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Research Article

Antibacterial Activities of Microgreens and Mature Extract of Kale and Red Spinach Against Selected Pathogenic Bacteria

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Abstract: Microgreen are currently in trending and gaining popularity due to high concentration of bioactive components that are generally related with human health. Due to scarce information available regarding antimicrobial compounds in microgreens, this study focused on determining the antimicrobial activity of kale and red spinach microgreens against *B. subtilis* and *E. coli* in comparisons with their mature plant. The plant extract antimicrobial activity was extracted using ethanol extraction method. Agar disc diffusion method was used to determine the diameter of inhibition zone of plant extracts. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) used microdilution with different concentration ranged from 1000 mg/ml to 31.25 mg/ml to evaluate the lowest concentration that can inhibit and kill the selected bacteria after incubation. Microgreens of kale and red spinach showed more effectiveness compared to their mature plants towards targeted bacteria in agar disc diffusion method. MIC and MBC value for all extract ranged from 62.5 mg/ml to 125 mg/ml. Hence, microgreens showed potential natural antimicrobial agent that can help to substitute the synthetic antimicrobial agent in preserving food.

Keywords: Microgreens, Antimicrobial activity, Agar disc diffusion, Minimum Inhibitory Concentration (MIC), Minimum Inhibitory Concentration (MBC).

INTRODUCTION

Nowadays, the reputation of plant extract has increase due to its flavouring and high in phytochemical [1] which could benefit the health and gives antimicrobial properties. The use of antimicrobials from natural sources can help to inhibit bacteria as well as to reduce the rate of diseases caused by microbiological pathogens [2]. In the recent years, consumption of microgreens has started to rise and trending among consumers that grown interest for diets, support healthy and longevity lifestyles. Microgreens or regularly called as "vegetable confetti" is a new class of speciality crop. Microgreens are defined as an immature green that are tender produced from vegetables, grains or herbs seeds including wild species [3] which harvested when first leaves have fully expanded and before true leaves developed. According to Xiao *et al*., [4], microgreens contain fortified phytonutrients and potential bioactive compounds that can provide many benefits to human health. Furthermore, Sun *et al*., [5] also stated that microgreens had more complex polyphenol profile and contained larger variety of polyphenol compounds as compared to their mature plant counterparts. On the other hand, microgreens can become one of the potential sources of natural antimicrobial compound due to high phenolics compound as mentioned in several other studies [6, 7]. Therefore, the aim of this study is to determine the effectiveness of microgreens as an antimicrobial agent compared with their mature plants against selected pathogenic bacteria. This study starts with obtaining plant extract from kale and red spinach both microgreen and mature plant by using ethanol extraction method. The antibacterial properties of the plant extracts were determined by testing the plants extracts against Gram-positive bacteria (*Bacillus subtilis*) and Gram-negative bacteria (*Escherichia coli*) using agar disc diffusion method, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The ratios of MBC/MIC were calculated to determine the microbicide or micro biostatic properties of the extracts.

MATERIAL AND METHODS

Samples preparation

Microgreens of kale (*Brassica oleracea var. sabellica*) and red spinach (*Amaranthus dubius*) were planted in pots in Kedah, Malaysia. During the germination process, the seed were sprayed with water twice daily. After five days of germination, the microgreens were exposed to undirect sunlight and

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watered twice a day. Microgreens were harvest after 14 days of germination. All the harversted microgreens were packed in airtight sealed bag and immediately into small foam box filled with ice pack to maintain the cold temperature when transported from plantation place to Negeri Sembilan, Malaysia. The mature plant counterparts of kale and red spinach were bought in local supermarket in Negeri Sembilan. The fresh and healthy leaves of microgreens and the mature leaves of kale and red spinach were washed and dried in drying oven at 45 °C overnight. The dried leaves were shredded into small pieces and ground by using Waring Laboratory Blender (Waring W-MX1100XTX, USA) to obtain fine powder [8].

Extraction of samples preparation

The sample materials were extracted by using ethanol following the method of Jacob *et al.,* [9] with some modification. The sample powder (20 g) was mixed with 100 mL of 95% ethanol with the ratio 1:5 respectively and left to soak for 48 hours. Next, the plant samples were filtered and vacuum dried at 40 °C by using the rotary vacuum evaporator. The crude extracts were weighed to calculate the extraction yield. The stock solution was stored at 4 °C until needed. The extract of kale and red spinach microgreens as well as the mature plants were adjusted 1000 mg/ml to 31.25 mg/ml.

$$Percentage\ yield = \frac{\text{weight of extraction crude extract}}{\text{weight of dried sample}} \times 100$$

Bacterial strains

Three bacterial strains *Bacillus subtilis*, and *Escherichia coli* were used as tested microorganisms. All the bacteria strain was obtained from Microbiology Laboratory of Faculty Science and Technology (FST), Universiti Sains Islam Malaysia (USIM). All the bacteria were inoculated into sterile Mueller-Hinton broth (MHB) and were incubated at 37 °C for 24 hours. The density of the bacteria was determined by using Biophotometer (Eppendorf, Germany) at 600 nm with optical density in range of 0.135 to 0.145.

