use as natural food preservatives in the food containing dried coconut.

KEYWORDS: *Cronobacter sakazakii*, antimicrobial activity, synergistic activity, minimum inhibitory concentration, agar well diffusion.

INTRODUCTION

Foodborne infections have been a major health issue in recent years. Most of the foodborne infections are traced to bacterial contamination of food. In the last two decades, antibiotic resistance has been an emerging problem worldwide.[1,2,3] This has led to the search for new, safe and effective antimicrobial agents from alternative natural resources like plant products. At the same time, there is a growing demand among consumers for natural preservatives or additives in processed foods.[3,4]

All *Cronobacter* species except *C.condimenti*, have been linked retrospectively to clinical cases of infection in either adults or infants. *Enterobacter sakazakii* is a member of the family Enterobacteriaceae, genus Enterobacter,and is a motile peritrichous, Gram negative bacillus.[5] The organism formerly known as *Enterobacter sakazakii* has been known to cause necrotizing enterocolitis, bacteremia & infant meningitis.[6] *Cronobacter sakazakii* has also been isolated from plant products and soil. Copra is the dried meat or kernel of the coconut. Since dried copra is an indigenous food ingredient in India, it is used in many of the food preparations, also in instant gravy masalas, chutneys available in market and other coconut containing food products. Cronobacter species are known to survive in low water activity environments. Many herbs & spices have been used to evaluate their antimicrobial activity against this organism. Plants have been a valuable source of natural products for a long period of time to maintain human health, especially with more intensive studies in the last decade for natural therapies.[7] Spices are used for flavour, colour, aroma and preservation of food or beverages. Spices may be derived from many parts of the plant namely bark, buds, flowers, fruits, leaves, rhizomes, roots, seeds, stigmas and styles or the entire plant tops. The term 'herb' is used as a subset of spice and refers to plants with aromatic leaves. Another option is to prepare extracts such as essential oils by distilling the raw spice material (wet or dry), or to use solvents to extract oleoresins and

other standardized products.[8] Since the 19th century antimicrobial activity of spices against many foodborne pathogens such as *Staphylococcus aureus, Bacillus cereus, Salmonella, Listeria monocytogenes* have been well documented. Also some other bacteria such as *Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus vulgaris, Enterobacter aerogenes, Escherichia coli* have been inhibited by spices. Many studies have reported antimicrobial activities of spices against foodborne pathogens. Methods have been evaluated in order to determine the antimicrobial activity of spice extracts. It was found that the disk diffusion method was appropriate only as a preliminary screening test prior to quantitative MIC determination with dilution methods.[9] Only few international studies are available on the antibacterial effects of spices against *Cronobacter sakazakii*. A study has investigated bactericidal action of cinnamon and its bioactive compound cinnamaldehyde against *Cronobacter sakazakii and C. malonaticus*.[10] Since there is rising demand for natural food preservatives than artificial ones, the objective of the present study was to evaluate antimicrobial activity of selected spices against *C. sakazakii* isolated in our laboratory from dried coconut along with a type strain of Cronobacter obtained from a culture collection.

MATERIAL AND METHODS

Sample collection

Spices such as red chilli (*Capsicum annuum L*), coriander (*Coriandrum sativum*), turmeric (*Curcuma longa L.*), fenugreek (*Trigonella foecum-graecum L.*), fennel (*Foeniculum vulgare*), mint leaves (*Mentha* piperita), asafoetida (*Ferula asafoetida* L), star anise (*Illicium verum hook*), bay leaf (*Laurus nobilis L.)* and poppy seeds (*Papaver somniferum L.*) were procured from Local markets in Mumbai.

Media and Chemicals

All bacteriological media were procured from Hi Media Laboratories Mumbai, India and Analytical grade solvents were procured from local chemical manufacturers. Curcumin (91% pure) was a kind gift from a local supplier**.** Ciprofloxacin was obtained from local medical store and extracted by standard methods.

Sample preparation:

Spices were washed with distilled water and dried in oven for 24 hours. Dried spices were crushed in sterile mortar pestle and used for further experimental work.

