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Article in International Journal of Current Microbiology and Applied Sciences · January 2020

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Antibacterial Activity of Beetroot Peel and Whole Radish Extract by Modified Well Diffusion Assay

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ABSTRACT

Screening of antimicrobial compound from waste and low cost plant material would be better alternative material for synthetic food preservatives. Hence, in the present study, beetroot peel and whole radish was chosen. Beet root peel extract (BPE) and Whole radish extract (WRE) was prepared using water and screened for the antibacterial compound against various food borne pathogen using modified well diffusion assay. Then the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) level was identified for the various food pathogens. Results showed that direct crude extract of both beetroot peel and radish having the antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi* and *Vibrio cholera*. WRE was more effective than the BPE. It is concluded that antibacterial activity of the plant derived material is truly depends upon the extraction method and the type of solvent used for extraction. Two to four fold higher concentration of water based extraction is needed to get the effect of fat solvent based extraction. BPE and WRE can be a promising source of antimicrobial agents to control the specific food borne pathogens. The modified well diffusion assay can be used for the initial screening of large number of unfiltered the plant extract and combined method of well diffusion assay with MIC would be more suitable procedure to identify the exact antibacterial activity of the crude unfiltered plant materials.

Keywords

Beetroot peel,
Radish, Agar well
diffusion, MIC and
MBC

Article Info

Accepted:
15 December 2019
Available Online:
20 January 2020

Introduction

Frequent reports of food borne illness were observed in humans due to consumption of fish and shell fish. Bacteria such as *Staphylococcus aureus*, *Salmonella*, *Vibrio cholerae* and *Escherichia coli* are causative agent for the disease outbreak (Novoslavskij *et al.*, 2016). Moreover, isolation of multidrug resistant pathogenic bacteria was regularly reported in the seafood by the researchers (Visnuvinayagam *et al.*, 2015; 2016; 2017; 2018). In order to overcome the problem, seafood industries are forced to use the synthetic antimicrobials to limit the growth of seafood borne pathogens (Wong *et al.*, 2006). Since, most of the chemical preservatives are proven hazardous and carcinogen on their long term use, most of the chemicals are under strict regulations (Bialonska *et al.*, 2010; Carocho *et al.*, 2015). Recently, plant materials like spices, aromatic herbs, fruit peels and other natural materials have been screened for the potential antimicrobial activities (Viji *et al.*, 2015; Visnuvinayagam *et al.*, 2019). Therefore, a growing commercial interest on plant-derived non-toxic antimicrobials can control bacterial and fungal growth in food systems (Gyawalli and Ibrahim, 2014). The present study is an attempt to make use of the low cost plant materials as an economic alternative for the costly synthetic chemicals, which can be used in the industry to control the pathogenic bacteria. So, two plants materials *viz.*, Beetroot peel (*Beta vulgaris*) and white radishes (*Raphanus sativus*) were chosen. Since, the beet root peel was discarded as a waste, so the present study was aimed for the utilization of waste and screening of the biomolecules. Similarly, radish was chosen owing to its availability throughout the year at cheaper rate.

Beetroot (*Beta vulgaris*) belonging to the *Chenopodiaceae* family is well known

powerful vegetable with respect to its antimicrobial properties and has great application in food industry as a natural colorant due to its betalain content (Kujala *et al.*, 2000; Canadanovic-Brunet *et al.*, 2011). Moreover, beetroot peel also possesses several bioactive compounds depicting the strong antimicrobial and antioxidant activity in comparison to other vegetable peel extracts (Miller *et al.*, 2000). Kujala *et al.*, (2002) has documented that the total phenolics content in beetroot decreases in the order peel (50%), crown (37%), and flesh (13%).

White radish (*Raphanu ssativus*) belongs to the *Brassicaceae* family contains phenolic acid, flavonoids and anthocyanins that act as potential antimicrobials. It is well documented that radish has many medicinal properties. Radish has strong antimicrobial property due to presence of isothiocyanates (ITCs) compounds such as methylthio-3-butenyl isothiocyanate, allyl-isothiocyanate, benzyl isothiocyanate, and phenethyl isothiocyanate (Dufour *et al.*, 2015).

Most of the previous studies on the plant extracts were carried out based on the organic solvent. So, in order to find the economic alternative for industrial application towards the control of food borne pathogens the non-solvent extraction method was followed in the present study to screen most of the food borne pathogen.

