Malaysian Journal of Microbiology, Vol 10(3) 2014, pp. 197-204
http://dx.doi.org/10.21161/mjm.60714

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Evaluation of antibacterial effects of *Rhizophora apiculata* pyroligneous acid on pathogenic bacteria

Darah Ibrahim[1], Jain Kassim[2], Sheh-Hong Lim[1]* and Wendy Rusli[2]

[1]Industrial Biotechnology Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia.
[2]School of Chemical Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia.
E-mail: limshehhong77@gmail.com

Received 27 January 2014; Received in revised form 27 January 2014; Accepted 9 April 2014

ABSTRACT

Aim: *Rhizophora apiculata* pyroligneous acid is a crude condensate produced from the distillation of smoke generated in the process of charcoal making has the potential to be used as antibacterial agent to treat bacterial infections. In this study, pyroligneous acid (PA), concentrated pyroligneous acid (CPA), Dichloromethane extract of CPA namely DCM A and B were tested against eight Gram positive and eight Gram negative pathogenic bacteria.
Methodology and results: The antimicrobial activities were studied by using disc diffusion method and broth dilution method. The effect of the extract on the growth profile of the bacteria was examined via time-kill assay. Moreover, microscopic observations using scanning electron microscopy (SEM) was done to determine the major alterations of *Bacillus subtilis* cells after treated with extract. The results exhibited significant inhibition zones within the range of 13.0-19.0 mm for PA, 19.0-23.0 mm for CPA, 15.0-17.0 mm for DCM A and 14.0-16.0 mm for DCM B. The results also revealed that extract DCM B of CPA was the most potential to be used as antibacterial agent with the minimum inhibitory concentration (MIC) values between 1.56-3.12 mg/mL. Scanning electron micrographs of DCM B treated *Bacillus subtilis* confirmed the damaged cells caused by the extract.
Conclusion, significance and impact of study: Data from this investigation revealed that *Rhizophora apiculata* pyroligneous acid may be a broad antimicrobial agent against pathogenic bacteria.

Keywords: *Rhizophora apiculata*, pyroligneous acid, antibacterial agent, minimum inhibitory concentration

INTRODUCTION

Pyroligneous acid, also called wood vinegar, is a crude condensate produced from the distillation of smoke generated in the process of wood carbonization. It is a complex mixture of compounds derived from the chemical break-down of the components in wood through the condensation of vapours and gases generated during the pyrolysis of a limited access of oxygen. It appears to be a clear reddish-brown liquid which resembles to the pleasing hue of black tea, beer or wine (Guille'n and Ibargoitia, 1998). The pH of pyroligneous acid is low ranging from 2-3, due to its high amount of volatile acids (8-10%) mainly formic and acetic acids (Sipila *et al*., 1998). Pyroligninеous acid is also well-known for its organoleptic properties and it is reported to have a strong smoky aroma (Guille'n and Manzanos, 2002). It is reported to contain a complex mixture of water, acetic acid, formic acid, guaiacols, catechols, syringols, vanillins, methanol, acetone, furan carboxaldehydes, isoeugenol, pyrone, ketones, esters, and more than 200 organic compounds including phenolic compounds which are pyrolytic products of lignin and hemicelluloses (Lee *et al*., 2010).

Pyroligneous acid offers a wide range of applications in food industry as food preservation (Youn *et al*., 2003) or food smoking (Holley and Patel, 2005), removal of odors and also used as a natural organic pesticide in agricultural sector to increase growth of plant roots as it can reduce the growth of phytopathogenic fungi such as the species of *Fusarium, Pythium* and *Rhizoctonia* (Jung, 2007). It has been traditionally used as sterilizing agent, deodorizer, fertilizer, antimicrobial and growth promoting agent (Zulkarami *et al*., 2011). It also possesses antioxidant activity (Mares, 1989; Loo *et al*., 2007) besides contains bioactive compounds that have synergistic effect among them which exhibit antimicrobial properties (Hwang *et al*., 2005; Darah *et al*., 2013a). It is believed that phenolic derivatives are one of the groups of compounds being responsible for the antimicrobial activity of pyroligneous acid (Cowan, 1999). Recently, the medicinal use of the pyroligneous acid has been studied

*Corresponding author

ISSN (print): 1823-8262, ISSN (online): 2231-7538

intensively in the field of oriental medical science (Kim *et al*., 2001; Park *et al*., 2003), where some natural resources has been used for investigating the biological activities (Lee and Seo, 2006; Lee *et al*., 2006).