Extraction of spices

Solvent and aqueous extracts of spices were made to determine the antimicrobial activity. Solvents such as methanol, ethanol, acetone and chloroform were used for the extraction of spices. 20g of each sample of spice was extracted with 90 % solvents (methanol, ethanol, acetone & chloroform) in a shaker at 30ºC for 8 hours. The residue obtained was extracted again twice with 90% methanol for 4 hours. The collected extracts was filtered through Whatmann filter no. 2. Extracts were allowed to evaporate at room temperature and were stored at 4ºC until further use.

Culture preparation & maintenance

The pure culture of *Cronobacter dublinensis spps dublinensis* was obtained from Microbial Type culture collection, IMTECH Chandigarh, India. The isolated culture of *Cronobacter sakazakii* was obtained from Microbiology laboratory, SNDT Women's University, Mumbai. The optical density of the cultures was adjusted to 0.5-0.6 at 600 nms. Cultures were maintained by sub-culturing on tryptic soy agar slants. Agar slants and plates were incubated at 37^0C for 24 hours and stored at 4^0C in refrigerator.

Antimicrobial assay

Agar well diffusion bioassay was used to determine antimicrobial activity of individual spices. A pilot experiment was out using varying concentrations of spice extracts at 100mg/ml, 10mg/ml, 1mg/ml, 0.1mg/ml, 0.01mg/ml. Using a sterile cork borer, wells were made in the seeded Mueller- Hinton agar plates and a 100 µl volume of spice extracts diluted appropriately in isopropanol were added in the wells. All the plates were incubated at 37^0 C for 24 hours. Antimicrobial activity was determined by measuring the clear zone of growth inhibition around the well.[11] The antimicrobial activity of spice extracts was compared with antibiotic ciprofloxacin using the similar concentrations. These tests were performed in duplicates and the mean of inhibition diameter was taken. Each experiment was repeated twice.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC of the spice extracts against test organism (*Cronobacter sakazakii*) was determined by the agar well diffusion method. The least effective concentration range was selected to determine MIC of spice extracts. The well containing least concentration of spice extracts showing clear zone of growth inhibition was considered as minimum inhibitory concentration of respective spice extracts.[12]

Determination of synergistic activity of spices

Synergistic activity of positive spice extracts was carried out using sterile Whatmann filter strips (1×7 cm). Effective concentrations of spice extracts were used to determine synergistic activity. Mueller- Hinton agar plates were swabbed with test culture using sterile cotton swabs. Whatmann strips were dipped in spice extracts & placed on the surface of swabbed plate in cross position. Synergistic activity was determined by measuring the zone of inhibition around the intersection of the strips which indicates synergistic activity of spices.[3]

Determination of thermal death point (TDP) and thermal death time (TDT) of *C. sakazakii*

Thermal death point and thermal death time of both isolated & pure culture of *C. sakazakii* was determined using well established procedures.[13]

Antibiotic susceptibility of *Cronobacter sakazakii*

Antibiotic susceptibility of *C. sakazakii* was determined using ciprofloxacin using agar well diffusion bioassay. Ciprofloxacin tablets were crushed & required concentration was prepared using sterile distilled water. Varying concentrations identical to that used for spice extracts were used by 10 fold dilution and 100µls of each dilution of the antibiotic was added in each well. Clear zone of growth inhibition indicated antibiotic susceptibility of *C. sakazakii*.

Effects of solvents against *C. sakazakii*

Solvents which were used for spices extraction such as methanol, ethanol, acetone and chloroform were subjected to antimicrobial activity determination. Agar well diffusion bioassay was used to determine the effects of individual solvents against both pure and isolated culture of *C. sakazakii* in order to confirm that activity of spices extracts was due to their bioactive constituents and not due to solvents used for extraction.

RESULTS AND DISCUSSION

Antibiotic susceptibility

Ciprofloxacin was used as control/reference to compare antimicrobial activity of spices against *C. sakazakii* (Figure 1).

