Bacterial inhibition and cell leakage by extract of *Polygonum minus* Huds. leaves

[1,2]Imelda, F., [1,3]Faridah, D. N. and [1,3*]Kusumaningrum, H. D.

[1]Food Science Study Program, Department of Food Science and Technology, Faculty of Agricultural Engineering and Technology, Bogor Agricultural University, Kampus IPB Darmaga, PO. Box 220, Bogor 16002, Indonesia
[2]Department of Agricultural Technology, Pontianak State Polytechnic, PO Box 1286, Pontianak 78124, Indonesia
[3]Southeast Asia Food and Agricultural Science and Technology Center, Jl. Puspa Lingkar Kampus IPB Darmaga, Bogor 16680, Indonesia

Abstract

Leaves of *Polygonum minus* Huds. contain some bioactive compounds which are potential as natural antibacterial agent. The performance of two extraction techniques, i.e. conventional maceration and ultrasound assisted extraction (UAE), for extraction of bioactive compounds of *P. minus* leaves were evaluated. The antibacterial activities of the extracts were also evaluated towards *Escherichia coli* and *Staphylococcus aureus* by agar disc diffusion in combination with TLC-bioautography and by observing the cell membrane leakage. Extraction with UAE provided higher extraction yields and reduced the extraction time in comparison to the conventional maceration. Ethanolic extract of *P. minus* showed the strongest inhibitory effect for both *E. coli* and *S. aureus*. The Minimal Inhibitory Concentration (MIC) of the ethanolic extract, determined by macro-dilution method, was 25 mg/mL for *E. coli* and 30 mg/mL for *S. aureus*. TLC-bioautography detected that fractions of ethanolic extract of *P. minus* with Rf_4 = 0.30 and Rf_5 = 0.37 demonstrated the most noticeable antibacterial activity for both *E. coli* and *S. aureus*. Leakage of cytoplasmic membrane was observed, indicated by the release of cells materials, measured at 260 and 280 nm using UV - Vis spectrophotometer.

Introduction

Polygonum minus Huds. leaves have unique taste and flavour and are commonly used as flavouring ingredient for local culinary in Southeast Asian countries such as Malaysia, Thailand, Vietnam and Indonesia. Its local name in Indonesia and Malaysia is Kesum (Kesom). In addition to enhancing the flavour of food dishes, kesum leaves are also used as traditional medicine to treat intestinal worms infection, stimulate menstruation, treat scurvy, prevent flatulence and gastric disorders and for recovery after childbirth (Almey *et al.*, 2010; Wasman *et al.*, 2010; Qader *et al.*, 2012). Kesum leaves contain high phenolic compounds (Almey *et al.*, 2010; Qader *et al.*, 2011), such as gallic acid, rutin, coumaric acid (Qader *et al.*, 2012), flavonoids such as myricetin and quercetin (Miean and Mohamed, 2001; Qader *et al.*, 2012), alkaloids, tannins and terpenoids (Wibowo *et al.*, 2009). Pharmacological studies have been conducted on *P. minus* leaves as antiviral, antibacterial and antifungal agents, as well as antioxidant, anticancer and antiulcer (Qader *et al.*, 2012). *P. minus* has also been reported exhibiting antibacterial activity against

Helicobacter pylori (Uyub *et al.*, 2010), *Bacillus subtilis* (Jamal *et al.*, 2011) and *Escherichia coli* (Wibowo, 2007).

Generally, the *P. minus* extracts have been obtained by conventional maceration procedure (Wibowo *et al.*, 2009; Almey *et al.*, 2010; Jamal *et al.*, 2011; Qader *et al.*, 2012). This technique is often time consuming and requires relatively large amounts of solvent and sometimes diminishes the active compounds (Mantegna *et al.*, 2012). UAE is one of the emerging technologies being developed in order to minimize the length of the extraction time, to reduce the cost, and to maximize the extract quality (Jabrak, 2013). UAE technique can improve the extraction performance of natural products from plant materials and has been used to extract phenolic compounds (Liazid *et al.*, 2010; Aspe and Fernandez, 2011), flavonoids (Velickovic *et al.*, 2007) and resveratrol (Mantegna *et al.*, 2012).

