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The Effect of Washing Methods on Hygienic and Quality Level of Industrial *Moringa oleifera* Leaves

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Abstract. *Moringa oleifera* had many functions, such as food, cosmetic and medical products. The leaves were washed by clean water and salt solution. The purpose of the study was to determine the effects of washing method to the hygienic and quality of industrial *M. oleifera* leaves. The results of the study will bring up the most effective washing method recommendation for the *M. oleifera* leaf industry. In this study, four types of washing method were applied to the *M. oleifera* leaves to compare several types of water sources, namely well water, the government-treated water, refilled water and bottled water. Further, two types of salt sources, namely commercial salt and raw salt, were also used. The parameter of *M. oleifera* hygiene was the total plate count, MPN coliform, *Salmonella* sp. presented, and *Staphylococcus aureus*' cell number. Meanwhile, the antioxidant capacity and flavonoid level were also determined as the quality level of *M. oleifera* leaves. The results showed that the most effective washing method was using a combination of bottled water and commercial salt with the total plate count, MPN, *S. aureus* value of 0.9×10^4 CFU/gram, 0.55/gram, 0.2×10^2 CFU/gram, respectively, all with positive results in *Salmonella* sp. test. The best *M. oleifera* quality was achieved by washing the leaves with refilled water and raw salt with a number of level of flavonoid of 8.57 mgQE/gram and antioxidant capacity percentage of 69%.

INTRODUCTION

Moringa oleifera leaves can be processed into herbal industrial products, such as medicine, tea, or herbal powder [1]. In its utilization for industrial products, *M. oleifera* leaves undergo several processing steps, from sorting, washing, drying, processing raw materials into finished materials and product packaging [2]. The washing process of raw materials is done to reduce impurities that stick to it, such as soil and dust. Besides, pathogenic microorganisms can also become dangerous contaminants if they enter the human body when consumed and cause infection [3].

The herbal industry in Sumenep Regency, Madura processes *M. oleifera* leaves into vegetable powder. In this industry, the washing process was carried out using well water or government-treated water. Using several washing methods, the result from the *M. oleifera* leaves industry in Sumenep Regency was still not in accordance with Indonesian National Standards (SNI) 7388 of 2009 concerning to the maximum limit of microbial contamination in food. SNI standards that must be appropriate is MPN Coliform, TPC, *S. aureus* test and *Salmonella* sp. test.

According to the industrial-scale washing standards, there are three stages of the washing process: the first stage of washing uses water to reduce impurities, the second stage is washing using a salt solution, and the third stage is rinsing [2]. The quality of water used for washing greatly affects the quality of raw materials. The water used for the washing process must be clean and does not contain contamination beyond the maximum limit. According to WHO (1971), the water used in the washing process is standard drinking water. Clean water conditions are effective for washing raw materials so they are not polluted by the washing water [4]. A salt solution is intended to kill pathogenic

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bacteria as the solution can cause bacterial cells to become lysis and die [5]. The salt used was easily found in Sumenep Regency, it was commercial salt and raw salt.

According to Government Regulation of the Republic of Indonesia number 66 (2014) article 8 paragraph 1 letter d concerning environmental quality standards for food media in maintaining healthy and hygienic food conditions, the food media has to be free from the dangers of biological, chemical and other contaminants. Proper processing is highly recommended to maintain food quality, especially in the washing process that serves to reduce pollution. In addition to reducing pollution, the water used was a polar compound that can dissolve polar compounds as well. Polar compounds in leaves are flavonoids [6].

Based on the explanation above, this study was done to find out the most effective washing method on the products, which were in the vegetable powder form, to reduce pollution and still maintain the quality of industrial *M. oleifera* leaves. Following the Indonesian National Standard, the tests included MPN coliform, TPC, *Salmonella* sp. and *S. aureus*. The leaf quality test included total flavonoid and antioxidant capacity. The results of this study will bring up the most effective washing method recommendation for *M. oleifera* leaf industry in Sumenep Regency, Madura.