Antimicrobial activity of spice extracts

The study shows that out of 10 selected spices 6 were found to have antimicrobial activity against *Cronobacter sakazakii*. Red chilli, turmeric, poppy seeds, asafoetida, bay leaf and mint leaves showed positive effects against *C. sakazakii*. Figure (2) shows the antimicrobial activity of spices against the test culture where highest activity of ethanolic extract of asafoetida as 1.95 mm & least inhibition by methanolic extract of turmeric as 1.575 mm per milligram of the spice extracts against the test culture.

Synergistic activity

Synergistic activity of effective spices was determined out of which only turmeric-poppy seeds combination was found to have synergistic activity. The highest synergistic activity was given with the 1:0.1 ratio of turmeric-poppy seeds combination as 23mm. The turmeric-poppy seeds combination showed synergistic activity in the range of 13mm- 23mm. Figure (3) shows synergistic activity of combination of spices showing inhibition.

Minimum inhibitory concentration

Figures (4,5) shows minimum inhibitory concentration of spices against pure culture of *C.dublinensis* and the isolated culture. Methanol extracts of spices (turmeric, red chilli, poppy seeds, asafoetida, bay leaf, mint leaves) were determined to have MIC as 0.1mg, 10mg, 0.6mg, 60mg, 100mg, 100 mg respectively. MIC of ethanol extract of spices (turmeric, red chilli, asafoetida) was 0.09mg, 10mg, 50mg respectively. MIC of acetone extracts of spices (turmeric, red chilli, poppy seeds, asafoetida, Bay leaf) was 3mg, 10mg, 0.9mg, 70mg, 90mg respectively. MIC of chloroform extract of spices (turmeric, red chilli, poppy seeds, asafoetida) was 0.01mg, 10mg, 50mg, 10mg respectively. Aqueous extract of spices did not show any activity against *C. sakazakii*.

Table (1) shows minimum inhibitory concentration of bioactive compound of turmeric, i.e curcumin. MIC of curcumin against pure culture of *C.dublinensis* was found to be 0.04mg whereas MIC of curcumin against isolated culture of *C. sakazakii* was found to be 0.06mg.

Thermal death time & thermal death point of *C. sakazakii*

Table (2) shows Thermal death point (TDP) & Thermal death time (TDT) of *C. sakazakii* were found to be 60^0C and 20 minutes respectively.

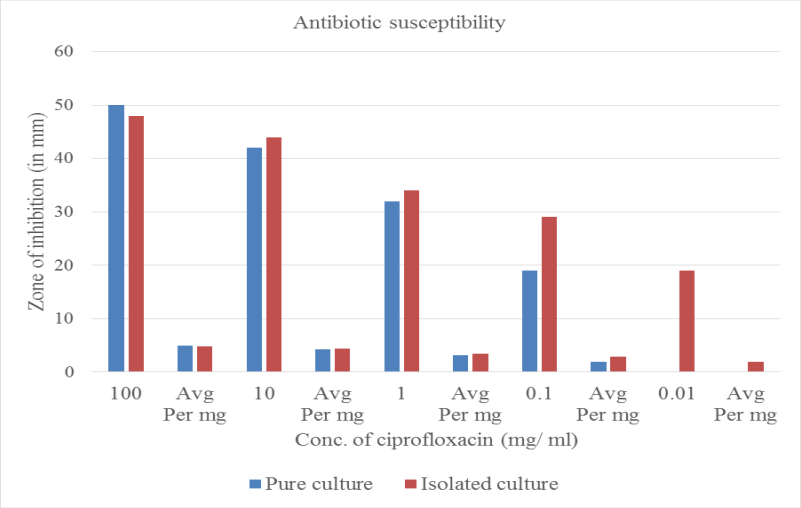

Figure 1: Antibiotic susceptibility of *Cronobacter dublinensis* and isolated culture to Ciprofloxacin