Most of the reported studies on pyroligneous acid came from beech, oak, thyme or sage woods (Demirbas, 2005; Jung, 2007). In Malaysia, pyroligneous acid is a by-product of charcoal making from the billets of *Rhizophora apiculata* or locally known as bakau minyak (Loo *et al*., 2007). Smoke from the vents of charcoal kilns is condensed in steel tubes and collected in plastic drums as raw distillate of pyroligneous acid. This byproduct is rarely used and often disposed off as waste. Based on this reason, a study on the *R. apiculata* pyroligneous acid and its dichloromethane extract were carried out in order to evaluate their effects against a series of pathogenic bacteria. The evaluations of their antibacterial activities as well as the effect of extract on the bacterial cell morphologies were studied.

MATERIALS AND METHODS

Pyroligneous acid

The *R. apiculata* pyroligneous acid was obtained from charcoal village in Kuala Sepetang, Taiping, Perak, Malaysia. The smokes that escape from the chimneys of the charcoal's kiln at temperatures of 240-500 °C were passed through a 30 m extension of air cooled stainless steel pipe for condensation purpose. The condensed smokes or known as pyroligneous acid or wood vinegar was collected in a polyethylene container and stored at room temperature (30±2 °C) and was used in the present study.

Preparation of concentrated pyroligneous acid

The pyroligneous acid was filtered through a Whatman No. 1 filter paper to eliminate any debris and oily phase. The filtrate was then concentrated using a rotary evaporator under reduced pressure at 80 °C until the volume of one tenth of its original volume obtained. The resulting concentrated pyroligneous acid (designated as CPA) which was free from water and some volatile components (such as methanol, acetone etc) was collected and kept at 4 °C until further used.

Preparation of the dichloromethane extract from concentrated pyroligneous acid

The extraction of the components of concentrated pyroligneous acid (CPA) was conducted using liquid-liquid extraction with dichloromethane (designated as CPM) as the extraction solvent to give DCM A extract. In order to isolate the phenolic derivatives from DCM A extract, an acid base treatment was conducted on DCM A extract by using aqueous NaOH solution, aqueous H_2SO_4 solution and $NaHCO_3$ to give DCM B extract (Amen-Chen *et al*., 1997).

Microorganisms and cultural maintenance

Sixteen pathogenic bacterial species which consisted of eight Gram positive (*B. cereus*, *B. subtilis*, *B. spizizenni*, *S. aureus*, MRSA, *S. epidermidis*, *Streptococcus pyogenes* and *S. faecalis*) and eight Gram negative (*Citrobacter freundii*, *E. coli*, *Erwinia* sp., *K. pneumonia*, *P. mirabilis*, *P. aeruginosa*, *Salmonella typhi* and *Yersinia* sp.) obtained from the Industrial Biotechnology Research Laboratory Culture Collection, School of Biological Sciences, Universiti Sains Malaysia were used throughout the study. The bacterial cultures were maintained on nutrient agar (Merck, Germany) slants at 37 °C for 24 h. All the cultures were kept at 4 °C until further used. Subculturing was done at every four weeks to maintain their viability.

Antibacterial activity

The antibacterial activity of the extract against the test bacteria were determined following the method described by Darah *et al*., (2013b) with slight modifications. Test bacteria were cultured on nutrient agar plates and incubated at 37 °C for 24 h. Bacterial suspensions were prepared by inoculating one loopful of a pure colony into 5.0 mL of sterile distilled water. Sufficient inoculums were added until the turbidity equal to 0.5 McFarland standards which approximately equivalent to $1.5x10^5$ cells per mL.