METHODOLOGY

Hygiene test

The methods used in this study followed the Indonesian National Standard (SNI) 7388 of 2009 concerning the maximum limit of microbial contamination in food. The hygiene tests conducted were MPN coliform test, TPC test, *Salmonella* sp. test, and *Staphylococcus aureus* test, as well as the total flavonoid content test and antioxidant activity percentage test.

Dilution of the sample was done by weighing 10 grams of the mashed sample then adding a 100 ml of a physiological solution as a 10^{-1} dilution. The solution was taken as much as 10 ml and added to the 90 ml of the physiological solution as a 10^{-2} dilution. The dilution was carried out up to 10^{-3}. For the MPN test, 10 ml of the 10^{-1} dilution was inoculated in a test tube containing 5 ml of medium double strength lactose broth, 1 ml of sample into a test tube containing 5 ml of medium single strength lactose broth, and 0.1 ml of sample into a test tube containing 5 ml of medium single strength lactose broth. The inoculated test tubes were then incubated at room temperature for 24 to 48 hours and observed [7]. For the TPC test, the solutions resulted from 10^{-1} up to 10^{-3} dilution were used. Each dilution was inoculated as much as 0,1 ml to NA medium in Petri dish. Samples were flattened and incubated for 24 to 48 hours at room temperature and observed. The observation was in the form of counting the number of colonies between 30 - 300 at the colony counter [8]. Total bacterial growth was calculated using the calculation formula. For the *Salmonella* sp. test using 10^{-3} dilution, the samples were taken 1 ml and added to 10 ml of lactose broth and incubated at 35 °C for 24 hours. After incubation, 1 ml pipette is taken, put in 10 ml of tetrathionate brilliant green broth, incubated at 43 °C for 24 hours (enrichment). Furthermore, the enrichment stage was taken by using ose and rub on selective media Salmonella Shigella Agar (SSA) as an affirmation test then incubated at 35 °C for 24 hours. The colony that was suspected was positive *Salmonella* sp. on SSA media will form pink colored with or without a black core in the middle [9]. For the *S. aureus* test using 10^{3} dilution, the samples were inoculated into a Petri dish containing sterile Mannitol Salt Agar (MSA) media. They were incubated for 24 to 48 hours at room temperature. The number of colonies growing and the media turning yellow indicated the presence of *S. aureus*. The change in color of the media to yellow is due to the ability of *S. aureus* to ferment mannitol [10].

Total Flavonoid Content Test

Quercetin 10 mg was dissolved in 10 ml of ethanol. The stock solution was pipetted 1 ml and ethanol was added up to 10 ml volume so that 100 ppm concentration was obtained. Then solutions with concentrations of 1 ppm, 2 ppm, 3 ppm, 4 ppm, and 5 ppm were made from the 100 ppm solution. Each solution was pipetted 1 ml and added by 3 ml of ethanol, 0.2 ml of 10% $AlCl_3$ and 0.2 ml of 1 M potassium acetate and sufficient with distilled water up to 10 ml. The sample was incubated for 30 minutes at room temperature, then the absorbance was measured using a UV-Vis spectrophotometry at a wavelength of 435 nm [12].

Extract of *M. oleifera* leaves was also pipetted 1 ml then added 3 ml of ethanol, 0.2 ml of 10% $AlCl_3$ and 0.2

ml of 1 M potassium acetate and sufficient with distilled water up to 10 ml. The sample was incubated for 30 minutes at room temperature, then the absorbance was measured using a UV-Vis spectrophotometry at a wavelength of 435 nm [12]. Samples were made in 3 replications for each analysis and the results were averaged. Total flavonoid levels can be calculated using the following formula [13]:

$$Flavonoid\ content = \frac{\mathrm{C} \times \mathrm{V}}{\mathrm{m}}$$

Note:
C : flavonoid concentration (mg/ml)
V : solvent volume (ml)
m : simplisia mass (gram)

Antioxidant Capacity Test

The antioxidant test was carried out by adding 0.3 ml of *M. oleifera* leaf extract to 0.9 ml of 0.1 M DPPH solution. Then it was incubated for 30 minutes at room temperature and measured on the UV-Vis spectrophotometry at a wavelength of 517 nm. The percentage of antioxidant activity was calculated using the formula [14]:

$$\%Antioxidant = \frac{Ac - A}{Ac} \times 100\%$$

Note:
Ac : Absorbance of the control
A : Absorbance of the sample

RESULT AND DISCUSSION

Analysis of Total Microbes in Samples of Industrial *M. oleifera* Leaves

The level of hygiene in the industrial *M. oleifera* leaves with several types of water and salt samples was observed through TPC test, MPN test, number of *S. aureus*, and the presence of *Salmonella* sp. (Table 1).

