OPEN ACCESS

J. Adv. Vet. Anim. Res., 2(2): 190-194.
Available at- *http://bdvets.org/JAVAR*

JAVAR Journal of Advanced Veterinary and Animal Research

ORIGINAL ARTICLE Volume 2 Issue 2 (June 2015)

DOI: 10.5455/javar.2015.b83

Efficacy of vinegar, sorbitol and sodium benzoate in mitigation of *Salmonella* contamination in betel leaf

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ABSTRACT

The present study was undertaken to mitigate *Salmonella* from betel leaf in Mymensingh. A total of 35 betel leaf samples were collected from 2 *baroujes* and 5 local markets in Mymensingh. The samples were sub-divided into two groups: (i) phosphate buffer solution (PBS) washed, and (ii) grinded sample. There was control and treated (with 1.5% vinegar, sorbitol, and sodium benzoate) sub-groups in both groups. Mitigation of *Salmonella* was determined by comparing Total Viable Count (TVC) and Total Salmonella Count (TSAC) of control with treated groups. No bacterial growth was observed in the betel leaf samples collected directly from *barouj* level. At market level, when grinded, there was no growth of bacteria in Plate Count Agar (PCA) and *Salmonella- Shigella* (SS) or Xylose Lysine De-oxy-chocolate (XLD) in both treated and untreated groups. But when the PBS washed samples were used, the TVC (mean log CFU±SD/mL) of betel leaf ranged from 5.16±0.82 to 5.96±1.11, whereas the TSAC value ranged from 4.87±0.58 to 5.56±1.00 for untreated group. In vinegar, there was no growth, but when treated with sorbitol, the TVC (mean log CFU±SD/mL) value reduced to 5.00±0.54 to 5.66±1.09, and TSAC (mean log CFU±SD/mL) value reduced to 4.28±0.71 to 4.78±0.64. When treated with sodium benzoate, the TVC (mean log CFU±SD/mL) value reduced to 5.06±0.53 to 5.75±1.02, and TSAC (mean log CFU±SD/mL) value reduced to 4.34±0.79 to 4.92±0.64. Data of this study indicates that all the three chemicals were effective in terms of reducing bacterial load but vinegar (1.5%) was found to be the most effective against *Salmonella* as well as some other bacteria when treated for 10 min.

Keywords

Betel leaf, *Salmonella*, Mitigation, Total Viable count, Chemical treatment

ARTICLE HISTORY

Received : 15 April 2014, **Revised**: 19 April 2015,
Accepted : 19 April 2015, **Published online**: 20 April 2015.

INTRODUCTION

The betel plant is an evergreen, shade loving perennial root climber belongs to the family Piperaceae and the scientific name is Piper betel (Chakraborty, 2011). It is available in most of South and Southeast Asia. In Bangladesh, about 60-70% of people usually consume *paan* frequently and all classes of people chew it. Significance of the leaves has been explained in relation to every sphere of human life including social, cultural, religious, medicinal and even day-to-day life like marriage, Puja, Sraddha ceremony. In Bangladesh, betel leaf farming yields vary by region and vine variety. A total of 2,825 hectares of land is dedicated to betel vine farming. The average production costs are about BDT. 300,000 per hectare, and the farm owners can earn a profit of over BDT. 100,000 per hectare (Annonymus, 2011). Bangladesh earns US$ 8 million per year by exporting betel leaf to European country which contributes a great to our national economy (Karim, 2014). But the European Union (EU) could detect the contaminated *Salmonella* in Bangladeshi betel leaves which was harmful for health and caused diarrhea and vomiting and other related serious illness to the people who consumed it, and ultimately the EU stopped the importation of betel leaf from Bangladesh (Chowdhury and Kallol, 2013).

Mitigation of microbial loads in betel leaf in Bangladesh is important from export and public health points of view. In Bangladesh, only the identification of bacteria from betel leaf has been performed (Haque, 2013) but study on mitigation of bacterial loads in betel leaf has not yet been done. The objectives of this study were to determine bacterial loads as well as the efficacy of vinegar, sorbitol and sodium benzoate to mitigate *Salmonella* contamination in betel leaf.