One milliliter of the suspension was added into 15.0 mL of sterilized molten nutrient agar aseptically. The mixtures were mixed well by swirling the plates left and right and then they were left on the bench to solidify. The commercial antibiotic disk GF A (Whatman, England) with 6.0 mm diameter was used to screen the antibacterial activity. Each of the sterile disks was then impregnated with 20 µL of the extracts that were pyrolineous acid (PA), concentrated pyroligneus acid, (CPA), Dichlromethane extracts (DCM A and DCM B), which corresponding to 100.0 mg/mL of extract stock. Chloramphenicol (Sigma, Germany) at the concentration of 30 µg/mL was used as a positive control. All the impregnated disks were air dried before placing them on the agar surface. The plates were incubated at 37 °C for 24 h and the antibacterial activity was determined by measuring the diameter of the inhibition zones formed around the disks. The experiments were carried out in triplicate and the results were expressed as means of three experiments.

Determination of minimum inhibitory concentrations

The determination of minimum inhibitory concentration (MIC) was performed using macrodilution method (Darah *et al*., 2013b). Briefly, different extract preparations were subjected to a serial dilution using sterile nutrient broth medium as a diluents to give final crude extract concentrations between 0.39 and 100.00 mg/mL. The test tubes were inoculated with the bacterial suspension (20 µL/mL broth), homogenized, and incubated at 37 °C for 24 h. The lowest dilution of the extract that retained its inhibitory effect resulting in no growth (absence of

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

turbidity) of a microorganism was recorded as the MIC value of the extract. The bacterial growth was indicated by the turbidity. Each test was performed in triplicate.

Time-kill study of *Bacillus subtilis* in the presence of DCM B extract

Bacterial suspension of *B. subtilis* was prepared as described previously and was harvested by centrifugation, washed twice with sterile distilled water and resuspended in sterile distilled water. The suspension was adjusted using the McFarland standard. The DCM B extract was added in to 25 mL of nutrient broth in a 50 mL Erlenmeyer flask to achieve concentrations of 0 (control), 1.56 (1/2MIC), 3.13 (MIC) and 6.25 (2MIC) mg/mL after addition of the inoculum. The experiments were conducted in triplicate and all the flasks were incubated in a shaker (Infors HT Ecotron) incubator at 37 °C with agitation at 150 rpm. One milliliter of the mixture within each flask was withdrawn at every 4 hourly intervals starting from 0 h until 48 h of cultivation and the bacterial cell growth was monitored by measuring optical density at 540 nm.

Scanning electron microscope observations

The bacterial suspension was prepared as described previously. To each sample, 1.0 mL of the 24 h old bacterial suspension was inoculated in a 50.0 mL Erlenmeyer flask containing 30.0 mL of sterilized nutrient broth and incubated in a shaker at 37 °C, 150 rpm for 18 h. The bacterial suspension was then added to the extract stock solution (the final concentration in each flask was at the MIC value) and incubated at the required incubation time (0, 12, 24 and 36 h). The SEM sample preparations were done following the method describes by Mares (1989). The prepared samples were then viewed under a scanning electron microscope (Leica Cambridge, S-360, United Kingdom).

RESULTS

Antibacterial activity and minimum inhibitory concentration (MIC) values of the extract

The pyroligneous acid (PA) in this study was concentrated under reduced pressure, using a rotary evaporator at 80 °C to give CPA. The removal of the volatile components in PA, such as methanol, acetone and some of the volatile acids, was another 'cleaning-up' procedure after filtration process. Those components were not the object of interests and the removal of those components would help to facilitate in the extraction and isolation of the components in PA. Additionally, at a temperature of 80 °C, the desired compounds would not degrade as they were of higher boiling points (semi-volatiles compounds) and would remain in CPA. Table 1 shows some of the physical properties of CPA.

Table 1: Physical properties of concentrated pyroligneous acid (CPA).