TABLE 1. The Results of Microbiological Analysis on Water and Salt.

Limit of contamination	Type of washing material					
	Bottled water	**Refilled water**	**Government water**	**Well water**	**Commercial salt**	**Raw salt**
TPC (CFU/ml or gram)	0.02×10^4	0.1×10^4	0.8×10^4	3×10^4	0.1×10^4	0.5×10^4
MPN (number of MPN/ml or gram)	0.03	0.03	0.036	0.036	0.03	0.036
S. aureus (CFU/ml of gram)	0	0.01×10^2	0.01×10^2	0.2×10^2	0	0
Salmonella sp.	(-)	(-)	(-)	(+)	(-)	(-)

In Table 1, we can see that the well water resulted in the lowest level of hygiene with a total microbe of 3×10^4 CFU/ml, MPN of 0.036/ml, the number of *S. aureus* of 0.2×10^2 CFU/ml and positive results on contamination of *Salmonella* sp. The low level of hygiene in well water is likely caused by the seepage of household wastewater through the ground [15]. According to the Decree of the Minister of Industry and Trade Republic of Indonesia Number 651 / MPP / Kep / l0 / 2004, government water, refilled water, and bottled water have been specially treated such as filtration and disinfection so that they have a higher level of hygiene.

Microbiological analysis results on the raw salt showed higher contamination compared to the table salt with a TPC value of 0.5×10^2 CFU/gram and MPN 0.036/gram. This is because the processing of raw salt does not include washing process after crystallization as in table salt [16]. In addition, high level of NaCl in commercial salt, which is > 94.7%, is more effective in inhibiting the growth of microorganisms than the raw salt with lower NaCl level (± 85%) [16] [17].

The industrial hygiene of *M. oleifera* leaves with several washing methods was analyzed microbiologically based on the Indonesian National Standard (SNI) 7388 of 2009, which states that the maximum limit of contamination in food consists of TPC (Total Plate Count), MPN (Most Probable Number) tests, *Staphylococcus aureus* and *Salmonella* sp. to find out contamination in the sample. The microbiological analysis results in the TPC test can be seen in Figure 1.

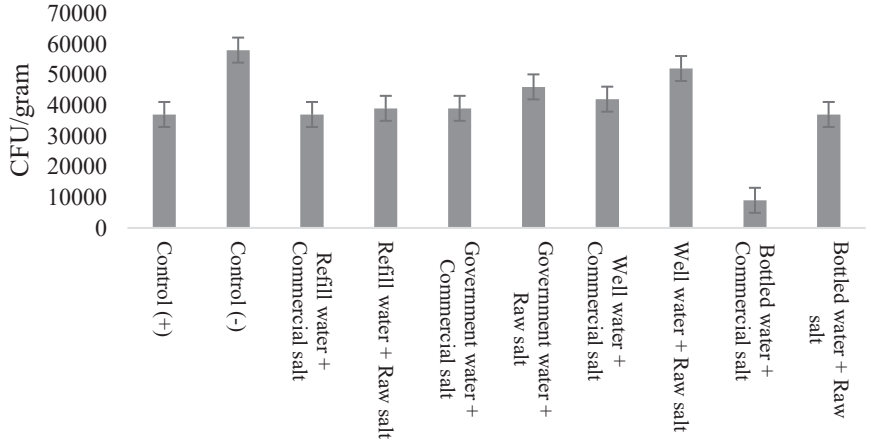

FIGURE 1. The Microbiological Analysis Result in the TPC Test.