Materials and Methods

Preparation of extract

Fresh beetroot peel and white radish were purchased from the market in Kochi and washed thoroughly with potable water. The raw materials were separated into two halves; first half of beetroot peel (*Beta vulgaris*) and white radish (*Raphanus sativus*) was ground for 2 min to obtain the crude juice and juices

were passed through muslin cloth in order to remove the coarse particles (Satish *et al.*, 2016). The second half of the raw materials were sliced and kept in solar drier at 50°C, 24 hr. for dry power preparation. Then the dried materials were ground into powder, sieved and stored in air tight container for further use.

Protein concentration

The crude extract of both beet root peel and whole radish were subjected to Kjeldahl protein assay in order to find the protein concentration and both extracts were stored at -20°C as a small volume aliquot was needed for further antibiogram analysis.

Modified well diffusion assay

Antibiogram was carried as per CLSI (2012) standard agar well diffusion technique with slight modification against *S. aureus*, *Escherichia coli*, *S.typhi*, *V. cholera*, *A. hydrophila* and *P. aeruginosa*. In the present study, instead of Mueller Hinton Agar (MHA) plate, a selective media plate for each of the bacteria was used (Table 1). Pre-set specific media was prepared and 5-6 mm diameter well was made using cork borer (#LA 373, HiMedia), then the bottom of the agar was sealed with sterile molten agar to avoid leakages.

Test bacterial culture was inoculated into Brain Heart Infusion (BHI) Broth and incubated at 37°C for 2-4 hrs. The grown cultures were adjusted with sterile normal saline solution until 0.5 Mc Farland standard turbidity appeared. Sterile cotton swab was immersed into test culture and spread over the plate. Beetroot peel and radish crude extracts of 100 µl were added into the each well along with the control and the plates were incubated at 37°C. The initial concentration of the BPE and WRE were 4.38 mg/100 µL and 0.6 mg/100 µL, respectively. After 24hrs halo

zone was measured using standard antibiotic zone scale (PW 096, Hi Media).

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC and MBC of crude extracts was determined by tube dilution method (EUCAST, 2003). The initial concentration of the BPE and WRE were 43.8 mg/mL and 6 mg/mL respectively. Serial dilution of the BPE and WRE extracts were carried out in test tubes. 1 mL Mueller Hinton Broth (MHB) was added into all the tubes, then 1mL of crude extract was added into the first test tube and mixed thoroughly and transferred the 1 mL into second test tube; similarly, 1 mL was serially diluted (2 fold dilution ratio) up to 11th tube. Then 50 µl of bacterial culture containing 5×10^5 cfu were added into all the test tubes and all the test tubes were incubated at 37°C for overnight for MIC and MBC determination.

After determination of MIC, later a loopful of inoculum taken from tubes having no bacterial growth and streaked on Mueller Hinton Agar (MHA) plates and incubated for further 24 h at 37 °C. The lowest concentration that can destroy all the bacteria is considered as MBC.

Results and Discussion

In the present study, crude extract of beetroot peel and whole radish were prepared and the antimicrobial activity was initially checked with the modified well diffusion assay, later it was confirmed by the MIC and MBC for the better application in the food industry. Based on the Kjeldahl protein assay, the concentration of protein in the extract was measured. The protein concentration of the BPE and WRE were 43.8 mg/mL and 6 mg/mL respectively.

Modified well diffusion assay

In the present study, initially the BPE and WRE was filter sterilized and the antibiogram activity was checked with the standard Muller Hinton Agar (MHA) medium with the specific bacterial cultures. But, no inhibition was found against any of the bacteria by both the extracts. It may be due to all the antimicrobial bio-molecule would be attached to the filter. Since, the extract was prepared from the water; it may be still intact with the filter. So, the well diffusion assay has to be carried out in the unfiltered material. But, in the unfiltered material a high level of other bacterial flora would grow around the well. So, in order to find the antimicrobial activity of unfiltered material in the present study a well diffusion assay was carried out with slight modification *i.e.*, instead of Mueller Hinton Agar, a specific media for the specific bacteria was prepared and the wells were made in the plate. So, the crude extracts were directly placed in agar well without filtration (unsterilized). Since, it is specific to each bacteria, it will allow only the inoculated bacteria to grow Eg. Mannitol salt agar was used for the *S. aureus*. Mannitol salt agar specifically will allow the growth of *S. aureus* due to higher concentration of salt and restricts the other bacterial growth (Table 1). So, pre-set plate was prepared and well was made with 5-6 mm diameter then the bottom was sealed with sterile molten agar. *S. aureus* culture of 0.5McFarland concentration was spread over the plate. Then, the crude extract was placed in the well and the inhibition was measured after the overnight incubation at 37°C. Similarly, other bacteria were also tested with this method, the details of the media with concern bacteria are listed in the table 1.