Physical properties	Appearances
Appearance	Liquid
Acidity (pH)	1.8
Color	Black liquor
Transparency	Dark
Odor	Strong smoky aroma

The antibacterial activity of the *R. apiculata* pyroligneous acid (PA), concentrated pyrligneous acid (CPA), dichloromethane extracts A and B (DCM A and B) against 16 bacterial species were examined in this study and their potency were assessed by the diameter of inhibition zones. The results are tabulated in Table 2. All the four extracts exhibited significant zone of inhibitions within the range of 13.0–19.0 mm for PA, 19.0 to 23.0 mm for CPA, 15.0 to 17.0 mm for DCM A and 14.0 to 16.0 mm for DCM B, whereas for commercial antibiotic, chloramphenicol at 30 µg/mL the inhibition zones were slightly bigger of 22.0 to 24.0 mm.

Table 3 shows the MIC values of the four extracts against bacteria and the results revealed that Gram positive bacteria are more susceptible to the extracts than Gram negative bacteria. The potency of the extracts against bacteria can be arranged as followed; DCM B> DCM A> CPA> PA. The MIC values of PA ranged between 6.25 to 12.5 mg/mL, CPA from 3.13 to 6.25 mg/mL, DCM from 3.13 to 6.25 and DCM B from 1.56 to 3.13 mg/mL.

Time-killed studies

Time-kill studies were performed over a period of 48 h with the *B. subtilis* cells being exposed to MIC (1.56 mg/mL), 1/2MIC (0.78 mg/mL) and 2MIC (3.13 mg/mL) values of the DCM B extract and the results are shown in Figure 1 DCM B extract was selected because it showed the lowest MIC values among the four extracts studied.

As shown in Figure 1, at 1/2MIC (0.78 mg/mL) the extract demonstrated a drastic drop in OD after 32 h, which leads to the death phase of the bacterial growth compared to the control. At the values of MIC (1.56 mg/mL) and 2MIC (3.13 mg/mL), the extract produced cell eradication after 16 and 12 h, respectively. Based on the results obtained from the time-kill studies, it was obviously seen the potency of the DCM B as antibacterial agents against pathogenic bacteria.

Structural degeneration of the untreated and extract treated cells

To have a clearer view in time-kill studies, the SEM studies were performed and the results revealed that the DCM B extract produced considerable morphological changes in the B. subtilis cells (Figure 2). Figure 2A shows the SEM micrographs of the bacterial cells without the DCM B extract treatment. The figure revealed the

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Table 2: Antibacterial activity of the *R. apiculata* pyroligneous acid and its dichloromethane extracts.

Bacteria	Diameter of inhibition zones (mm)				
	PA (100 mg/ mL)	CPA	DCM A	DCM B	Chloramphenicol (30 mg/ mL)
Gram positive					
Bacillus subtilis	19	20	16	14	23
Bacillus cereus	18	20	16	16	23
Bacillus spizizenni	19	22	16	16	24
Staphylococcus aureus	13	23	16	16	24
Methicillin-resistant *S. aureus*	13	21	15	14	22
S. epeidermidis	16	22	16	16	24
Streptococcus pyogenes	14	21	15	15	23
Streptococcus faecalis	13	22	16	15	24
Gram negative					
Citrobacter freundii	13	22	16	14	24
Escherichia coli	12	20	16	14	22
Erwinia sp.	15	20	17	15	23
Klebsiella pneumoniae	15	20	16	15	22
Proteus mirabilis	17	21	16	14	23
Pseudomonas aeruginosa	15	20	15	14	22
Salmonella typhi	10	19	15	14	22
Yersinia sp.	13	21	16	15	24

PA, pyroligneous acid; CPA, concentrated pyroligneous acid; DCM, dichloromethane extract

Table 3: Minimal inhibitory concentrations (MIC) of various extract on bacteria.

