Anti-*Salmonella* and anti-biofilm activity of *Catharanthus roseus* (CR) extract against endophytic *Salmonella sp.* isolated from raw salad vegetables.

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Abstract

Endophytic Salmonella sp. were Isolated (n= 52) from raw salad vegetables (n= 550). Among all isolated Salmonella sp. 6 species were observed with multidrug resistance and they were selected for further studies. Catharanthus roseus plants are studied for anti-Salmonella and anti-biofilm activity against isolates, and the present study was conducted to prevent contamination of endophytic Salmonella sp. to raw salad vegetables. So keeping this as an aim, we studied the antibiofilm and antibacterial activity of different concentrations of Catharanthus roseus against isolated endophytic Salmonella sp. Based on observation during the study, we found Catharanthus roseus has antibacterial as well as antibiofilm activity against Salmonella sp.

Keywords: Endophytic Salmonella, Anti-*Salmonella*, anti-biofilm, *Catharanthus roseus*, Anti-bacterial

Introduction

The intake of raw salad vegetables all over the globe is a chief attribute of nourishment. Throughout time, as people became more urbanized, diet behaviors became further distinct, and diet utilization patterns altered from the diet for appetite to the diet for healthiness and welfare. The alteration in the ingestion pattern has been marked amongst both rural and metropolitan households [1]. Escalating urbanization is an additional key ingredient promoting the alteration in utilization patterns. India is the second chief producer of vegetables in the globe with a yearly production of nearly 94 metric million tons. Due to its diverse climatic circumstances, India is one of the rare countries producing nearly all - tropical and exceptional vegetables. This has led to amplifying the popularity of formulated nominally treated vegetables comprising pre-cut salads, shredded vegetables, prepared fruit salads, sprouted seeds, etc. Rvealed that a diet rich in raw vegetables led to a considerable 40% decrease in cardiac troubles and a 45% decrease in death. The above research is one of countless such findings that highlight the significance of vegetables in the diet. Raw vegetables are globally appreciated as a valuable constituent of a nourishing diet because it is a source of vitamins, minerals, fiber, and antioxidants. As the utmost produce is grown in a natural situation, it is susceptible to contamination through pathogens [2]. Circumstances that may affect the episode of such contamination involve agricultural water property, the application of manure as fertilizer, the occurrence of animals in areas or parking spaces, and the health hygiene of employees managing the product throughout production, packing, processing, transportation, distribution, or preparation. The above reasons as well as the fact that produce is often consumed raw without any type of intervention (that would reduce, control, or eliminate pathogens) before consumption contribute to its potential as a source of food-borne diseases [3]. Over the last decade, the epidemiology of food-borne diseases in developing as well as developed countries has changed due to the emergence of newer pathogens believed to be due to major changes in the global economy (facilitating the rapid transport of spoilable foods, raising the possibility for acquaintance to foodborne pathogens from other portions of the globe as well as another aspect [4].

Salmonella in foods

Salmonella sp. is the most common pathogenic bacteria associated with a variety of foods. Although myriad foods can serve as *Salmonella* sources, meat and meat products, poultry and poultry products, and dairy products are significant sources of foodborne pathogen infections in humans. The presence of *Salmonella* sp. in fresh raw products can vary widely [5]. Frequency usually ranges from 1 to 10 %, depending on a range of factors including organism, farming, and/or food production practices and geographical factors. Research on *Salmonella* frequency in different countries is extensive and *Salmonella* serotypes have been isolated in a variety of foods. *Salmonella* can contaminate eggs on the shell or internally and egg shells are much more frequently contaminated than the white/yolk. Furthermore, egg surface contamination is associated with many different serotypes,

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while infection of the white/yolk is primarily associated with *S*. Enteritidis. A range of fresh fruit and vegetable products have been implicated in *Salmonella* infection, most frequently lettuce, sprouted seeds, melons, and tomatoes. *Salmonella* sp [6]. are often isolated during routine surveys of produce such as lettuce, cauliflower, sprouts, mustard cress, endive, and spinach bean sprouts, alfalfa sprouts, unpasteurized juices, and fresh salad fruits and vegetables [7].

