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Der Pharmacia Lettre, 2017, 9 [6]:86-93
[http://scholarsresearchlibrary.com/archive.html]

ISSN 0975-5071
USA CODEN: DPLEB4

Comparison of Different Washing Methods for Bacteriological Quality of Freshly Eaten Leafy Vegetable *Centella asiatica*

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ABSTRACT

Different protocols have been used for washing and disinfecting leafy vegetables. Traditional methods and chemical methods are used for washing and disinfecting leafy vegetables in Sri Lanka. However its effect on microbial reduction was barely studied. The aim of this study was evaluated the effectiveness of washing and disinfecting protocols used by the people in Sri Lanka on Centella asiatica. The leaf samples were collected from cultivated land (Manipay) and Market (Thirunelveli). The samples were microbiologically analyzed. Based on the results market sample contained high numbers of bacteria than cultivated land sample. Shigella sp. found in market sample which was absent in cultivated land sample. Market samples were subjected to different washing and disinfecting treatment techniques as Dipping and washing in portable tap water (T_1); Dipping in turmeric solution and washing in portable tap water (T_2); Dipping in Sodium chloride solution and washing in portable tap water (T_3); Dipping in mixture of Sodium chloride and turmeric solution and washing in portable tap water (T_4); Dipping in lime solution and washing in portable tap water (T_5); Dipping in vinegar solution and washing in portable tap water (T_6). After treatments, the samples were microbiologically evaluated to measure bacterial reductions. LSD test was performed to identify the significance between the treatments and to found the most effective treatment method. Results revealed that there was a significant difference among treatments. Based on the results, treatment T_6 was the most effective method and treatment T_5 was completely removing the Shigella sp. MIC test was done for lime and vinegar solution based on their effectiveness on bacterial reduction than other treatments on leafy vegetable Centella asiatica. According to the MIC results 1% (v/v) of lime solution and Vinegar solution well enough to inhibit the growth of E. coli, Shigella sp., test isolate-3, test isolate-4 which were isolated from the Centella leaves sample from market.

Keywords: *Centella asiatica*, *Shigella* sp., *E. coli*, Coliforms, Faecal coliforms, MIC Test

INTRODUCTION

Fresh leafy vegetables provide mankind with an abundance of benefits. Leafy green vegetables are valued highly for their vitamin and iron content. Vegetables are helpful in neutralising the acid substances produced in the course of digestion of meat, cheese and other foods. These play an important role in health and have the ability to prevent many diseases such as heart disease, cancers and diabetes [1,2]. Green leafy vegetables play a major role in the Sri Lankan diet probably due to its health benefits, low cost, availability and active promotion of fresh vegetables as a part of healthy diet [3]. *Centella asiatica* is a commonly freshly eaten leafy vegetable in Jaffna and also it has several health benefits. It is regarded as one of the most important rejuvenative herbs in ayurvedic medicine. It is said to fortify the immune system, both cleansing and feeding it and to strengthen the adrenals. It has been used as a pure blood tonic and for skin health. It has also been used to promote restful sleep. *Centella asiatica* can relieve high blood pressure and helps the body defend against various toxins. It is used to treat rheumatism, blood diseases, congestive heart failure, urinary tract infections, venereal diseases, hepatitis and high blood pressure. It is a mild diuretic that can help shrink swollen membranes and aid in the elimination of excess fluids. It hastens the healing of wounds and it has ability to promote memory power [4].

However, the recent increase in reports of foodborne illnesses associated with freshly eaten leafy vegetables. They are vulnerable to contaminated by pathogens [5-7] at several points throughout the pre-harvest and post-harvest systems via soil, water, animals, insects, equipment and human handling [5,8]. While growing, vegetables may be exposed to many sources of contamination like contaminated sewage used in watering the gardens from where these vegetables are grown. *Salmonella* species and other pathogens are derived from raw and treated sewage. Bacterial pathogens are considered as the most common agents of causing foodborne illnesses, such as *Salmonella* sp., *Shigella* sp., Coliforms, *E. coli* etc. [6,9-11]. In developing countries like Sri Lanka, foodborne illnesses caused by contaminated vegetables are frequent [10]. However lack of foodborne disease investigations and standard diagnostic procedures, cause outbreaks to go undetected and they are hardly reported in scientific literature. Therefore, conducting a scientific research on determining the hygienic condition of fresh vegetables is essential and timely. So the study was done for the freshly eaten leafy vegetable *Centella asiatica* [12-15].

MATERIALS AND METHODS

Sample collection

Samples were collected from cultivated land (Manipay) and the market (Thirunelveli) by using sterile polythene bags. Samples were brought to the laboratory as early as possible for further examinations.