In the present study, one set of crude extract was directly prepared from the plant; other set of extract was prepared directly by adding the

water in the powder of the beetroot peel and whole radish. Results showed that direct crude extract of both beetroot peel and radish has the antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi* and *Vibrio cholera* (Figure 1). This finding was in agreement with the result of Pavlovic *et al.*, (2013); who reported the crude extract of beetroot has been the most effective against *Staphylococcus aureus* and *K. pneumoniae*. Ironically, the powder (powder dissolved in water) of beetroot and radish did not exhibit any antibacterial activity. It may due to the loss of activity while preparing the powder. *i.e.*, beetroot and radish powder was exposed to the drying temperature of 50°C that might have caused the loss of activity. Pavlovic *et al.*, (2013) also stated that loss of antibacterial was observed while extracting the bio-molecule at temperature more than 50°C; they also suggested that low temperature in the extraction process would prevent the thermal deprivation of bioactive compounds.

Crude beetroot peel showed the inhibition zone ranging from 12.61±0.17 mm to 14.66±0.25 mm. Highest zone of inhibition (14.66±0.25 mm) was found against *E. coli* and the minimum (12.61±0.17mm) inhibition zone was found against *Salmonella typhi* (Table 2 and Figure 1).Narender *et al.*, (2018) also reported the highest inhibition zone by beetroot against *E. coli* and *S. aureus* was 26 mm and 25 mm respectively. But, in the present study, the zone size was less compared to other previous report, it may be due to water was used for the extraction of the bio-molecule, it is much less efficient to extract the bio-molecule using the fat solvent extraction. Nisa *et al.*, (2015) also has reported the antimicrobial activity of beetroot dye against the *E. coli* and other specific microorganisms like *Salmonella spp.*, *Staphylococcus aureus*, *Pseudomonas spp.*, yeast and moulds. Reason for the antibacterial

activity of beetroot peel may be due to the presence of bioactive compounds such as betanin and isobetanin (John *et al.*, 2017).

Similarly crude whole radish extract showed the inhibition range from 13.10 ± 0.53 mm to 21.33 ± 0.17 mm. The radish extract was effective against the *S. aureus* with the maximum zone of inhibition (21.33 ± 0.17 mm) and depicted the lowest zone (13.10 ± 0.53 mm) of inhibition against the *V. cholera*. Ahmad *et al.*, (2012) has reported the lesser values of zone inhibition (7-9 mm) by crude extract of radish as compared to our findings. Reason of antimicrobial activity of radish can be attributed to presence of flavonoids which are found to be effective against a wide range of microorganisms; presence of complex soluble proteins may react with the bacterial cell wall thus causes antibacterial activity (Parekh *et al.*, 2008).

The overall results indicated that the crude whole radish extract was more effective than the beetroot peel extract. Based on the present observations it is depicted that the antibacterial activity of the plant derived material truly depends upon the extraction method and the type of solvent used for extraction. In the present study no antimicrobial activity was observed for the both extracts against the *P. aeruginosa* and *A. hydrophila*.

MIC and MBC

Among the four bacteria, lowest (better) value was observed for the beetroot extract on *Pseudomonas aeruginosa* that is 13.13 ± 4.38 mg compared to other three pathogens (Table 2). Similarly, the lowest (better) MBC value was observed for the *Pseudomonas aeruginosa* i.e., 13.13 ± 4.38 compared to other three pathogens. Based on the MIC and MBC value the order of susceptibility was observed for the BPE as follows *Pseudomonas*

aeruginosa < *Aeromonas hydrophila* < *Staphylococcus aureus* and *Salmonella typhi*. The present study results are in agreement with the findings of Vulic *et al.*, (2015) reports. Baydar *et al.*, (2004) also reported that the beetroot extract exhibited antibacterial activity against *Staphylococcus aureus* (MIC = 0.75 mg/ml. In the present study, it was observed that, a weak antimicrobial activity was found against *Escherichia coli* (MIC = 1.5 mg/ml) and *Pseudomonas aeruginosa* (MIC = 4.5 mg/ml) i.e., higher amount of BPE is needed to control the pathogens. Inhibitory behaviour of beetroot peel may be attributed to presence of phenolic compounds that exhibit antimicrobial activity e.g. carvacrol, oxygenated derivatives (thymol methyl ether) and its precursor *sp*-cymene and γ -terpinene (Baydar *et al.*, 2004).