Bacteria	MIC (mg/ mL)			
	PA	CPA	DCM A	DCM B
Gram positive				
Bacillus subtilis	6.25	3.13	3.13	1.56
Bacillus cereus	6.25	3.13	3.13	1.56
Bacillus spizizenni	6.25	3.13	3.13	1.56
Staphylococcus aureus	6.25	3.13	3.13	1.56
Methicillin-resistant *S. aureus*	12.5	6.25	6.25	3.13
S. epeidermidis	6.25	3.13	3.13	1.56
Streptococcus pyogenes	6.25	3.13	6.25	3.13
Streptococcus faecalis	6.25	3.13	6.25	3.13
Gram negative				
Citrobacter freundii	6.25	3.13	3.13	1.56
Escherichia coli	6.25	6.25	6.25	3.13
Erwinia sp.	6.25	6.25	6.25	3.13
Klebsiella pneumoniae	6.25	6.25	6.25	3.13
Proteus mirabilis	6.25	3.13	3.13	1.56
Pseudomonas aeruginosa	6.25	6.25	6.25	3.13
Salmonella typhi	6.25	6.25	6.25	3.13
Yersinia sp.	6.25	3.13	3.13	1.56

PA, pyroligneous acid; CPA, concentrated pyroligneous acid; DCM, dichloromethane extract

ISSN (print): 1823-8262, ISSN (online): 2231-7538

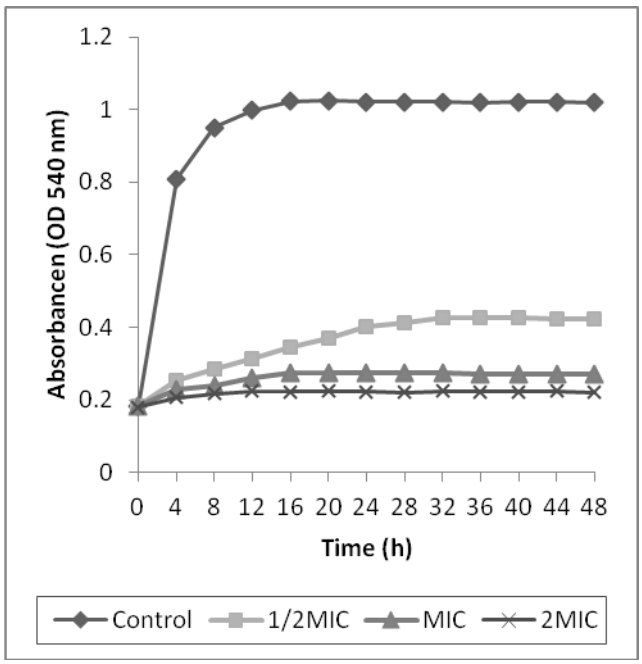

Figure 1: Effects of CPA extract on the growth of *Bacillus subtilis* at different concentrations.

normal rod shape cell structure without any shrinkage or cavity formation as the surface was smooth and regular. Figure 2B shows the morphology of the cell after 12 h of treatment with the extract. The bacterial cells started to show multiple defects with many of cells exhibited crumpled or shrunken cell surface. Figure 2C revealed more formation of crumpled cells and some the cells formed cavities. After 36 h of exposure (Figure 2D), the bacterial cells were seemed to be totally deformed and collapsed cells were seen. The cells were collapsed, clumping together and hence lost their original rod shape as compared to the control cells.

DISCUSSION

R. apiculata pyroligneous acid is a by-product of charcoal making industry from the billets of *R, apiculata*, a mangrove plant which were planted widely for charcoal industry in Malaysia. The freshly collected reddish-brown distilled solution from the charcoal kiln at the temperature of 240-500 °C has a smoke aroma (Loo *et al*., 2008). *R. apiculata* pyroligneous acid has been reported to consist of 5.5% acetic acid, 3.4% methanol and 6.5% wood tar. Due to its high amount of volatile acids (8-10%), pyroligneous acid is acidic with pH ranging from 2-3. These acids contribute to its mild corrosive properties.