Based on Figure 1, washing using well water with raw salt showed the highest number of colonies in the TPC test among other treatments, which was 5.2×10^4 CFU/gram. This shows that the low quality of washing is caused by the amount of microbial contamination obtained exceeding the maximum contamination limit set by SNI 7388 in 2009, which is 5×10^4 CFU/gram. The low quality of well water used to wash *M. oleifera* leaves can be caused by contamination of seepage of wastewater from the houses [15].

Well water and raw salt contaminated by microbes can cross-contaminate the leaves of *M. oleifera* that have been washed [18]. Meanwhile, the lowest number of colonies in the TPC test was obtained in washing using bottled water with salt, which was 0.9×10^4 CFU/gram. This is because drinking water and commercial salt as washing agents have the lowest contamination compared to other types of salt, which is equal to 0.2×10^4 CFU/ml and 1×10^4 CFU/gram. That is because the bottled water has previously been carried out special treatment such as filtration and disinfection to reduce the amount of water pollution [19]. Salt can inhibit the growth of microorganisms due to the differences in the osmotic pressure in cells [20].

The Closest Approximate Number Coliform in Samples of Industrial *M. oleifera* Leaves

The results of the closest estimated number (MPN) coliform in samples of industrial *M. oleifera* leaves are shown in Figure 2.

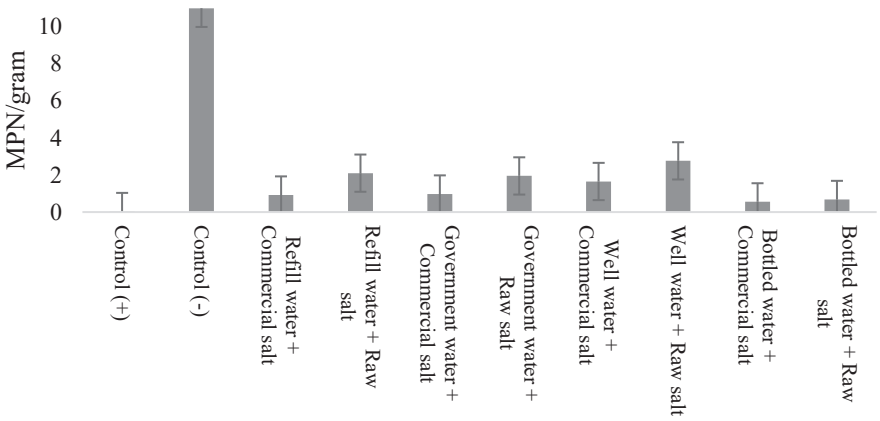

FIGURE 2. The Result of Microbiological Analysis in The MPN Test.

Based on Figure 2, the results of the coliform MPN test show that all samples have lower MPN values than the standards set by SNI 7388 in 2009, which is 10 MPN/gram. This is supported by the results of microbiological analysis on the type of washer which show the low amount of contamination in the MPN test on all types of water and salt used to wash *M. oleifera* leaves. In the MPN test, the bacteria detected were coliforms. a positive result in this test is indicated by the presence of lactose fermented bubbles from coliform bacteria [21].

Analysis of Enteric Bacterial Contamination in Samples of Industrial *M. oleifera* Leaves

Salmonella sp. and *S. aureus* tests were carried out to determine enteric bacterial contamination in several industrial *M. oleifera* leaf washing methods. Microbiological analysis results on the *S. aureus* and *Salmonella* sp. shown in Figure 3 and Table 2.