MATERIALS AND METHODS

Collection of samples: A total of 35 betel leaf samples were collected from two *baroujes* (namely Bottola bazaar and Lokkhipur) and five local markets namely Kamal-Ranjit (KR) market, Jobbermor, Sheshmor, Kewatkhali and Ganginarpar (**Table 1**).

Processing of sample

Grinding of betel leaf samples: One of the betel leaf collected from each area was weighted with a digital weighing machine and grinded with a sterile mortar and pastel. A ten-fold serial dilution of the grinded sample was prepared in sterile phosphate buffer solution (PBS).

Washing of betel leaf samples: Each betel leaf collected from each area was weighted with a digital weighing machine and washed with sterile PBS. A ten-fold serial dilution of the washed samples was prepared in sterile PBS in test tube.

Determination of Total Viable Count (TVC) and Total *Salmonella* Count (TSAC)

For control (untreated) group: At first a ten-fold dilution of the betel leaf sample was made with sterile PBS. Then 0.5 mL of the diluted sample was taken and a ten-fold serial dilution (10^{-1} to 10^{-6}) were made with 4.5 mL sterile PBS and spreaded onto PC agar and XLD or SS agar and incubated at 37°C for 24-48 h. The colonies were counted from the plate containing 30-300 colonies of a particular dilution and then the number was multiplied by dilution factor and reciprocal value to calculate Total Viable Count and Total *Salmonella* Count which was expressed as mean log CFU±SD/mL.

For treated group: A ten-fold serial dilution (10^{-1} to 10^{-6}) were made with 0.5 mL ten-fold diluted betel leaf sample in 4.5 mL sterile PBS (containing 0.07 mL vinegar, sorbitol and sodium benzoate respectively). Then spreaded onto PC agar and XLD or SS agar and incubated at 37°C for 24-48 h and the TVC and TSAC were calculated which was expressed as mean log CFU±SD/mL.

Isolation of bacteria: The processed betel leaf samples were enriched in nutrient broth at 37°C for overnight. The overnight cultures were streaked on XLD or SS agar for isolation of *Salmonella* and incubated at 37°C for 24 h. Single colony was further subcultured until pure culture was obtained.

Identification of bacteria: Identification of bacteria was performed on the basis of colony morphology (shape, size, margin, elevation and colour), Gram staining reaction, motility test and biochemical tests (Carter, 1986). A genus specific PCR was performed to amplify 16S rRNA of *Salmonella* using previously used primers (Noah et al., 1993).

RESULTS AND DISCUSSION

Total viable count (TVC) and Total *Salmonella* count (TSAC) of betel leaf in control and treated groups

***Barouj* level:** No bacterial growth in Plate Count (PC) agar and *Salmonella-Shigella* (SS) agar or Xylose Lysine De-oxy-chocolate (XLD) agar in both control and treated group in grinded and PBS washed group.

Market level: No bacterial growth on PC agar and SS or XLD agar in both control and treated group in grinded case.

PBS washed sample: Bacterial growth occurred on PC agar and SS or XLD agar. The TVC and TSAC of betel leaf from different markets in control and treated group are presented in **Table 1** and **Table 2**. The TVC of betel leaf (mean log CFU±SD/mL) ranged from 5.16±0.82 to 5.96±1.11, and the TSAC value ranged from 4.87±0.58 to 5.56±1.00 mean log CFU±SD/mL in untreated group. In the case of vinegar, no growth was observed, but when treated with sorbitol, the value of TVC reduced to 5.00±0.54 to 5.66±1.09, and TSAC value reduced to 4.28±0.71 to 4.78±0.64. When treated with sodium benzoate, the TVC value reduced to 5.06±0.53 to 5.75±1.02, and TSAC value reduced to 4.34±0.79 to 4.92±0.64. The highest TVC and TSAC values were found at Sheshmor market in both control and treated groups, and the lowest TVC values were recorded at Kewatkhali market in treated group. On the other hand, the lowest TSAC value was found at Kewatkhali market. The average of TVC and TSAC values in untreated and treated groups of betel leaf from five local markets are shown in **Table 3** and **Table 4**.