Agar disc diffusion

Disc diffusion method was carried out by following the method of Al-Talib *et al.,* [10] with some modifications. The disc was sterilized by using autoclave at 121 ºC for 1 hour and a half. A 15 µL of plant extract concentration (1000, 500 and 250 mg/ml) were infused in each of the sterilized disc. The bacterial cultures with the quantity of 0.1ml were inoculated on Mueller-Hinton agar (MHA) and spread evenly using sterile cotton swab. The extract disc with different concentration (1000, 500 and 250 mg/ml) were transferred on the surface of MHA and were incubated at 37 °C overnight. Streptomycin was used as positive control while DMSO solution was used as negative control.

Minimum Inhibitory Concentration

Micro dilution method was done for MIC based on Golus *et al*. [11]. A 100 µL stock solution of tested plant extract with different concentration (1000, 500, 250, 125, 62.5, and 31.25 mg/mL) were added into the well of 96-microtiter plate. After that, overnight bacterial inoculum (100 µL) was also added into the well. Streptomycin were added to serve as positive control while bacterial inoculum with DMSO were acted as negative control. Then, the microtiter plates were further incubated overnight at 37°C. The turbidity was observed via visual assessment for visible growth of bacteria in the wells. Turbidity appeared denoted presence of bacteria whereas absence of turbidity indicates the inhibition of microbial growth. The MIC value were determined at wells with the lowest dilution or concentration with no detectable growth of bacteria.

Minimum Bactericidal Concentration and of MBC/MIC ratio

The tested plant extract from MIC test wells were taken and were streak onto the MHA. The plates then were incubated for 24 hours at 37 °C. The lowest concentration of the tested plant extract which express no bacterial growth on Mueller-Hinton agar (MHA) plates after incubation were recorded as MBC values [12]. The MBC/MIC ratio were determined to detect whether the antimicrobial effects were microbicide or micro biostatic. Puteri, [13] stated that it is considered bactericidal if the MBC/MIC ratio is less than or equal to 4 and bacteriostatic if the ratio is greater than 4 and less than 32.

$$Ratio = \frac{\text{MBC value}}{\text{MIC value}}$$

RESULTS AND DISCUSSION

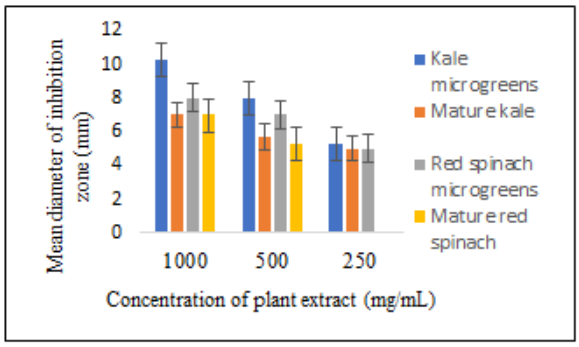
The results on yield of the extract with 95% ethanol is shown in Table 1.

Table-1: Percentage yield of the ethanolic samples extract

Type of sample	Percentage yield
Kale microgreen	21.30 %
Red spinach microgreen	20.55 %
Mature kale	20.80 %
Mature red spinach	19.15 %

Percentage yield = [weight of extraction crude extract / weight of dried samples] X 100

The results of inhibition zone of four samples were analysed triplicate and were recorded in Figure 1 and Figure 2.



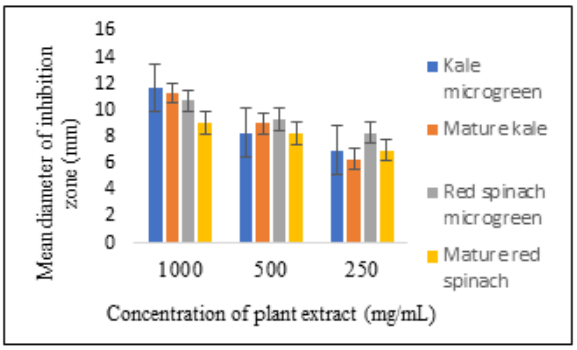


Fig-1 (left) Figure 2 (right) : Mean diameter of inhibition zone (mm) for ethanolic samples extract against *Bacillus subtilis* (left) *and Escherichia coli* (right)

It was observed that kale and red spinach microgreens extract showed highest mean diameter of inhibition zone against *B. subtilis* at concentration 1000 mg/ml which is 11.7 mm and 10.7 mm, respectively compared to its mature plant which is 11.3 mm and 9.0 mm, respectively.