Treatment method

The following method was done for market sample and cultivated land sample. 25 g of *Centella asiatica* leaves were weighed. Leaves were cut into small pieces and were put into a sterile duran bottle which contained 225 ml of sterile saline water. Then it was shaken at 200 rpm for 60 seconds. 1ml of sample was taken and serial dilution was carried out up to 10^{-5} dilution. 1ml of sample from each dilution was placed in the center of the sterile petridishes by using sterile pipettes [14,15].

Enumeration of bacteria

Determination of total aerobic bacteria

For total aerobic count, 1 ml of leaf sample from different dilutions was pipetted in the center of the sterile petridish. Then pour plate technique was carried out by using sterile plate count agar as medium. The plates were allowed to settle down. Then plates were incubated in an inverted position in the incubator at 37°C. Total aerobic bacterial count was done after 24 hours and 48 hours separately.

Determination of total coliforms

For total coliform count, 1 ml of leaf sample from different dilutions was pipetted in the center of the sterile petridish. Then pour plate technique was carried out by using sterile MacConkey agar as medium. The plates were allowed to settle down. Then plates were incubated in an inverted position in the incubator at 37°C temperature. Total coliforms count was done after 24 hours and 48 hours separately [16-18].

Determination of total faecal coliforms

For total faecal coliforms count, 1ml of leaf sample from different dilutions was pipetted in the center of the sterile petridish. Then pour plate technique was carried out by using sterile MacConkey agar as medium. The plates were allowed to settle down. Then plates were incubated in an inverted position in the incubator at 44.5°C temperature. Total faecal coliforms count was done after 24 hours and 48 hours separately.

Determination of *E. coli*

For total *E. coli* count, 1ml of leaf sample from different dilutions was pipetted in the center of the sterile petridish. Then pour plate technique was carried out by using sterile Endo agar as medium. The plates were allowed to settle down. Then plates were incubated in an inverted position in the incubator at 37°C temperature. Total *E. coli* count was done after 24 hours and 48 hours separately.

Check for the presence of *Salmonella* sp. and *Shigella* sp.

For the presence of *Salmonella* sp. and *Shigella* sp., 1ml of leaf sample from different dilutions was pipetted in the center of the sterile petridish. Then pour plate technique was carried out by using sterile SS agar (*Salmonella* and *Shigella* agar) as medium. The plates were allowed to settle down. Then plates were incubated in an inverted position in the incubator at 37°C temperature. Then the plates were observed for the presence of *Salmonella* sp. and *Shigella* sp. after 24 hours and 48 hours.

The following treatment techniques were done for the market sample.

Treatment techniques

T_0- *Centella asiatica* leaves without any treatment

T_1- Leaves were dipped in potable tap water for 10 minutes.

T_2- Leaves were dipped in 2.5% (w/v) of turmeric solution for 10 minutes.

T_3- Leaves were dipped in 2.5% (w/v) of NaCl solution for 10 minutes.

T_4- Leaves were dipped in 2.5% (w/v) of (NaCl [1.25 g] +Turmeric [1.25 g]) solution for 10 minutes.

T_5- Leaves were dipped in 10% (v/v) of lime solution for 10 minutes.

T_6- Leaves were dipped in 10% (v/v) of vinegar solution for 10 minutes.

In the case of treatments T_2, T_3, T_4, T_5 and T_6 leaves samples were rinsed in potable tap water for 30 seconds, after the treatment before undergoing microbiological analysis.

Isolation of bacterial cultures

1ml of market leaf samples from 10^{-3} dilution were pipetted in the center of the sterile petridishes. Then pour plate technique was carried out by using sterile SS agar, Endo agar and MacConkey agar separately. Then plates were allowed to settle down. Plates with Endo agar and SS agar incubated in an inverted position in the incubator at 37°C temperature. Plates with MacConkey agar incubated in an inverted position in the incubator at 44.5°C temperature.

Confirmation of bacterial isolates

Isolate 1-Red colour colony with red colour surrounding was isolated from endo agar by using streak plate method. It was confirmed as *E. coli.*

Isolate 2-Teal colour colony was isolated from S.S. agar (*Salmonella* and *Shigella* agar) by using streak plate method. Then it was confirmed as *Shigella* sp. according to the following test results. Pink colour rod shape bacterial colonies were observed for the Gram's staining, it was given negative result for the oxidative test, it was given positive result for the catalase test, it was given positive result for the nitrate test and it was fermented glucose without gas production.

Isolate 3-Reddish colour colony was isolated from MacConkey agar which was incubated at 44.5°C by using streak plate method.

Isolate 4-Reddish colour colony with yellow center was isolated from MacConkey agar which was incubated at 44.5°C by using streak plate method.

Isolate-3 and Isolate-4-Faecal coliforms; further studies have to perform to confirm these test isolates.