The average TVC of bacteria in betel leaf of five local markets in control group was 5.69±0.75 mean log CFU±SD/mL. After the treatment with vinegar, no growth of microorganism was found. When treated with sorbitol, the average value was recorded as 5.35±0.66 mean log CFU±SD/mL, and when treated with sodium benzoate, the value was 5.43±0.67 mean log CFU±SD/mL. The bacterial load recorded in the treated group was insignificant as compared to the value of control group.

The average TSAC of bacteria in betel leaf of five local markets in control group was 5.20±0.71 mean log CFU±SD/mL. After the treatment with vinegar, there was no growth of microorganism. When treated with sorbitol, the average value was 4.67±0.69 mean log CFU±SD/mL, and when treated with sodium benzoate, the average value was recorded as 4.78±0.72 mean log CFU±SD/mL. The bacterial load recorded in the treated group was insignificant as compared to the value of control group.

Cultural and morphological or staining characteristic: The cultural characteristics of *Salmonella* was similar to the findings of other authors (Freman, 1985; Hossain, 2002; Cheesbrough, 2006; Muktaruzzaman et al., 2010).

Gram staining characteristics: The morphological or staining characteristic of *Salmonella* was similar to the findings of other authors (Freman, 1985; Gene, 2002; Samad, 2005). The Gram staining revealed Gram-negative, pink colored, small rod shaped appearance, arranged in single or paired under the microscopic examination with 400X objectives.

Table 1. Total viable count in untreated and treated betel leaves obtained from five local markets

Markets Name	TVC (mean log CFU±SD/mL)			
	Control group	Treated group		
		Vinegar (1.5%)	Sorbitol (1.5%)	Sodium benzoate (1.5%)
KR market	5.72±0.63	-	5.30±0.51	5.58±0.61
Shesmor market	5.96±1.11	-	5.66±1.09	5.75±1.02
Jobbermor market	5.96±0.61	-	5.46±0.52	5.56±0.58
Kewatkhali market	5.16±0.82	-	5.00±0.54	5.06±0.53
Ganginarpar market	5.66±0.58	-	5.31±0.62	5.21±0.55

TVC= total viable count; CFU= colony forming unit; - = No growth of bacteria.

Table 2. Total *Salmonella* count in untreated and treated betel leaves obtained from five local markets

Markets Name	TSAC (mean log CFU±SD/mL)			
	Control group	Treated group		
		Vinegar (1.5%)	Sorbitol (1.5%)	Sodium benzoate (1.5%)
KR market	5.03±0.73	-	4.28±0.71	4.34±0.79
Shesmor market	5.56±1.00	-	5.23±1.02	5.21±1.09
Jobbermor market	5.31±0.62	-	4.58±0.59	4.72±0.59
Kewatkhali market	4.87±0.58	-	4.48±0.52	4.72±0.48
Ganginarpar market	5.25±0.62	-	4.78±0.64	4.92±0.64

TSAC= total *Salmonella* count; CFU= colony forming unit; - = No growth of bacteria.

Table 3. Average of TVC in untreated and treated group of betel leaf from five local markets.

Group		Average TVC (mean log CFU±SD/mL)
Control		5.69±0.75
Treated	Vinegar (1.5%)	-
	Sorbitol (1.5%)	5.35±0.66
	Sodium Benzoate (1.5%)	5.43±0.67

TVC= total viable count; CFU= colony forming unit.

Table 4. Average of TSAC in untreated and treated group of betel leaf from five local markets.

Group		Average TSAC (mean log CFU±SD/mL)
Control		5.20±0.71
Treated	Vinegar (1.5%)	-
	Sorbitol (1.5%)	4.67±0.69
	Sodium Benzoate (1.5%)	4.78±0.72

TSAC= total *Salmonella* count; CFU= colony forming unit.