Pearson correlation were used to compare the significant between each type of sample. The result shows there are no significance different between kale microgreens and mature kale towards tested bacteria except for *E. coli* where the P-value is 0.03 which < 0.05. Meanwhile, red spinach microgreens successfully displayed significance different with mature red spinach against all targeted bacteria. The P-value of tested sample extract were showed in Table 2.

Table-2: P-value of tested sample against tested bacteria

Test bacteria	P-value	
	Kale microgreens and mature kale	Red spinach microgreens and mature red spinach
B. subtilis	0.075	0.001
E. coli	0.030	0.002

The minimum inhibitory concentration for four samples were analysed in triplicate. MIC results of microgreens of kale and red spinach with mature kale and red spinach are shown in Table 3.

Table-3: MIC of ethanolic samples extract on selected pathogenic bacteria

Type of sample extract	Bacteria	The concentration of samples extract (mg/ml)						Positive control	MIC value (mg/ml)
		31.25	62.5	125	250	500	1000		
Kale microgreens	*B. subtilis*	+	-	-	-	-	-	-	62.5
	E. coli	+	-	-	-	-	-	-	62.5
Mature kale	*B. subtilis*	+	+	-	-	-	-	-	125
	E. coli	+	-	-	-	-	-	-	62.5
Red spinach microgreens	*B. subtilis*	+	-	-	-	-	-	-	62.5
	E. coli	+	-	-	-	-	-	-	62.5
Mature red spinach	*B. subtilis*	+	-	-	-	-	-	-	62.5
	E. coli	+	+	-	-	-	-	-	125

- = no colony growth; + = presence of colony growth

From the experiment findings, it was observed that the MIC value of tested sample extract against targeted bacteria ranged from 62.5 mg/ml to 125 mg/ml. The MIC value of kale microgreens showed lowest at 62.5 mg/ml for both bacteria. Meanwhile, the mature plant counterpart of kale showed lowest MIC value at 62.5 mg/ml against *E. coli* only. Kale microgreens successfully displayed highest inhibition towards *B. subtilis* compared to mature kale. The MIC value of red spinach microgreens displayed lowest at 62.5 mg/ml for both gram positive and negative bacteria while mature red spinach showed lowest MIC value at 62.5 mg/ml against *B. subtilis*. This result proved that kale and red spinach microgreens explicit higher microbial inhibition characteristic towards *E. coli and B. subtilis* after overnight incubation compared to mature their mature plant.

The MBC is used to measure the ability of samples extract to kill the tested bacteria. The lowest plants extract concentration were reported as the minimum bactericidal concentration (MBC) whether there is growth of bacteria on Mueller-Hinton Agar (MHA) plates. The minimum bactericidal concentration of four samples was analysed in triplicate. Table 4 show the results of MBC for microgreens and mature kale and red spinach.

Table-4: MBC of ethanolic samples extract against selected pathogenic bacteria

Type of sample extract	Bacteria	The concentration of samples extract (mg/ml)						Positive control	MBC value (mg/ml)
		31.25	62.5	125	250	500	1000		
Kale microgreens	*B. subtilis*	+	-	-	-	-	-	-	62.5
	E. coli	+	+	-	-	-	-	-	125
Mature kale	*B. subtilis*	+	+	-	-	-	-	-	125
	E. coli	+	-	-	-	-	-	-	62.5
Red spinach microgreens	*B. subtilis*	+	-	-	-	-	-	-	62.5
	E. coli	+	+	-	-	-	-	-	125
Mature red spinach	*B. subtilis*	+	+	-	-	-	-	-	125
	E. coli	+	+	-	-	-	-	-	125

- = no colony growth; + = presence of colony growth

From this outcome, kale microgreens have showed highest abilities to inhibit *B. subtilis* with lowest MBC value (62.5 mg/ml) more than mature kale (125 mg/ml). However, mature kale showed lowest MBC value towards *E. coli* (62.5 mg/ml) compared to kale microgreens (125 mg/ml). Meanwhile, red spinach microgreens explicit higher inhibition activities towards *B. subtilis* (62.5 mg/ml) compared to mature red spinach (125 mg/ml). From Table 5 below, it can be concluded that all samples extracts are bactericidal against both bacteria. It is considered bactericidal if the MBC/MIC ratio less than or equal to 4 and bacteriostatic if the ratio is greater than 4 and less than 32 [15].

