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COMPARATIVE STUDY OF MEDICINAL PLANT EXTRACTS AND PROBIOTIC AGAINST MULTIDRUG RESISTANCE *Salmonella* ISOLATES

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ABSTRACT:

Medicinal plants and probiotics both have potential antimicrobial activity against antibiotic-resistant pathogens which causes gastrointestinal diseases in human. Multi drug resistance is a big issue in medical field and these multi drug resistance enteric pathogenic bacteria generally found in sewage water. The microorganisms have developed resistance to many commercial antibiotics due to the various uses of antibacterial drugs. This study involved isolation, identification and molecular characterization of multidrug resistance *Salmonella* strains from sewage water treatment plants located in Delhi. Main aim of this study was to identify anti-microbial efficacy of *Murraya koenigii*, *Piper betle*, *Tagetes erecta* and *Aegle marmelos* leaves extract and their combination of with *Lactobacillus plantarum* which is commonly used probiotic against

gastrointestinal disorder. Combination of *Murraya koenigii* leaf extract and *Lactobacillus plantarum* has shown effective against both *Salmonella* strains isolated from sewage water in the study. This combination has shown significant results as compared to rest of three plant leaf extract used for the study. Thus combination therapy may be effective for treatment of gastrointestinal diseases and could be suggest as an alternative medicine.

Key Words: *Salmonella* sp**.,** *Murraya koenigii, Piper betle, Tagetes erecta, Aegle marmelos, Lactobacillus plantarum*

INTRODUCTION:

Salmonella enteric, Salmonella typhi and paratyphi A, B or C causes severe gastrointestinal disease in humans. Gastrointestinal disease generally transmitted through faecal-oral route by contamination of water and food, specially by food-handling carriers. Human enteric pathogens are mainly found in Sewage water which contains human feces and therefore contains human enteric pathogens i.e. *Clostridium difficile, Salmonella typhymurium, Shigella dysenteriae, Salmonella enteric, , Klebsiella spp., Vibrio spp., Helicobacter pylori* and *Campylobacter jejuni*. These pathogens can promote gastrointestinal disease and cause water borne endemic. For combat against these pathogens invention of new medicines is necessary.Now a days treatment against antibiotic resistance Salmonella strains has become challenging task for medical field. These strains are widely resistance against fluoroquinolones and third-generation cephalosporin. The problem has become serious in many developing countries **(**Tsobou R *et al*, 2015**)**. Due to less side effects and less toxicity, it is now a trend to use herbal medicines as an alternative treatment (Anita Joshi *et al*, 2009). Many plant extracts have shown promising microbiocidal activities against human enteric pathogenic bacterial strains. Medicinal plant extracts contains bioactive phytochemicals. Due to the wide range of medicinal value *Murraya Koenigii, Piper betel, Aegle marmelos, Tagetes erecta* are frequently used in ethno pharmacology field.

Murraya koenigii, commonly known as curry leaf is under the member of *Rutaceae* family. These leaves have natural flavour and contains so many medicinal properties such as antimicrobial, antifungal, antidiabetic, antioxidant, anti-inflammatory and hepato-protective activities. These leaves are very useful in gastrointestinal diseases like diarrhoea, dysentery (Manisha Vats *et al*., 2011). *Piper betle* is the leaf which belonging to the Piperaceae family. *Piper betle* plant leaves are rich in so many secondary metabolites and has been