Interaction of Salmonella with foods

Salmonella serotypes can grow and survive on a large number of foods. Their behavior in foods is controlled by a variety of environmental and ecological factors, including water activity, pH, chemical composition, the presence of natural or added antimicrobial compounds, and storage temperature; as well as processing factors such as heat application and physical handling. For example, the optimum pH for growth in *Salmonella* is approximately neutral, with values > 9.0 and < 4.0 being bactericidal. Minimum growth in some serotypes can occur at pH 4.05 (with HCl and citric acids), although this minimum can occur at pH as high as 5.5, depending on the acid used to lower pH [8]. Growth in *Salmonella* can continue at temperatures as low as 5.3 °C (*S.* Heidelberg) and 6.2 °C (*S.* Typhimurium) and temperatures near 45 °C (temperatures ≥ 45 °C are bactericidal). In addition, available moisture inhibits growth at values below 0.94 in neutral Ph media, although higher aw values are required as pH declines to near the minimum growth values **[9].**

Food as Cause of Human Salmonellosis

Foodborne salmonellosis is still today a serious public health issue: very common in poor developing countries, due to the bad general hygiene conditions, it is also largely widespread in developed countries. In the latter, 95% of recorded clinical cases are foodborne. According to in the European Union (EU) *Salmonella* is the second cause of the foodborne disease after *Campylobacter* and it is still first in many EU States, such as Italy. Unlike *Campylobacter*, *Salmonella* often causes very large *multistate outbreaks* of food infection; this proves the greater resistance of this pathogen in the external environment and food. In developed countries, the main source of salmonellosis is still today food of animal origin, particularly meat (fresh and processed) and shell eggs. Also, fresh fruits and vegetables can convey bacteria to humans, as well as undrinkable water. *Salmonella* is quite resistant to adverse conditions and this allows them to persist in the environment and spread along the food chain, from the animals to the food of animal origin, or to plants that are fertilized with animal manure. Two species are currently registered in the genus *Salmonella*: *S. enterica* and *S. bongori*. The former is better adapted than the latter to live in the intestine of man and warm-blooded animals, whereas *S. bongori* travels in the external environment and is detectable in the intestinal contents of warm-blooded animals, so it is rare for it to be found in food for human consumption. The dangers for human health mainly arise from food contaminated with *Salmonella enterica*, which is often present in the intestines of livestock and pets, without causing any infection to the animals ("healthy carrier" condition). Humans can be healthy carriers of *S. enterica* in the intestine too. This may be a potential hazard to food hygiene if the healthy carriers are the people involved in producing and handling the food. Usually, a healthy carrier eliminates *Salmonella* in their faeces for several months after the episode of gastroenteritis through which they become a carrier. In the case of *Salmonella* ser. Typhi, however, has been demonstrated that humans can be asymptomatic carriers of the bacterium for decades [11]. The genus *Salmonella* has more than 2,500 serotypes, and over 1,600 of these are within the *enterica* species, but not all serotypes have the same affinity for humans and/or animals and they are not all found in the food that humans consume. Some serotypes (Typhi, Paratyphi A, and C, some clones of Paratyphi B and Sendai) travel almost exclusively among men and express their pathogenicity only when they infect a human being. Few serotypes travel exclusively among animals and do not infect humans, if not seldom (e.g. Abortusovis in sheep and Gallinarum-Pullorum in poultry). On the contrary, approximately 150 serotypes travel more or less constantly between the animal reservoir, the environment, food, and man, starting from *Salmonella* ser. Typhimurium [12].

Preventive approaches

Organic acids have been documented to possess antimicrobial activities against different pathogenic bacteria such as *Salmonella* spp., *E. coli*, and *Listeria monocytogenes*. Several studies have proved the antimicrobial ability of organic acids. For instance, reported that citric acid was an antimicrobial chemical that significantly affected the biofilm elasticity of pre-formed *Pseudomonas* biofilms [13]. Another study demonstrated that gallic acid has the potential to inhibit bacterial motility and thus prevent and control biofilms of these pathogenic bacteria Malic acid was found to be effective in the inhibition of *S. enterica* serovar *Typhimurium* biofilm in carrot and food contact surfaces. There have been reports of copper being an effective and broad-spectrum antimicrobial agent against a wide range of pathogenic bacteria. The mechanism of antimicrobial action of copper begins with the rupture of the bacterial outer membrane on contact followed by the entry of copper ions into the cell and obstructing cell metabolism. Copper has shown its antimicrobial action against a broad range of pathogens including *E. coli* O157:H7 and *Salmonella [14]*.