Compared to previous report the higher (Poor) MIC and MBC value was observed for the both beet root peel and whole radish extract. The reason may be due to that water was used in the present study for the extraction of the bio-molecule. Approximately double time concentration of MIC and MBC was noticed in the present study. Based on the MIC and MBC value of the crude whole radish extract it was observed that the *P. aeruginosa* was susceptible compared to other three pathogens. 0.11 ± 0.04 mg of crude radish extract was able to inhibit the *P. aeruginosa* and 0.23 ± 0.08 mg was able to completely destroy the same bacteria.

In the present study, some of the bacteria showing susceptible to modified well diffusion assay, but were not able to detect in the MIC and MBC i.e., *E. coli* was able to show the susceptibility in the modified well diffusion assay, but were not detected in the MIC and MBC assay. Similarly, organism like *Pseudomonas* and *Aeromonas* showed susceptibility in the MIC and MBC assay, but

was not inhibited in the modified well diffusion assay. The ambiguity of result may be due to some contaminant may grow in the modified well diffusion assay and some organism may grow in the broth or the bio-molecule in the BPE can be dissolved in the broth (MIC) so the contact of the bio-molecule is possible in the water medium; in case of well diffusion the contact is not

possible. So, based on this observation, to identify the exact antimicrobial activity, a combined method of well diffusion assay and MIC would be better result. Since, the modified method is easy and not need use the filtration process it can be recommend for the initial screening of the more number of plant extract.

Table.1 Specific media’s used for the growth of bacteria

S. No.	Name of the Bacteria	Specific Media
1	<i>Staphylococcus aureus</i> (ATCC-25923)	Mannitol Salt agar (MSA)
2	<i>Escherichia coli</i> (ATCC-10536)	Eosin methylene blue (EMB) agar
3	<i>Salmonella typhi</i> (ATCC-9150)	Hektoen enteric agar (HEA)
4	<i>Vibrio cholera</i>	Thiosulfate citrate bile salt sucrose (TCBS) agar
5	<i>Aeromonas hydrophila</i> (ATCC-35654)	Trypticase soya agar with Ampicillin
6	<i>Pseudomonas aeruginosa</i> (ATCC-10145)	Trypticase soya agar with Cephalothin-sodium Fusidate-Cetrimide (CFC) supplement

Table.2 Zone of inhibition zone for the BPE and WRE in modified well diffusion assay

Pathogenic microorganism	Inhibition zone (mm)	
	Beetroot peel extract (BPE)	Whole radish extract (WRE)
<i>Staphylococcus aureus</i>	13.15±0.13	21.33±0.17
<i>Escherichia coli</i>	14.66±0.25	16.00±0.20
<i>Salmonella typhi</i>	12.61±0.11	20.34±0.02
<i>Vibrio cholera</i>	NI	13.10±0.03
<i>Aeromonas hydrophila</i>	NI	NI
<i>Pseudomonas aeruginosa</i>	NI	NI