conventionally used as compost, carminative, antiseptic agents, antifungal and antibacterial. It has also been reported for the cure of stomach problems. *Aegle marmelos* (Linn.) commonly known as bael, is under *Rutaceae* family. It is found all over India and is well known from ancient time. These leaves extract as medicine for relieving gastrointestinal disorder, peptic ulcer and respiratory infections (Nadkarni A. K *et al* 2000). Previous research of *Aegle marmelos* showed that the plant has antidiarrhoeal properties (Shobha F.G.,*et al* 2001). *Tagetes erecta* which belongs to family *Asteraceae. Tagetes erecta* is generally used for garnishing houses and Idols of God during the festivals in India. Since pre-historic time this plant has been used for medicinal field against gram negative as well as gram positive bacteria (Roberta Piccaglia *et al* 1998). Now a day combination therapy with well known herbal medicine and probiotics is gaining popularity for controlling gastrointestinal disorders. Among various commonly prescribed probiotics *Lactobacillus* bacterium are most prominent. *Lactobacillus plantarum* is a gram positive aero tolerant bacteria which is commonly used as probiotics. *Lactobacillus plantarum* produce antimicrobial substances which is very beneficial against gram negative enteric bacteria. Several *Lactobacillus plantarum* strains have been shown to produce different compounds having antimicrobial activity (Herreros *et al*, 2005, Tharmaraj *et al*, 2009, Cortés-Zavaleta *et al*, 2014). Some studies have been shown the combined effects of *Aloe vera* and *Lactobacillus plantarum* against food borne pathogens (Jiang *et al*, 2016). The combination of medicinal plants and probiotics as functional feeds has not been widely studied. Although individually they are highly valuable and their combinations can be enhance their effectiveness and usefulness in terms of medicinal value through synergism.

The objective of present study was to evaluate the synergistic antimicrobial activity of herbal leaf extract and *Lactobacillus plantarum* against *Salmonella* sp. isolated from sewage water. The study involves; (a) Isolation and identification of *Salmonella* sp. from sewage water (b) Preparation of four medicinal plant (*Murraya koenigii, Piper betle, Tagetes erecta* and *Aegle marmelos*) leaf extracts and their efficacy against isolated *Salmonella* sp. (c) Efficacy of four antibiotics against isolated *Salmonella* sp. (d) Evaluation of combinational antimicrobial efficacy of each medicinal plant leaf extract (*Murraya koenigii, Piper betle, Tagetes erecta* and *Aegle marmelos*) along with *Lactobacillus plantarum* against isolated *Salmonella* sp. by agar well diffusion method (e) Interpretation of the data which can helpful to assist in formulation of new herbal implications in ayurvedic medicine.

MATERIALS AND METHODS:

a) Isolation and identification of *Salmonella* isolates from sewage water sample:

250 ml of sewage water sample collected from sewage treatment plants of Delhi, was filtered through 0.45 micron filter paper and then filter paper was inoculated in Buffer peptone water for incubation at 37^0C for 24 hours. 0.1 ml of above was inoculated in 10 ml of Rappaport vassiliadis medium and then incubated at 42^0C for 24 hours. Streaked on the Brilliant green agar plates and Bismuth sulphide agar plates. Characteristic colonies was observed on plates. Further confirmation was done by Gram's staining and HiMedia IMViC biochemical kit for *Salmonella* as per IS: 5887(Part-3) 1999, Reaffirmed 2018. Molecular identification of Salmonella isolates were done by 16srRNA sequencing (Figure 1 and Figure 2).

b) Preparation of plant leaf extract:

Four medicinal plants leaves i.e. *Murraya koenigii, Piper betle, Tagetes erecta* and *Aegle marmelos* were selected for this study. 10g of each leaves powder were added to 100 mL of 70% aqueous methanol solution (w/v) covered with filter paper, then kept on rotary shaker for 24 hours and stored in dark at room temperature for three days. The supernatant was collected and the solvent was evaporated to make the final volume of the leaves methanol extract for this experiment. Final concentrations having 100 mg/mL of each leaves extract were used for the study.

c) Antibiotics and their solutions:

Four antibiotics which are generally prescribed in gastrointestinal disorder i.e. Metronidazole, Ampicillin, Nitroimidazole and Norfloxacin were used to evaluate susceptibility and resistance pattern of *Salmonella* isolates. Final working solution having 100 mg/ml concentration of each antibiotic was used for the study.