MIC test for test isolates with lime solution and vinegar solution

Sterile petridishes were divided into 4 portions and each portion was labelled with each test isolates. Same concentrations of bacterial inoculums were prepared from the test isolates by using McFarland standard. Double strength sterilized Nutrient agar medium was prepared. 10ml of Nutrient agar medium was added into each boiling tube and kept in 45°C. Lime solution was sterilized by filter sterilization.

Different concentrations of lime solution were prepared by using following dilution technique. 40 ml of sterilized lime solution was taken in a sterilized beaker. Then 60 ml of sterilized water was added into it. Then it was mixed well by using sterilized glass rod. 10 ml of the above lime solution 40% (v/v) was added into the boiling tube which was contained 10ml of double strength sterilized nutrient agar medium and mixed well from this 20% (v/v) lime solution was obtained.

This dilution method was carried out to get different dilutions of lime solution (0.1%, 0.2%, 0.3% ...up to 1% and 1%, 2%, 3%...up to 10% and 20%). Same dilution method was followed for the vinegar solution also. Then pour plate method was carried out. Plates were allowed to settle down. Then 5 µl of bacterial inoculums from each test isolate was inoculated into their corresponding portion on petridishes which was contained nutrient agar medium. Then plates were incubated in an incubator at 37°C and 44.5°C separately. Then plates were observed for the growth of bacteria (*Shigella* sp., *E. coli*, Test isolate-3 and test isolate-4) after 24 hours and 48 hours.

RESULTS AND DISCUSSION

The following table showed that means bacterial numbers were counted after 48 hours for the untreated leaf samples of market and cultivated land for the 10^{-3} dilution (Table 1).

Numbers of bacteria × 10^4/ml	**Sample from cultivated land**	**Sample from market**
Total aerobic bacteria	6	120
Total coliforms	5	48
Total faecal coliforms	8	50
Total *E. coli*	0	13

Table 1: Mean bacterial numbers for the untreated samples of market and cultivated land.

Present (+) or absent (-)	**Sample from cultivated land**	**Sample from market**
Salmonella sp.	-	-
Shigella sp.	-	+

Table 2: Presence or absence of *Shigella* sp., and *Salmonella* sp.

Results were showed (Tables 1 and 2) that market sample has high number of bacteria than cultivated land sample and also that bacteria are abundant on the surface of the leafy vegetable *Centella asiatica* (The total aerobic count, total Coliform count, total faecal coliform count and total *E. coli* count). Viable bacterial count (VBC) is an important indication of bacteriological quality of food products and high bacterial load in foods could pose a health risk to consumers [18] (Table 2).

Shigella sp. also observed in the market sample, but this species was absent in the cultivated land sample. This high counts could be attributed to the unhygienic practices right from the cultivated land to the market, such as washing the leaves by using the water which was contained domestic sewage, handling practices and bacterial load on leafy vegetables increase with the time during storage [18]. During the current study the presence of pathogenic bacteria and a high VBC in *Centella* leaves were observed from market sample. The previous research on leafy vegetables such as cabbage, *Centella* and lettuce from market also supported the same findings [14] (Tables 3 and 4).

The condition of sales makes the leafy vegetables predisposed to contamination especially as practiced in Zaria where the source of water in the garden and in the market is questionable [19]. Most of the leafy vegetables are easily contaminated by soil and they provide increased surface area for bacterial colonization. Infiltration of pathogens into cracks, wounds, crevices, stomatal

cavities and intercellular spaces of fruits and vegetables has been demonstrated by several researchers [7,20]. The study provides clear evidence of contamination of fresh leafy vegetable from common foodborne pathogens *Shigella* sp. and *E. coli*, Coliforms are usually indicators of intestinal contaminants from man and animals. This may not be too surprising since most often the source of watering the gardens is usually sewage from domestic sources and runoff water that is mostly used for irrigation purposes in this community [13] (Tables 5 and 6).

Salmonella sp. is a group of bacteria (*Salmonella typhi***)** which causes typhoid fever. The latter is commonly known for causing salmonellosis which is a type of foodborne intestinal infection contracted after eating food contaminated with the Salmonella bacteria. These types of intestinal infections are more likely in children or the elderly. *Shigella* is a family of bacteria that cause an infectious intestinal disease known as shigellosis. It is mainly transmitted through contact with an infected person and contaminated food and water. Shigellosis can occur in any age group but is more commonly seen in children. It is one of the common causes of outbreaks of bacillary dysentery. Therefore even though thorough washing can be effective more vigorous processing is needed to reduce the pathogen number and make vegetable safe for consumption. These results emphasize the necessity for awareness among consumers regarding the microbiological quality of freshly eaten leafy vegetables and the potential health hazard of pathogenic bacteria present in leafy vegetables should not be underestimated. If the counts are these high then they pose dangers to consumers (Tables 7 and 8).