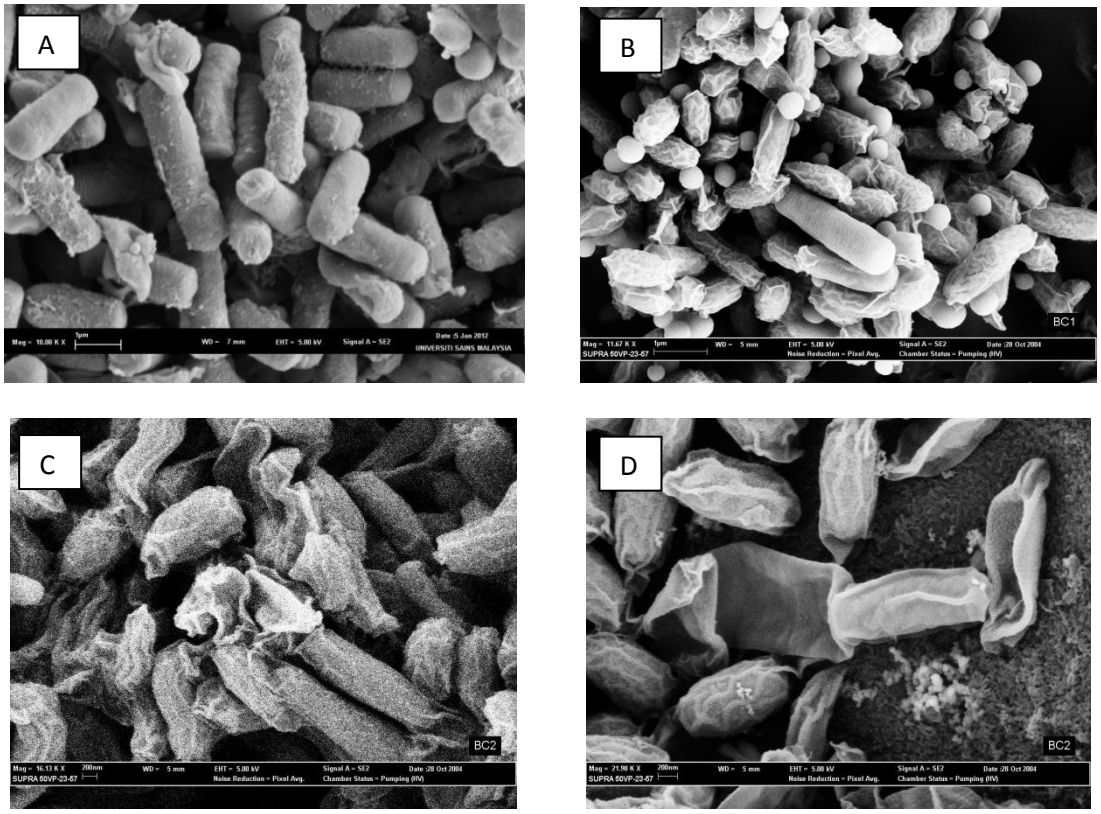

Figure 2: SEM micrographs showing the morphological changes of *Bacillus subtilis* after treated with DCM B extract at 1.56 mg/mL. (A) Untreated cells (control), (B) 12 h, (C) 24 h and (D) 36 h of exposure to the extract

ISSN (print): 1823-8262, ISSN (online): 2231-7538

R. apiculata pyroligneous acid from Matang, Malaysia has been studied for its phenolic content and antioxidant properties (Loo *et al*., 2007). However, the detail study on antibacterial activity of the *R. apiculata* pyroligneous acid is scarce. The present study has shown that pyroligneous acid has promising antibacterial activity and this is probably it is widely used in traditional oriental medicine (Park *et al*., 2003).