c) Agar Well Diffusion Assay (Zone of Inhibition Evaluation):

Antimicrobial activity of leaves extract was assessed by agar well diffusion method. Sterile Muller Hinton Agar (MHA) plates were inoculated with *Salmonella* isolates. 100µl of respective antibiotic solutions, *Lactobacillus plantarum* (1 x 10^8 cfu/ml) and standardized leaves extract (100 mg/mL) were added in wells. Plates were incubated overnight at 37ºC. Zone of inhibition were observed in plates where diameter of zones were calculated using Vernier callipers.

d) Statistical analysis: Data were represented as means ± SD and results were statistically analyzed using SPSS software by means of independent one-way ANOVA tests. The differences were assessed by means of the least significant difference (LSD) and multiple comparison tests ($P < 0.05$). 95% confidence level was used to determine statistically significant differences are dependent on the p value.

RESULTS & DISCUSSION:

During this study *Salmonella enteric* and *Salmonella typhimurium* these two strains were identified. The susceptibility patterns of these two strains were evaluated against four commonly prescribed clinically significant medicines (Metronidazole, Ampicillin, Nitroimidazole and Norfloxacin), four plant leaves extract and combination of leaves extract with *Lactobacillus plantarum* by agar well diffusion method. The antimicrobial activity in terms of average zones of diameter considering three plates for bacterial isolates were calculated and shown in Table 1 and Table 2. Susceptibility patterns were shown in Figure 4A to 4F. In this study, it has shown that minimum 100mg/ml concentration of medicinal plant leaves extract have strong antimicrobial activity against both multi drug resistant *Salmonella* strains. These two strains were shown resistance against Ampicillin, Nitroimidazole and Norfloxacin as there was no zone of inhibition was observed against these three antibiotics.

In comparing the inhibition zones against *Salmonella enteric* and *Salmonella typhimurium* it has been observed that minimum zone of inhibition was 11.46 ± 0.07 for *Aegle marmelos* leaf extract and maximum zone of inhibition was 16.65 ± 0.14 for *Murraya koeniggi* leaf extract. *Lactobacillus plantarum* also showed effective ($P < 0.05$) against these two strains as zone of inhibition varies between 11.72 ± 0.27 to 12.24 ± 0.06. Some of the previous study has shown that the minimum inhibitory concentration of *Aegle marmelos* extract for *Salmonella typhi* was the lowest with methanol extract suggested that the smallest amount of this extract was required and was most active. *Murraya koeniggi* leaf extracts have shown the strongest inhibition zone against *Proteus mirabilis* (18mm), *Staphylococcus aureus, Corynebacterium pseudotuberculosis* (15mm), *Klebsiella pneumoniae* (15mm), *Pseudomonas aeruginosa* (14mm), *Enterobacter aerogenes* (13 mm) and a moderate level zone of inhibition was observed with *Salmonella enteric* (11mm) and *Streptococcus pyrogens* (10 mm) respectively (Rajendran MP *et al*, 2014). Our study has shown comparable results with the inhibition zone for two *Salmonella* strains ranging from 16.46 ± 0.14 mm to 16.65 ± 0.14 mm. The zone of inhibition of *Piper betle* against *Salmonella enteric* was shown 13.48 ± 0.11 mm and for *Salmonella typhimurium* was 13.40 ±

0.14 mm. Some previous research showed that extraction in ethanol of *Piper betle* leaves had a high inhibition zone on 100% concentration (Abd El-Hack, M.E et al 2016). Another research have been shown that the inhibition zone of aqueous extracts on beetle leaves is better than extraction in methanol (Pasha c et al 2009). *Tagetes erecta* leaf extract shown that zone of inhibition 11.47 ± 0.06 mm against *Salmonella enteric* and 14.57 ± 0.05 mm against *Salmonella typhimurium*. Our results are similar to those of the methanolic extract of *Tagetes erecta*, since it gives rise to zones of 10 mm inhibition (Vega-Menchaca, 2013). On the other hand, zones of inhibition ≥ 9 mm of the ethanolic extract of *Tagetes erecta* on *Salmonella typhi*, *Shigella dysenteriae*, *Salmonella enteritidis*, and *Shigella flexneri* were detected (Capunzo *et al*., 2003).