Regulation mechanism of biofilm formation

Biofilm formation is majorly regulated by the CsgD protein, a regulator belonging to the LuxR family. CsgD has an N-terminal receiver domain with a conserved aspartate (D59) as a putative target site for phosphorylation and a C-terminal LuxR-like helix-turn-helix DNA binding motif. Multiple factors bind to the promoter sequence of csgD and regulate its transcription, such as OmpR, RpoS, RpoE, integration host factor (IHF), histone-like nucleoid structuring protein (H-NS), and MlrA. OmpR is one of the first discovered to be required for csgD transcription. Six binding sites (D1–D6) for OmpR are identified in csgD promoter regions. The binding of OmpR-P to D2 centered immediately upstream of D1 is proposed to repress promoter activity. IHF competes with OmpR-P for binding

at its upstream site IHF1, which overlaps with D3–D6 and thereby activates the transcription of csgD [15].

Objectives of the present research experiment

- To prevent endophytic contamination of *Salmonella sp*. by preventing Biofilm production
- *in vitro* screening of *Catharanthus roseus* plant extracts for potential antibacterial and anti-biofilm activity
- To detect MIC, MBC, and MBIC values of *Catharanthus roseus* plant extract against isolated endophytic *Salmonella sp.*

Materials and Methods

All the materials used in this study were purchased from authorized firms in India. Media, chemicals, and reagents were purchased from Hi-Media PVT. LTD. Mumbai - 400 086. Glassware was purchased from Borosil Glass Works Limited, Mumbai- 400 018 (India), plasticware was procured from M/S Tarsons Products PVT LTD. New Delhi - 110001.

Preparation of plant extracts

Shade-dried plant material was fully ground into powder and extracted using the maceration extraction method. Methanol was used as an extraction solvent. The solvent-to-sample ratio was 10:1 (v/w). 100 gm of dried plant powder of each plant part was soaked separately in 1000 ml of methanol in an Erlenmeyer flask. The flasks were covered with aluminum foil and allowed to stand in the dark for 72 hours on a rotary shaker at 150 rpm for extraction. The extract suspensions were filtered through Whatman filter paper No. 1. Filtrate or solvent was then evaporated at 40°C-45°C in a hot air oven until crude extract was obtained and its percentage yield was calculated. Stock solutions of extracts in dimethyl sulphoxide (DMSO) (10.0 % v/w) were prepared and stored at 4°C for further experiments **(Figure 1)**

Determination of Anti-Salmonella activity

In the present study, the anti-*Salmonella* activity of various plant extracts in methanol was screened for MIC by Resazurin-based microdilution assay A stock solution (1g/ml) of each plant extract was prepared in 10% DMSO (Dimethyl sulphoxime) & and tested for their antimicrobial efficacy. The prepared agar plates were marked with the organism and extract code.

Determination of MIC by Resazurin-based microdilution assay

The MICs of various plant extracts were evaluated by Resazurin-based microdilution assay (RMDA) with slight modifications. RMDA was performed using 96-well plates under sterile conditions. Each plant extract is prepared in Different concentrations of test extracts ranging from 1000 µl/ml in 1000.0 µl of LB broth by successive dilution method in test tubes and 100 µl aliquates transferred to 96-well plates. Then, 10.0 µl of resazurin indicator solution and 10.0 µl of bacterial suspension were added (1×10^6 CFU/ml) to each well consecutively. Each plate had growth control as well as sterility control. The plates were prepared in triplicate and incubated at 37°C for 18-24 h at 100 rpm. After incubation, the colour change was recorded. The change of blue colour to pink was recorded as a positive result. The viable colonies of bacterial culture transform the blue dye, resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide) to a pink-colored compound, resorufin. The MIC value is the lowest concentration at which the dye colour remained unchanged. Each experiment was conducted in triplicates and the average was recorded as the MIC value of the test extract against bacterial strain **(.** Negative (media only) and positive (media with bacterial inoculum) controls were also assessed. Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation. The tubes were examined for visible growth (cloudy) and recorded growth as (+) and no growth as (-). The concentration at which no growth was described as the MIC of the extract