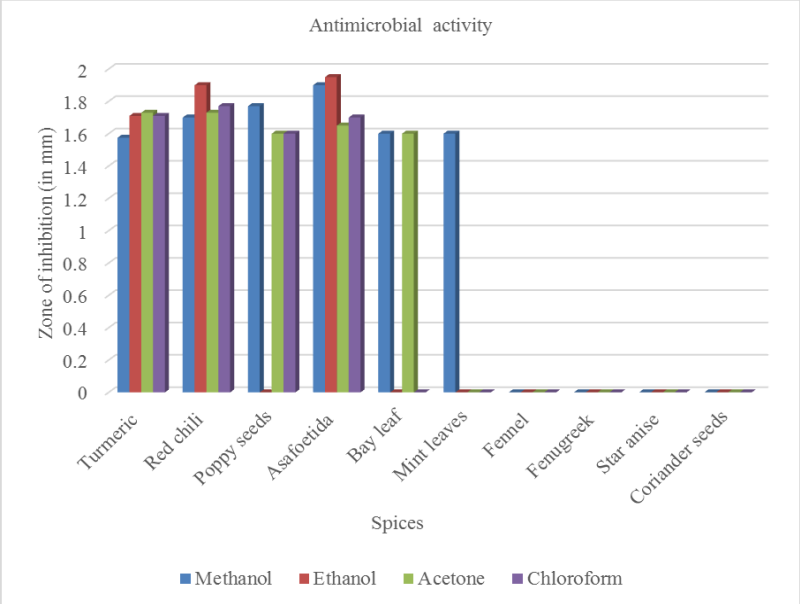

Figure: 2 Antimicrobial activity of spice extracts in different solvents

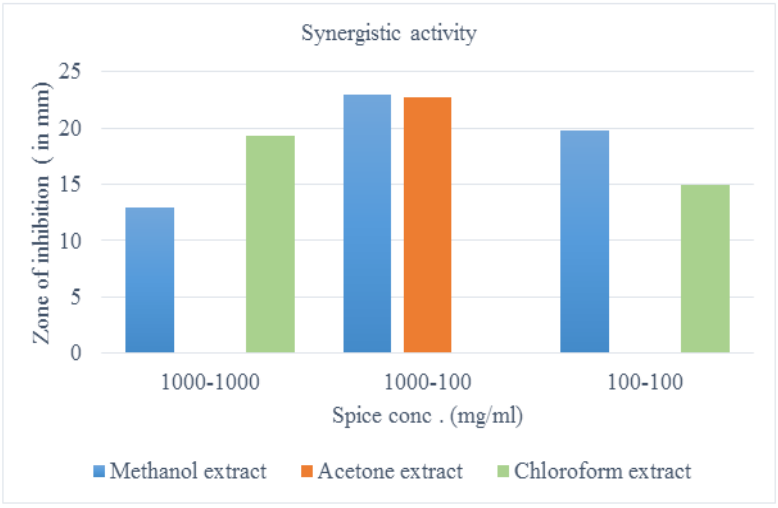

Figure 3: Synergistic activity of turmeric and poppy seed extracts in different ratios using different solvents

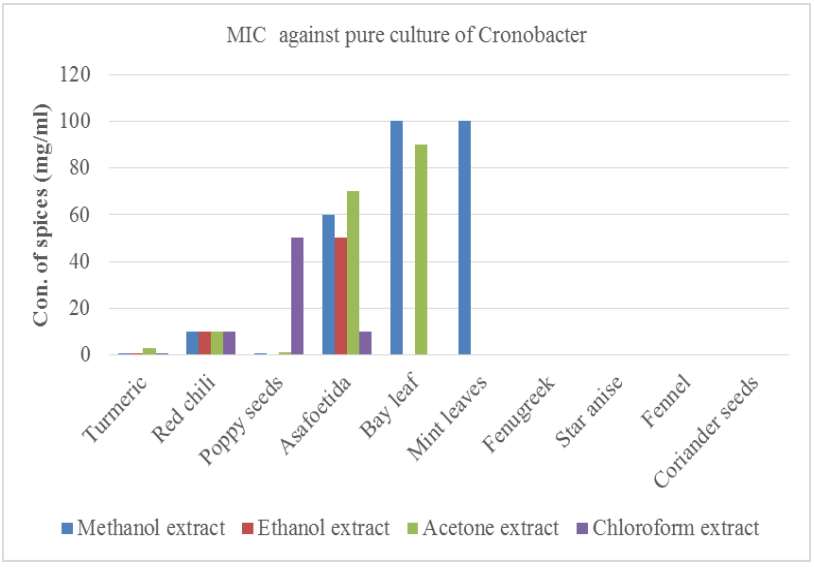

Figure: 4 Minimum inhibitory concentration against pure culture of *C. dublinensis*