On the other hand combination of plant leaves extract with *Lactobacillus plantarum* has shown better efficacy ($P < 0.05$) against isolated strains. Zone of inhibition has shown between 18.44 ± 0.15 mm to 22.44 ± 0.17 mm (Figure 3). *Murraya koenigii* leaves extract was found to be very promising medicinal plant and the combination of this plant leaf extract with *Lactobacillus plantarum* has shown more effective compare to rest of three plant extracts against *Salmonella typhimurium* and *Salmonella enteric* in our study and has shown significant results ($P < 0.05$). Oil extracts from this plant have shown (Rajendran MP *et al*, 2014) the strongest inhibition zone against *Proteus mirabilis* (18mm) and a moderate level zone of inhibition was observed with *Salmonella enterica* (11mm). Previously studies showed that medicinal plant (Babak D *et al*, 2014) and probiotics (Yoon W.J *et al* 2009) had antioxidant and anti-inflammatory effects. The significant inhibitory effects of oil and the probiotic on microorganism were observed alone and in combination together.In our study it has been observed that the antimicrobial activity of *Murraya koenigii* leaf extract with combination of *Lactobacillus plantarum* on *Salmonella enteric* and *Salmonella typhimurium* have shown to be more profound and this combination can be used as alternative medicines. **CONCLUSION:**

In this study the combinational formulation will be a gate way of new herbal implication in ayurvedic medicine. Combining the effect of *Murraya koenigii* leaf extract with *Lactobacillus plantarum* may reveal a new approach concerning their complementary antibacterial effects. The synergistic effect of the *Murraya koenigii* leaf extract and *Lactobacillus plantarum* will be necessarily greater than utilizing them alone as healthcare products. This new combination could contribute for the development of new safe and effective therapeutic agents. It may be the best natural alternative to antibiotic therapy for tested pathogenic bacteria. Therefore, these products may be very beneficial for the treatment of gastrointestinal diseases.

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CONFLICT OF INTEREST:

The authors declare that there is no conflict of interests regarding the publication of this article.

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Table 1: Antibacterial activity against *Salmonella* isolates

Name of Pathogen	Antibacterial activity Zone of inhibition* (in mm)								
	MET	NOR	AMP	NIT	MK	PB	TE	AM	LP
Salmonella enteric	19.56 ± 0.07	NZI	NZI	NZI	16.65 ± 0.14	13.48 ± 0.11	11.47 ± 0.06	11.46 ± 0.07	12.24 ± 0.06
Salmonella typhimurium	19.81 ± 0.09	NZI	NZI	NZI	16.46 ± 0.14	13.40 ± 0.16	14.57 ± 0.05	14.52 ± 0.17	11.72 ± 0.27

NZI: No zone of inhibition**, * Zone of inhibition (in mm):** average zones of diameter considering 3 plates, diameter including well diameter 6 mm

MET: Metronidazole, **AMP:** Ampicillin, **NIT:** Nitroimidazole**, NOR:** Norfloxacin, **LP:** *Lactobacillus plantarum***, MK:** *Murraya koeniggi***, PB:** *Piper betle***, TE:** *Tagetes erecta***, AM:** *Aegle marmelos*

Table 2: Efficacy of herbal combination with probiotic against *Salmonella* isolates

Name of Pathogen	Antibacterial activity Zone of inhibition* (in mm)			
	LP + MK	LP + PB	LP + TE	LP + AM
Salmonella enteric	22.44 ± 0.17	20.32 ± 0.13	19.43 ± 0.21	19.77 ± 0.20
Salmonella typhimurium	23.32 ± 0.23	20.45 ± 0.13	18.44 ± 0.15	19.23 ± 0.30