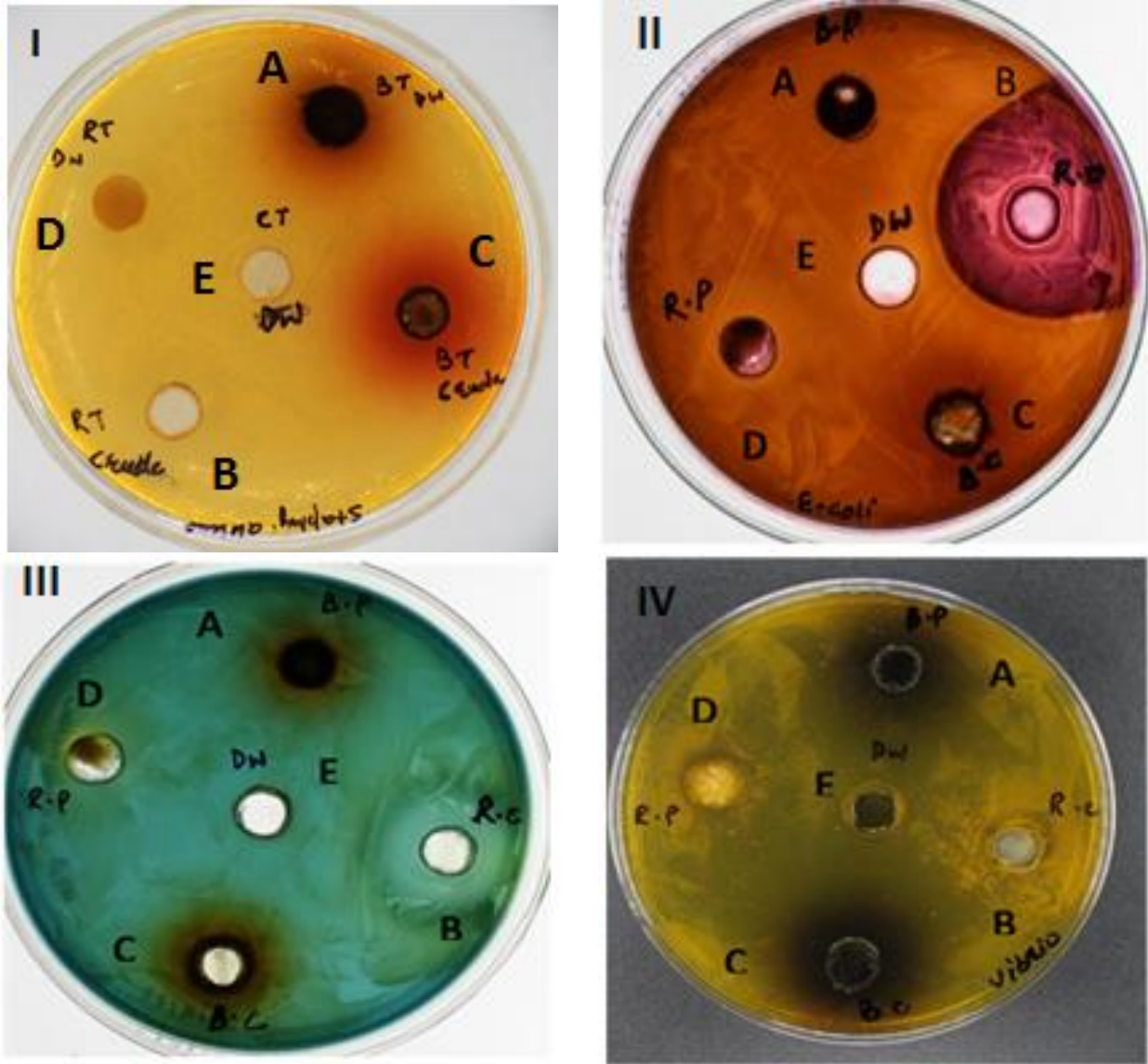
NI- No inhibition, Results are given as mean± standard deviation

Table.2 Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of BPE and WRE against pathogenic microorganism

Pathogenic microorganism	Beetroot peel extract (BPE) mg/100µL		Whole radish extract(WRE) mg/100µL	
	MIC	MBC	MIC	MBC
<i>S. aureus</i>	26.25±8.75	35±0.0	2.3±0.8	2.3±0.8
<i>S. typhi</i>	26.25±8.75	35±0.0	2.3±0.8	2.3±0.8
<i>P. aeruginosa</i>	13.13±4.38	13.13±4.38	1.1±0.4	2.3±0.8
<i>A. hydrophila</i>	26.25±8.75	26.25±8.75	2.3±0.8	2.3±0.8

Results are given as mean± standard deviation

Figure.1 Modified well diffusion assay for the unfiltered crude extract
Where= I: Inhibition zone against *I.S. aureus*; II. *E.coli*, III. *S. typhi*, IV. *V. cholerae* A: beetroot peel powder in distilled water B: crude extract of radish C: crude extract of beetroot peel D: radish powder in distilled water E: distilled water as control



Based on the experimental outcome, it can be concluded that both the beetroot peel and whole radish can be a promising source of antimicrobial agents but much need to be explored regarding the activity of solvent free natural extracts. Since the modified well diffusion assay is rapid and economic and

suitable of the most of the crude extract analysis it will be a more useful to screen the large number of plant extract. The combined method of well diffusion assay with MIC would be more suitable procedure to identify the exact antibacterial activity of the crude unfiltered plant materials.

Acknowledgement

Authors are highly thankful to Prof. A. Ramachandran, Vice-Chancellor Kerala University of Fisheries & Ocean Studies (KUFOS) and Dr. Ravishankar, C.N., Director, ICAR-Central Institute of Fisheries Technology, Cochin for providing valuable support to carry out this research work.

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How to cite this article:

Hafsa Maqbool, S. Visnuvinayagam, A. A. Zynudheen, M. P. Safeena and Sathish Kumar. 2020. Antibacterial Activity of Beetroot Peel and Whole Radish Extract by Modified Well Diffusion Assay. *Int.J.Curr.Microbiol.App.Sci.* 9(01): 1222-1231.
doi: <https://doi.org/10.20546/ijcmas.2020.901.135>