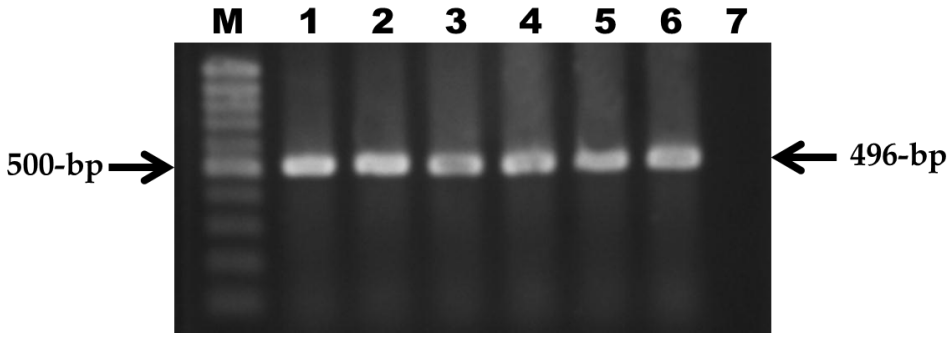

Figure 1. PCR assay to amplify 16S rRNA of *Salmonella* from betel leaf sold at 5 local markets in Mymensingh. **M**: 100 bp DNA marker; **Lane 1**: Positive control of *Salmonella*; **Lane 2:** *Salmonella* isolates of betel leaf at KR market; **Lane 3:** *Salmonella* isolates of betel leaf at Sheshmor; **Lane 4:** *Salmonella* isolates of betel leaf at Jobbermor; **Lane 5:** *Salmonella* isolates of betel leaf at Kewatkhali; **Lane 6:** *Salmonella* isolates of betel leaf at Ganginarpar; **Lane 7:** negative control without DNA template.

Motility profile: All the isolates were found to be motile when examined using hanging drop slide under microscope.

Biochemical tests: The biochemical characteristic of *Salmonella* was similar to the findings of other authors (Buxton and Fraser, 1977; Dhruba et al., 1999; Hossain, 2002). In sugar fermentation test, among the five basic sugars used, all the isolates of *Salmonella* fermented Dextrose, Maltose and Mannitol and produced acid and gas whereas Lactose and Sucrose were negative.

Other biochemical tests: All isolates were Methyl-red positive, Voges-Proskauer and indole negative. All the isolates of *Salmonella* were catalase negative that was confirmed by the formation of no bubble in H_2O_2.

Molecular detection of *Salmonella* by PCR: DNA extracted from five *Salmonella* isolates were used in PCR assay. PCR primers targeting 16S rRNA of *Salmonella* amplified 496-bp fragments of DNA confirming the identity of *Salmonella* shown in **Figure 1**, as reported by Noah et al. (1993).

Treatment with chemicals: For the purpose of mitigation of *Salmonella* from betel leaf, different chemicals were tested such as vinegar, sorbitol and sodium benzoate. The results of the effect of these chemical treatments on *Salmonella* of betel leaf revealed that each of the chemicals individually reduced microbial count to various degrees. In case of vinegar there was no growth on PC agar and no growth of *Salmonella* on SS agar. Sorbitol and sodium benzoate reduced microbial count to various degrees. Sorbitol was not effective against many bacteria and has been reported by Shalaby and El-Raliman (2006). Among the three chemicals used vinegar was most effective in terms of reducing bacterial load and this might be due to inherent chemical nature of the vinegar as it is acidic in nature and bactericidal (Entani et al,1998), while sodium benzoate and sorbitol are alkaline and bacteriostatic in nature (Ostergaard, 1994). In terms of toxicity, vinegar, sorbitol and sodium benzoate used in this study are generally recognized as safe (Frazier and Westhoff, 1998).

CONCLUSION

The occurrence of *Salmonella* in betel leaf is alarming as they cause public health hazard, and 1.5% vinegar can be used as the most effective tool for mitigating *Salmonella* contamination in betel leaf.

ACKNOWLEDGEMENT

We are thankful to the authority of National Science and Technology (NST) for providing fund which was helpful for the research work.

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