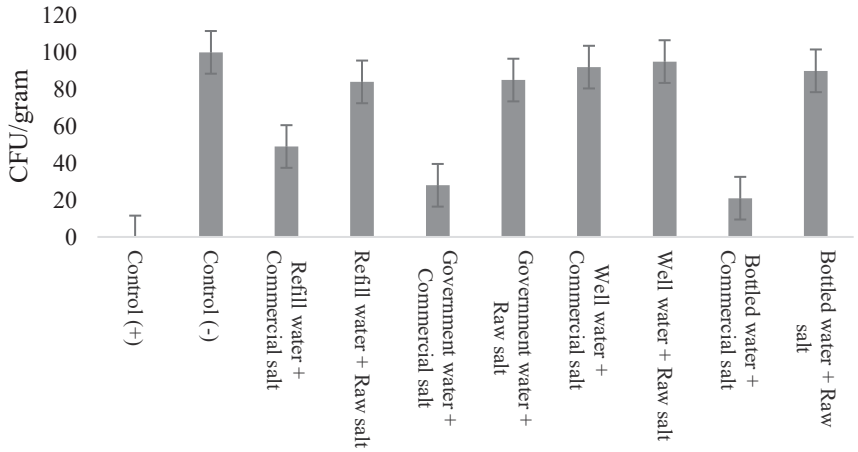

FIGURE 3. The Result of Microbiological Analysis in *S. aureus* Test

Based on Figure 3, the *S. aureus* test results show that all samples have lower values than the standards set by SNI 7388 of 2009, which is equal to 1×10^2 CFU/gram. This is consistent with the results of microbiological analysis on the types of water and salt washers which showed a low amount of contamination in the *S. aureus* test in all types of water and salt used to wash the leaves of industrial *M. oleifera*.

S. aureus test results have a low contamination value because the composition of the medium used can only be utilized by certain groups of bacteria. The *S. aureus* test was carried out on MSA selective media with positive results in the form of a yellow colony produced by mannitol fermentation by *S. aureus* [12]. *S. aureus* is a pathogenic

bacterium that can be found on leaf surfaces. Its excessive presence in food ingredients can cause epidermal necrolysis disease due to the presence of exfoliative toxin and toxic shock syndrome due to exotoxin [22] [23].

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TABLE 2. The Result of Microbiological Analysis in *Salmonella* sp. Test

Samples	The result of *Salmonella* sp. test
Control (+)	(-)
Control (-)	(+)
Refill water + Commercial salt	(+)
Refill water + Raw salt	(+)
Government water + Commercial salt	(+)
Government water + Raw salt	(+)
Well water + Commercial salt	(+)
Well water + Raw salt	(+)
Bottled water + Commercial salt	(+)
Bottled water + Raw salt	(+)

Table 2 shows that all washed samples were contaminated by the pathogenic *Salmonella* sp. The characteristics of the colonies growing on SSA match the characteristics possessed by *Salmonella* sp., which are turbid or colorless with or without a black core in the middle [24]. The existence of *Salmonella* sp. can reduce sodium thiosulfate to H_2S gas. The H_2S gas which is insoluble from iron sulfide and reacts with ferric ions to form a black colony [25]. *Salmonella typhi* is relatively weak in reducing sodium thiosulfate so that the colony's color is not black [26].

Salmonella sp. is a gram-negative bacteria that are included in pathogenic bacteria and can grow at temperatures of 5 – 47 ^{o}C, pH 4 – 9, and Aw 0.94 - 0.99 [27]. *Salmonella* sp. is able to survive in NaCl solution up to a concentration of 5% so that it still can grow in the samples of *M. oleifera* leaves that have been soaked in a 1% of salt solution [28]. The existence of *Salmonella* sp. in food can cause Salmonellosis disease, which is a disease caused by infections in the digestive tract and intestine [29].

Analysis of The Quality in Samples of Industrial *M. Oleifera* Leaves

The effect of washing method on the quality of *M. oleifera* leaves can determine through the level of flavonoids and the percentage of antioxidant inhibition in the sample as shown in Figure 4.