TLC-bioautography is a specific technique to detect the spots on the TLC chromatogram towards microbial test response, based on the biological activity of the bioactive compounds of the extracts as antibacterial, antifungal and antiprotozoal agents

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*Corresponding author.
Email: *h_kusumaningrum@ipb.ac.id*
Tel: +62 251 8626725; Fax: + 62 251 8626725

(Choma, 2005; Kusumaningtyas *et al.*, 2008). This technique can rapidly detect and separate the active compounds of the plant extract (Gu *et al.*, 2009). It is also cheaper and the interpretation of the results is relatively easy and accurate (Kusumaningtyas *et al.*, 2008).

The present paper was subjected to evaluate the performance of UAE technique in comparison to the conventional maceration technique to extract active compounds of *P. minus* leaves; and to detect the active fractions of ethanolic extract of *P. minus* leaves by TLC-bioautography against *Staphylococcus aureus* and *Escherichia coli*. Furthermore, the effect of the ethanolic extract of *P. minus* on the cell membrane of *S. aureus* and *E. coli* was also studied.

Materials and Methods

Plant materials and extraction

P. minus (Kesum) leaves were collected from Pontianak, West Borneo, Indonesia. Kesum leaves were freeze dried (LABCONCO, UK), ground into fine powder (300 μm), and were extracted using different solvents, i.e. n-hexane, ethyl acetate and ethanol (Merck, Darmstadt, Germany) by multilevel techniques using UAE procedure. Twenty five g leaves powder in 250 mL solvent was extracted at 40 kHz frequency for 20 min at 40 ± 1℃ in ultrasonic bath (BRANSONIC Ultrasonic cleaner 8510E-MTH, USA). After that, the mixture was filtered and the supernatant was evaporated using vacuum evaporator at 40℃ (BUCHI Rotavapor RII, Switzerland) and subsequently blown with N_2 to remove residual solvent (Velickovic *et al.*, 2007 with some modifications). The extraction using the same solvents was also conducted by maceration process for 24 h (for each solvent) at room temperature (28℃), with shaking at 200 rpm (New Brunswick Scientific, Innova 2100, USA). All extracts were then kept at -4℃ until further analysis.

Microorganism preparation

E. coli ATCC 25922 and *S. aureus* ATCC 25923 were maintained on Tryptone Soy Agar (TSA) (Oxoid, Hampshire, UK) slants at 4℃. The bacteria were grown separately in Brain Heart Infusion Broth (BHIB) (Oxoid, Hampshire, UK) and incubated at 35-37°C for 18-24 h. One loop-full of bacterial suspension was subsequently streaked on TSA plates and incubated at 35-37°C for 48 h. A single colony was transferred to 10 mL of fresh Tryptone Soy Both (TSB) (Oxoid, Hampshire, UK) and incubated at 35-37°C for 18-24 h. This culture was used for antibacterial assay (Oonmetta-aree *et al.*, 2006).

Antibacterial activities test

Hexane, ethyl-acetate and ethanolic extracts of *P. minus* obtained by UAE were dissolved in DMSO (dimethylsulfoxide, Merck, Darmstadt, Germany) to final concentration and sterilized by filtration through 0.45 μm membrane filters (Sartorius, Gottingen, Germany). The antibacterial activity was determined by Kirby and Bauer disc diffusion method (Sharififar *et al.*, 2007). Cultures were centrifuged for 10 min at 10000 rpm, resuspended in sterile sodium chloride solution (0.85 g/100 mL) and the optical density (OD) was adjusted to the standard of McFarland No. 0.5 to achieve a concentration of approximately 10^8 CFU/mL. Bacterial suspensions were spread over Mueller Hinton Agar (MHA) plates (Oxoid, Hampshire, UK) using sterile cotton swab. The sterile disc (ø 6 mm, Oxoid, Hampshire, UK) were then placed on the inoculated agar and saturated with 10 μL of extracts (500, 400, 300, 200, 100 and 50 mg/mL). A disc with 10 μL of Streptomycin (1 mg/mL) and a disc with 10 μL of DMSO were also placed as positive control and as negative control, respectively. The inoculated plates were then incubated at 37℃ for 24 h. The antibacterial activity was evaluated by measuring the zone of inhibition (including the diameter of disc) against the test bacteria.