Bacterial number ($\times 10^4$/ml)	**Treatments**						
	T_0	T_1	T_2	T_3	T_4	T_5	T_6
Total aerobic bacteria	UnC	30	23	27	41	22	6
Total coliforms	UnC	15	12	32	26	2	3
Total faecal coliforms	40	38	13	25	18	5	5
Total *E. coli*	45	6	13	24	21	3	3

Table 3: Mean bacterial numbers were counted for the market sample after subjected to different treatment techniques.

Bacteria	**T_0**	**T_1**	**T_2**	**T_3**	**T_4**	**T_5**	**T_{6-}**
Salmonella sp.	-	-	-	-	-	-	-
Shigella sp.	+	+	+	+	+	-	+

Table 4: Presence and absence of *Salmonella* sp. and *Shigella* sp. for the market sample after subjected to different treatment techniques.

Different treatments had different effects on bacterial numbers of the market sample. Untreated one usually has the high bacterial number than the treated ones. *Salmonella* sp. was completely absent in all treated samples and *Shigella* sp. was only absent in the treatment T_5.

Statistical analysis

Differences in Mean value for each treatments ($\times 10^4$/ml)	**T_3 (27)**	**T_4 (26.5)**	**T_1 (22.25)**	**T_2 (15.25)**	**T_5 (8.0)**	**T_6 (4.25)**
T_3	-	0.5	4.75	11.75	19	22.75
T_4	0.5	-	4.25	11.25	18.5	22.25
T_1	4.75	4.25	-	7	14.25	18
T_2	11.75	11.25	7	-	7.25	11
T_5	19	18.5	14.25	7.25	-	3.75
T_6	22.75	22.25	18	11	3.75	-

Table 5: LSD analysis for the total bacterial numbers for different treatment techniques.

Treatments	**T_1 (μ_1)**	**T_2 (μ_2)**	**T_3 (μ_3)**	**T_4 (μ_4)**	**T_5 (μ_5)**	**T_6 (μ_6)**
Mean value ($\times 10^4$/ml)	22.25	15.25	27.00	26.50	8.0	4.25
Note: μ=Mean value. $\mu_3 > \mu_4 > \mu_1 > \mu_2 > \mu_5 > \mu_6$						

Table 6: Mean total bacterial numbers for different treatment techniques

According to the LSD test all the treatments have significant different between each other (P=0.5). Treatment T_6 was more effective than other treatments. Treatment T_5 has small different compare to T_6, but this treatment completely removed the *Shigella* sp. So the MIC test was done for Vinegar and lime treatments.

Bacteria	**Concentrations % (v/v)**													
	20	**15**	**10**	**5**	**1**	**0.9**	**0.8**	**0.7**	**0.6**	**0.5**	**0.4**	**0.3**	**0.2**	**0.1**

Shigella sp.	-	-	-	-	-	-	-	+	+	+	+	+	+	+
E. coli	-	-	-	-	-	-	-	+	+	+	+	+	+	+
Test isolate-3	-	-	-	-	-	+	+	+	+	+	+	+	+	+
Test isolate-4	-	-	-	-	-	-	-	+	+	+	+	+	+	+

Table 7: MIC test with lime solution.

Bacteria	**Concentrations % (v/v)**													
	20	**15**	**10**	**5**	**1**	**0.9**	**0.8**	**0.7**	**0.6**	**0.5**	**0.4**	**0.3**	**0.2**	**0.1**
Shigella sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. coli	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Test isolate-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Test isolate-4	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Note: + Present - Absent														

Table 8: MIC test with vinegar solution.

Results of MIC (Minimum inhibitory concentration) test

MIC value differs for different bacteria treated with lime and vinegar solution. Results showed (Tables 6 and7) that low concentrations of lime solution and vinegar solution were well enough to inhibit the growth of above bacteria which were mentioned in the table isolated from *Centella* leaf sample. 1% (v/v) lime solution was needed to inhibit the growth of Test isolate-3, 0.8 % (v/v) lime solution was needed to inhibit the growth of *Shigella* sp., test isolate-1 and test isolate-4. 1% of vinegar solution was well enough to inhibit the growth of all test isolates. The previous research on lettuce leaf treated with 20% vinegar showed microbial reduction [21,22].

CONCLUSION

Market sample contained more bacterial numbers than cultivated land sample. Treatment T_6 was more effective than other treatments. Treatment T_5 has small different compare to T_6, but this treatment completely removed the *Shigella* sp. The MIC value for *Shigella* sp.=0.8% (v/v) lime solution. The MIC value for *E. coli* =0.8% (v/v) lime solution. The MIC value for Test isolate-3=1% (v/v) lime solution. The MIC value for Test isolate-4=0.8% (v/v) lime solution. MIC test was done for vinegar solution upto 1% (v/v). Bacterial growth was not observed up to this concentration. Further studies have to perform with the concentrations of vinegar solution less than 1% (v/v) to find out the MIC.

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