***Zone of inhibition (in mm):** average zones of diameter considering 3 plates, diameter including well diameter 6 mm

LP: *Lactobacillus plantarum*, **MK:** *Murraya koeniggi*, **PB:** *Piper betle*, **TE:** *Tagetes erecta*, **AM:** *Aegle marmelos*

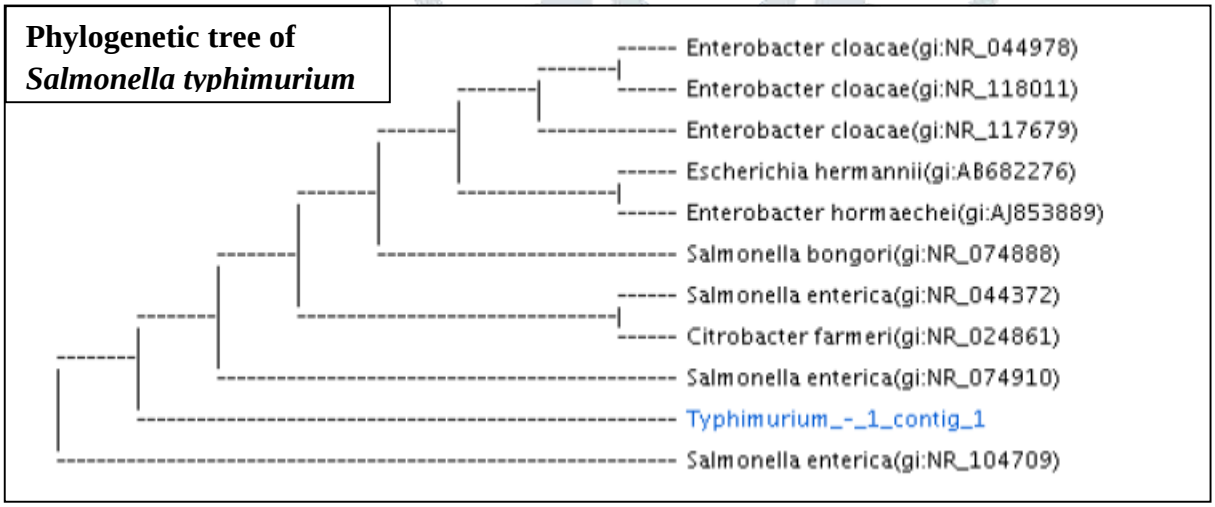

Fig 1: Molecular identification of *Salmonella typhimurium*

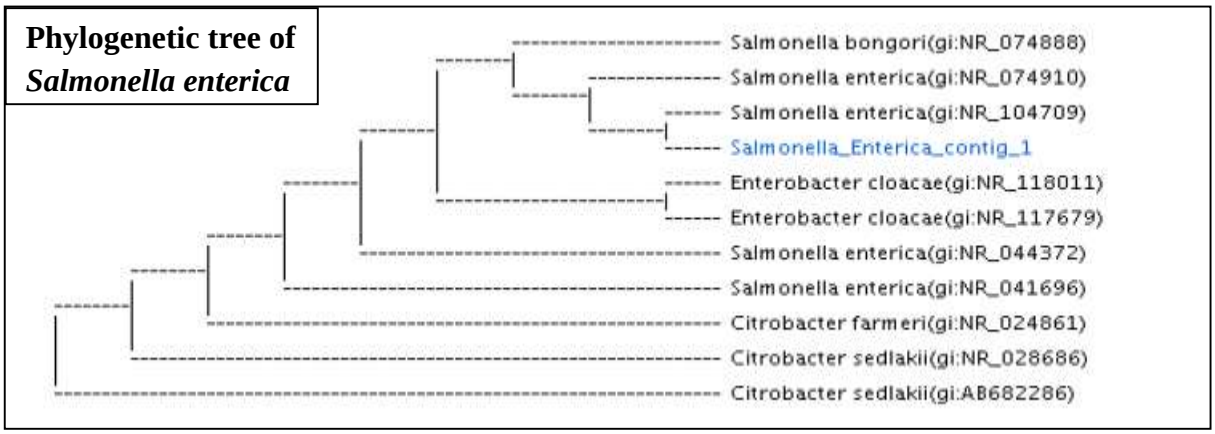

Fig 2: Molecular identification of *Salmonella enterica*

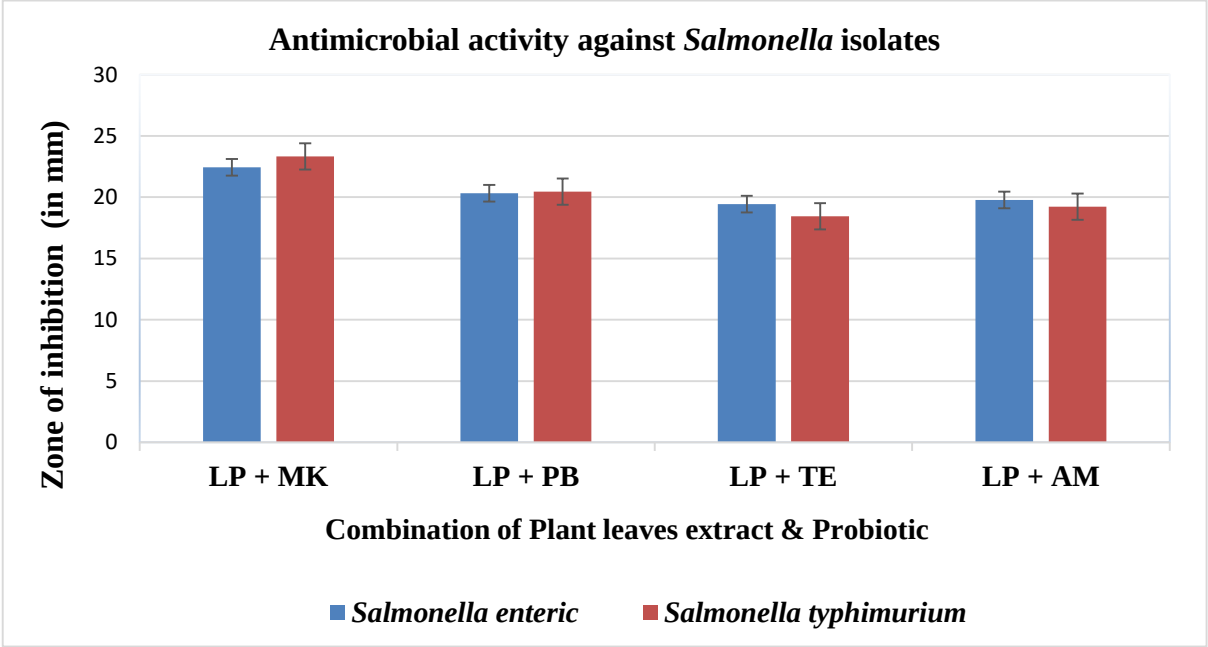

Fig 3: Graphical representation of efficacy of herbal combination with probiotic against *Salmonella* isolates

(A)

(B)

(C)

(D)

(E)

(F)

Fig 4 A to 4F : Zone of inhibition of *Salmonella* isolates against antibiotics, probiotic and herbal extracts

MET: Metronidazole, **AMP:** Ampicillin, **NIT:** Nitroimidazole**, NOR:** Norfloxacin, **LP:** *Lactobacillus plantarum***, M:** *Murraya koeniggi***, P:** *Piper betle***, T:** *Tagetes erecta***, A:** *Aegle marmelos*