Table-5: MBC/MIC ratio

Test bacteria	MBC/MIC ratio			
	Kale microgreens	Mature kale	Red spinach microgreens	Mature red spinach
B. subtilis	1	1	1	2
E. coli	2	1	2	1

According to the achieved results, kale and red spinach microgreens displayed highest antimicrobial activity compared to their mature plant counterpart. This result can be supported by a study from Sun *et al.* [5] where five microgreens of *Brassica* species which is mizuna, purple kohlrabi, red cabbage, red and purple mustards were tested to identify and profiling polyphenols presence in the microgreens. The study reported that, there are 165 phenolic compounds were present and identified by using matching information from UHPLC-PDA-HRMSn which is large numbers in kaempferol, glycosylated and acylated quercetin than their mature plants. Phenolic compounds are secondary metabolites that are able to display antimicrobial properties. There are many subclasses in this group compounds including phenolic acids, phenols, flavones, quinones, flavonols, coumarins, flavonoids and tannins [14]. Furthermore, Ghoora and Srividya [15] also noted that fenugreek microgreen has higher total polyphenols content which are 136 mg gallic acid equivalents/ g DW (dried weight) compared to the fenugreek mature leaves which are 109 mg gallic acid equivalents/ g DW (dried weight). The total polyphenols were determined by the Folin-Ciocalteau method with some modifications. Therefore, containing high amount and numerous compounds of phenolics in microgreens could contribute to the antimicrobial activity of these young plants in this study. Xiao *et al*. [16] stated microgreens are pack with nutrients because microgreens are harvest right after gemination where all the essential nutrients for growing are still very concentrated. In their study, they evaluated four groups of vital nutrients, including vitamin K, vitamin C, vitamin E, lutein, and beta-carotene, in 25 different commercially grown microgreens where the results showed microgreens possess significantly higher nutrient densities than mature leaves. They discovered microgreens like red cabbage, cilantro, and radish contain up to 40 times higher levels of vital nutrients than their mature counterparts.

The type and biological activity level exhibited by the plant material including antimicrobial ability are subjected or can be influenced by numerous factors, including the plant part, geographical source, soil conditions, content of moisture, harvest time, drying method, storage conditions and process of post-harvest as discussed by Wendakoon *et al.*, [17]. Moreover, according to Verma *et al*., [18], the antimicrobial activity of plant extract can be affected due to several variables such as types of solvents and the bacterial strain used in the study. In an antimicrobial study done by Okunola *et al.*, [19] using fresh and dried leaves of *Carica papaya* extract against selected bacteria and fungi showed that higher antimicrobial activity displayed by ethanolic plant extracts than acetone extract of both fresh and dried samples.

Based on the present study, the data showed Gram-positive bacteria *B. subtilis* was more susceptible bacteria compared to Gram-negative bacteria *E. coli*. This is aligned with previous studies stated by Chanda and Kaneria [20] that Gram-positive bacteria are more susceptible towards plants extract as compared to gram-

negative bacteria. This is because Gram-negative bacteria make antibiotic less effective compared to Gram-positive bacteria by reason of gram-negative bacteria have complex cell wall construction which decrease the capability of extract and antibiotic to enter the cell of bacteria [21]. In spite of that, certain of plant extract were effective against *E. coli* compared to *B. subtilis* as it shown for the MBC value of mature kale against targeted bacteria. The same result also happened in antibacterial properties study by [22] on ethanolic mushroom extract against *B. cereus, S. aureus, E. coli* and *S. typhimurium* where the data exhibited *E. coli* as the most susceptible bacteria when tested on sample extract. This may due to the presence of alpha and beta-unsaturated aldehydes compound in the plant extract. This disclosed that some plant extract with high antimicrobial effects towards Gram-positive bacteria does not essentially have low antimicrobial activity against Gram-negative bacteria [23].

CONCLUSION

In this present study, the ethanolic extract of microgreens and its mature plant counterpart of kale and red spinach were competently exhibited antimicrobial activity against selected Gram-positive (*B. subtilis*) and Gram-negatives bacteria (*E. coli*). Microgreens of kale and red spinach has significantly antimicrobial activity compared to their mature plants against selected pathogenic bacteria. In summary, kale and red spinach microgreens extract with the highest effectiveness of antimicrobial activity could be useful for subsequent application in food preservation against bacteria. However, further research and studies is needed to investigate the screening bioactive compound that are responsible for antimicrobial activity of microgreens by using GC-MS. This study could be served as reference base for other researcher to carry out more comparative studies on antimicrobial activity especially in microgreens. Growing, harvesting and postharvest handling condition could effect on the phytonutrient. Additional studies are required to evaluate the effect of these agriculture practices on the antimicrobial compounds.

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