Determination of MBC

The minimum bactericidal concentration (MBC) is the lowest concentration of an antibacterial agent required to kill a particular bacterium. It can be determined from broth dilution minimum inhibitory concentration (MIC) tests by subculturing agar plates that do not contain the test agent. The MBC is identified by determining the lowest concentration of antibacterial agent that reduces the viability of the initial bacterial inoculum by ≥99.9%. Antibacterial agents are usually regarded as bactericidal if the MBC is no more than four times the MIC. For the determination of MBC, the bactericidal concentration was then found by subculturing the contents of selective tubes into a series of Mueller Hinton

Figure 1: *Catharanthus roseus (CR).*

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broths. GC tube containing culture was serially diluted up to 10^{-8} and plated 10^{-4}, 10^{-5}, $10^{-6,}$ and 10^{-7} diluted materials on respective properly labeled nutrient agar plates to check the total viable count of the initial inoculums used to determine MIC, MBC. The number of bacterial colonies in each plate was counted properly and recorded. The dilution at which no colonies appeared was considered as MBC of the extract.

Determination of Antibiofilm activity

Determination of MBIC by microtitre plate assay

In a microtitre plate containing 200 µl of Tryptone Soybean broth in each well, the test isolates including *Salmonella sp*. were inoculated in separate wells and incubated at 37°C for 18-24 h. The content of the wells was removed after incubation and washed with 0.2 ml of phosphate buffer saline (PBS pH 7.2) to remove the planktonic cells. The wells were then stained with 200 µl of 0.4% crystal violet. Excess stain was rinsed off by thorough washing with distilled water, and 100 µl of ethanol was added as a destainer. The level of adhered bacteria was read as Optical density (OD) at 580 nm using an ELISA auto reader.

Statistical analysis

To study the significant difference was found between the results of ANOVA and chi-square and the difference between the groups was significant at the significance level of $p < 0.05$. All the values were expressed as mean ± SD (standard Deviation). The statistical significance of differences between the control and experimental groups was assessed by one-way ANOVA. The value of probability less than 5% ($P < 0.05$) was considered statistically significant**.**

Results and Discussion

Determination of MIC , MBC by microtiter plate resazurin test

To evaluate the plant extract anti *Salmonella* efficacy MIC and MBC assays are performed **(Figure 2).** Plant extract against isolated endophytic *Salmonella sp.* (DP1) varied from 500 µl /mL and 1000 µl/mL. For *Salmonella sp.* (DP1) the most efficient anti-*Salmonella* activity was observed in CR extract which was determined to range between 500.0 µl/mL-1000.0 µl/mL, which indicates that even lower concentrations of CR extract can inhibit the growth of *Salmonella sp*. The bacteriostatic characteristic of the extracts was evaluated using the MIC assay, whereas the bactericidal concentration of the CR extracts was determined between 650-780 µl/mL. CR extract showed efficient anti-*Salmonella* activity. Therefore further dilution was studied from CR extracts by the same method as above between a range of MIC (500-1000 µl/mL) to obtain accurate MIC and MBC. The results of these dilutions determined MIC of the CR plant is 650 µl/mL whereas the MBC of the CR plant is 780 µl/mL.

Determination of MBIC by micro-titer plate assay

To evaluate the plant extract anti-biofilm efficacy of various plant extracts MBIC assay was performed **(Figure 3).** For *Salmonella sp.* (DP1) the most efficient anti-biofilm activity was observed in CR extract which was determined to range between 250 µl/mL- 500 µl/ml. CR extract showed efficient

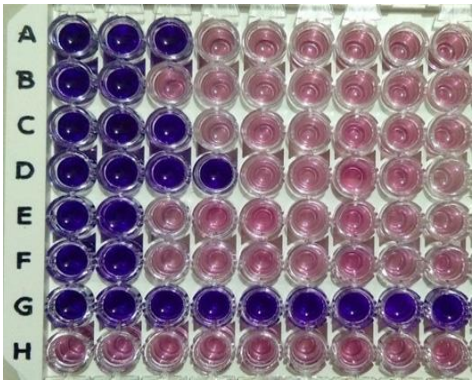

Figure 2: *Microtitre plate resazurin test.*

Figure 3: *Crystal violet binding Micro-titer plate assay.*

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anti-biofilm activity. Therefore further dilution was studied from CR extracts by the same method as above between a range of MBIC (250-500 µl/mL) to obtain accurate MBIC. The results of these dilutions determined that the MBIC of the CR plant is 330 µl/mL.

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