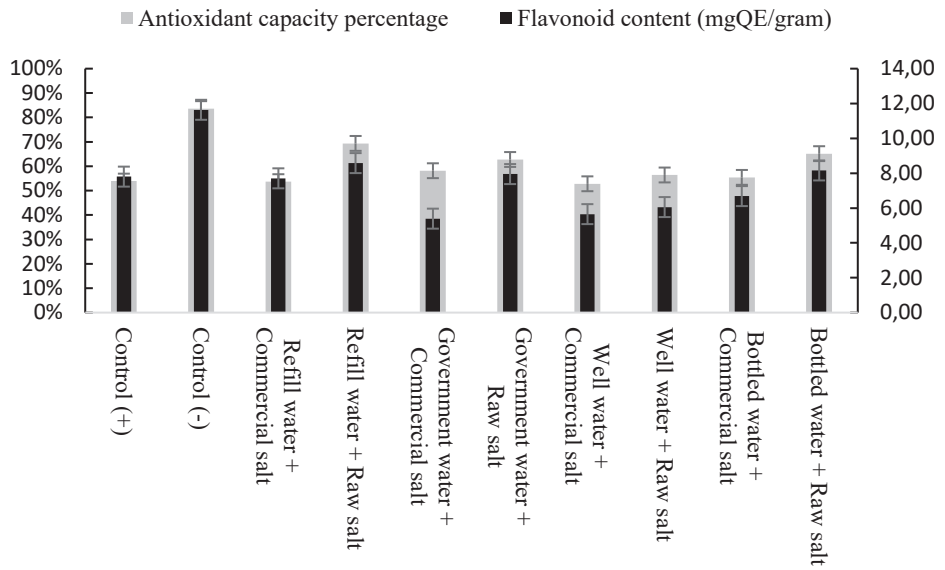

FIGURE 4. The Result of Flavonoid Content and Antioxidant Capacity Test

Based on Figure 4, it is known that the highest flavonoid level was found after being washing using raw salt solution with refilled water at 8.579 mgQE/gram, while the lowest value was after washing using commercial salt solution with government water at 5.391 mgQE/gram. Flavonoid levels in all washing treatments were lower than the negative control. The low flavonoid level is affected by washing treatment using water and salt. This is because flavonoids generally have a bond with a sugar group (glycosides) which causes flavonoids to be more soluble in water or polar solvents [30]. Water is a solvent that has a high dielectric constant so that it is able to dissolve polar compounds [31].

In addition, in the treatment using a salt solution, the level of flavonoids in samples washed with the raw salt solution was higher than the samples washed with a solution of commercial salt. This can occur because the NaCl content in table salt is higher than the same mass of raw salt. Commercial salt has a NaCl level of higher than 94.7%, while raw salt has a NaCl level of ± 85% [16] [17]. This affects the osmotic pressure that occurs in the sample. The higher level of NaCl makes a bigger difference in osmotic pressure between the cells inside and outside of the cell so that the osmosis process of solvent and solute is higher [32].

The highest percentage of antioxidant capacity was found in washing treatment using raw salt solution with refilled water by 69%, while the lowest was in washing treatment using commercial salt solution with well water by 53%. The percentage of antioxidant capacity in all treatments decreased and had a lower value than the negative controls. This is caused by the compounds that play a role as a soluble antioxidant during the washing process by water and salt solution [31] [32].

CONCLUSION

Based on the results of this research, we can conclude that the most appropriate washing method that meets the SNI 7388 (2009) for *M. oleifera* leaves was the washing method using commercial salt solution combined with bottled water, which achieved the highest hygiene level for each parameter. Meanwhile, the results for *Salmonella* sp. test for each sample did not meet the SNI 7388 (2009) with a positive result for each treatment. The best result of flavonoid content test and antioxidant capacity was achieved by the variable of raw salt solution combined with refilled water with a value of 8,579 mgQE/gram and the antioxidant capacity percentage of 69%. Whereas the lowest result of washing was shown from the variable of commercial salt solution combined with government water, which was 5,391 mgQE/gram. The lowest antioxidant capacity was shown by the variable with commercial salt solution combined with well water for washing method with the antioxidant capacity percentage of 53%.

From the results in this study, *Salmonella* sp. contamination still cannot be treated. Therefore, for the next research, *M. oleifera* needs to be planted far away from the environment with high contamination aspect of *Salmonella*

sp. Beside that, for cost efficiency of industrial process production, washing the leaves with well water with pre-treatment method can be applied to reduce *Salmonella* sp. contamination. Further research will be carried out on well water to adjust the standards specified in the industrial washing method.

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