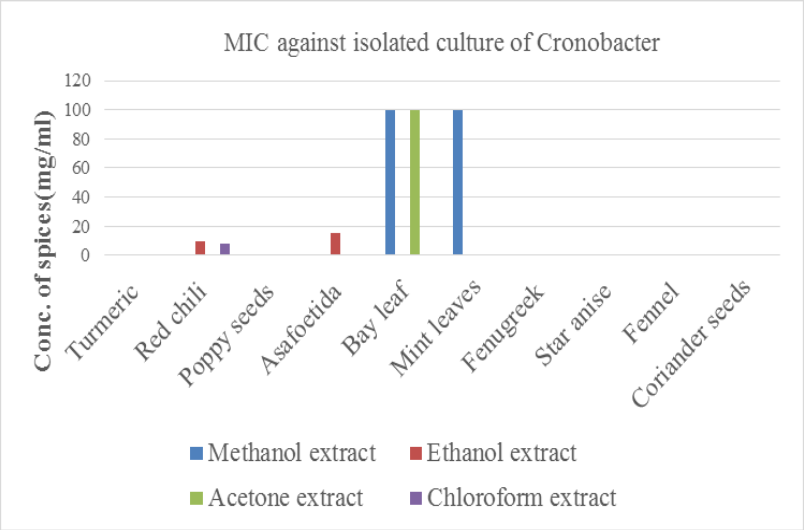

Figure: 5 Minimum inhibitory concentration against isolated culture of *C. sakazakii*

Table 1: Minimum Inhibitory Concentration of Curcumin against *Cronobacter* cultures

	Pure culture (mg/100 µl)	Isolated culture (mg/100 µl)
Curcumin	0.04±0.00	0.055±0.07

Table 2: Thermal death point & thermal death time of *C. sakazakii*

	Pure culture	Isolated culture
Thermal death point	60^0 C	60^0 C
Thermal death time	20 minutes	20 minutes

Although dried spices and herbs have been reported to be carriers of Cronobacter species bacteria, their presence in not often detected in products of this type.[14] Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic value. Recently, the acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the available antibiotics has led to investigations into the antimicrobial activity of medicinal plants[15].[16] have recently shown that plant extracts of piper nigrum and cinnamon verum showed inhibiton of quorum sensing biofilm formation in Cronobacter species using light and scanning microscopy. Similarly[17] have reported the use of citral a food flavor additive, as being effective in controlling Cronobacter sakazakii strains especially in powdered infant formulae. In an earlier study by[18] thirty three components including tocopherol, organic acids and bacteriocins were tested for their ability to inhibit Cronobacter and they report the effect of combinatory approaches to inhibit Cronobacter species in powdered infant formula In the current study, spices were selected on the basis of their existing reports available against other bacteria and other known food pathogens. Spices such as cinnamon, garlic, ginger, cumin was shown to have the highest positive effects against other bacteria and food pathogens. Also cinnamaldehyde from cinnamon have found to inhibit Cronobacter species and *Listeria monocytogenes*. Capsaicin from chili peppers enabled selection of red chili to determine its activity against *C. sakazakii*. Effectivity of turmeric against other food pathogens have been determined and found to give highest inhibition. A study has investigated bactericidal action of cinnamon & its bioactive compound cinnamaldehyde against *Cronobacter sakazakii* and *C. malonaticus.*[10] These are also the first reports available on the antimicrobial effects of turmeric and asafoetida and poppy seeds and synergistic activity of turmeric and poppy seeds against *C. sakazakii*.

CONCLUSION

Based on the present study, it can be concluded that spices can be used alone or in combination to preserve the foods including dried coconut (copra) as an ingredient. The use of spices can enable the application of food preservatives in the food products in order to prevent food infections caused by the emerging pathogen *Cronobacter sakazakii* at the same time enhancing the taste and flavor of these foods.

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