The activity of the four extracts (PA, CPA, DCM A and DCM B) against both Gram positive and Gram negative bacteria can be indicative of the presence of broad spectrum antibiotic compounds or simply general metabolic toxins in the solution. The antibacterial activity of the extracts may be due to the presence of lignans (like phyllanthin and hypophyllanthin), flavonoids (like quercetin), astragalin, triterpenoids, glycosides, and tannins (ellagitannins), in the extract. Phytochemical constituents like flavonoids are known to prevent gastric ulcer due to the astringent and antimicrobial properties, which appear to be responsible for gastro-protective activity, as reported by Okolo *et al*., (2012). P-cymene, a monoterpenoid has also been reported to have a good antimicrobial property (Paithankar *et al*., 2011; Selvamohan *et al*., 2012).

In this study eight Gram positive (*B. cereus, B. subtilis, B. spizizenni*, *S. aureus*, MRSA,*S. epidermidis*, *Streptococcus pyogenes* and *S. faecalis*) and eight Gram negative (*Citrobacter freundii*, *E. coli*, *Erwinia* sp., *K. pneumonia*, *P. mirabilis*, *P. aeruginosa*, *Salmonella typhi* and *Yersinia* sp.) bacteria were used as test microorganisms. All these are pathogenic bacteria that are known to cause several diseases and infections in humans and animals. For instance, *S. aureus* and *P. aeruginosa* are most common pathogens causing serious infections while *E. coli* is an opportunistic pathogen at the site of cut wound. The MIC values of the extract against all the test bacteria were determined using macrodilution method and the results showed that MIC values for Gram positive bacteria were between 1.56 to 6.25 mg/mL which were more susceptible than Gram negative bacteria which exhibited the MIC values of 1.56 to 12.50 mg/mL.

In the present study Gram positive bacteria were found to be more susceptible to the extracts than Gram negative bacteria which corroborated the previous reports that event terrestrial and marine plant extracts are more active against Gram positive bacteria (Darah *et al*., 2011; Nalubega *et al*., 2011; Nor Afifah *et al*., 2012). This may be attributed to the fact that these two groups differ in their structure of the cell wall components. The same characteristics were observed in other antimicrobial studies of other natural resource extracts against pathogenic bacteria (Darah *et al*., 2011; Narayan, 2012; Nor Afifah *et al*., 2012).

Hyde *et al*., (2006) suggested that the morphological changes of the antibiotic-treated bacteria occur when the antimicrobial agent attacked the cell membrane. In this case, the bioactive compound of the extract that locked on the cell surface structure had permeabilized the bacterial membranes. Any disruption in cell wall integrity will have a great influence in bacterial growth. This prediction was coincided well with the findings of Sasidharan *et al*., (2010) who reported the methanolic extract of macroalgae *Gracilaria changii* exerted its inhibitory effect on the cell wall of the bacterial cells which led to the complete damage of the cells. Various studies were reported to investigate the mechanism of actions involved in bacterial killing process. Among them are the interactions of antibacterial compound with the cell membrane (Hyldgaard *et al*., 2012).

Gram negative bacteria are considered to be more resistant due to their outer membrane which acting as a barrier to many environmental substances including antibiotics (Narayan, 2012). This outer membrane includes the asymmetric distribution of the lipids with phospholipids and lipopolysaccharide (LPS) located in the inner and outer leaflets, respectively (Inovye *et al*., 2001). This characteristic that is absent in the Gram positive bacteria might have acted as the additional barrier that hinders the movement of foreign substance into the cell (Darah *et al*., 2011). In addition, the cell wall of Gram-positive bacteria contains lipotheichoic acids (LTA) that represent unique and essential structural components to the cells and should be good drug targets to the bioactive compounds of the extract (Cabeen and Jacobs-Wagner, 2005).

The present investigation concluded that *R. apiculata* pyroligneous acid and its extracts have a great potential as antibacterial agent to treat infectious diseases caused by a range of pathogenic bacteria. The study provides support for the use of pyroligneous acid in the management of infectious diseases. These findings can form the basis for further studies to prepare an optimize preparation of the pyroligneous acid to further evaluate them against a wide range of bacterial strains.

ACKNOWLEDGEMENT

The authors are grateful to Ministry of Science, Technology and Innovation of Malaysia (MOSTI) (203/PBIOLOGI/6730001) for supporting this research.

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