Determination of Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

The MIC was determined by classical method with successive dilution (Mazzola *et al.*, 2009 with some modifications). A series of two fold dilution of highly active extract of *P. minus*, ranging from 6.25 mg/mL to 100 mg/mL, was prepared in 1 mL of TSB. Cultures were adjusted to the standard of McFarland No. 0.5 to achieve a concentration of approximately 10^8 CFU/mL. One hundred μL of the bacterial suspension was then transferred into 1 mL of TSB mixture, to obtain the final concentration of approximately 10^6 CFU/mL. Thereafter, suspension were incubated with shaking at 37°C for 24 h. The MIC of the extract was confirmed by spreading of one loop-full of suspension from all tubes on TSA plates and incubated at 37°C for 24 h. The MIC was described as the lowest concentration of the extract which reduced 90% of the number of the initial inoculum (Cosentino *et al.*, 1999). Subsequently, the MBC was determined as the lowest concentration of the extract that reduced 99.9% or more of the initial inoculum (Cosentino *et al.*, 1999; Oonmetta-aree *et*

al., 2006).

Phytochemical analysis

The qualitative test for the identification of phytochemical compounds such as phenols, steroids, triterpenoids, tannins and flavonoids were carried out according to standard procedures (Harborne, 2006).

Thin layer chromatography (TLC)

Highly active extract of *P. minus* were fractionated using TLC technique. Twenty µL of 200 mg/mL extract was loaded on TLC plate silica gel GF254 (Mecrk, Darmstadt, Germany). Toluene:ethyl acetate (93:7) was used as mobile phase. Separated fractions were visualised under ultraviolet light at 254 and 366 nm.

TLC-Bioautography

Agar overlay assay (Rossi *et al.*, 2011; Kannan *et al.*, 2013 with some modifications) was used to detect the antibacterial active fractions of *P. minus* extract. Cultures were adjusted to the standard of McFarland No. 0.5 to achieve a concentration of approximately 10^8 CFU/mL and diluted to obtain final concentration of the cell number of approximately 10^6 CFU/mL. One hundred µL of the bacterial suspension was then transferred into 10 mL of sterile MHA. MHA plates were prepared freshly and chromatogram plates which had been sterilized with UV light for 1 h was placed on the surface of the agar (the fractions facing upside). Thereafter, the above setup was over-layed with MHA which was inoculated with the culture. After solidified, plates were incubated at 37°C for 24 h. After incubation, plates were sprayed with 2,3,5 triphenil tetrazolium chloride (TTC) and incubated at 37°C for 2 h. Clear zones indicated the presence of active fractions of the extract that inhibited the growth of test bacteria.

Cellular leakage measurements

Cultures were transferred to 100 mL of fresh sterile TSB and incubated at 37°C for 18-24 h. After incubation, cultures were centrifuged at 10000 rpm for 10 min, resuspended in sterile sodium chloride solution (0.85 g/100 mL). Suspensions were then adjusted to achieve a concentration of approximately 10^{10} CFU/mL. The extract at concentration of 4x MIC was put into each test tube containing 4 mL of the above bacterial suspensions. These suspensions were incubated at 37°C for 0, 30, 60, 90, 120, 150 and 180 min. After incubation, cultures were centrifuged at 10000 rpm for 10 min and bacterial pellets were removed. The absorbance of the supernatant was subsequently measured at 260 and 280 nm using

UV - Vis spectrophotometer (Shimadzu UV-1800, Japan) (Oonmetta-aree *et al.*, 2006 with some modifications).

The protein content of the supernatant was determined using Bradford reagent. One mL of Bradford reagent was added to 1 mL supernatant and incubated at room temperature for 5 min. The absorbance was measured at 595 nm using UV - Vis spectrophotometer (Shimadzu UV-1800, Japan). Bovine serum albumin (BSA) was used as protein standard (Henie et al., 2009; Klotz *et al.*, 2010).

Data analysis

Studies were performed in triplicate and analysed by Student's t-test and one way analysis of variance (ANOVA) followed by Duncan's Multiple Range using SPSS software package version 20.0 for windows. The results were expressed as mean ± SD. P values < 0.05 were considered as significant.

Results and Discussion

Yields of extraction

Multilevel extraction was performed in this research since it has been known as an efficient procedure for extraction of limited number of samples and effective to separate the active components of extract based on its polarity (Houghton and Raman, 1998). The extraction process was carried out by order from non-polar condition using hexane to polar condition using ethanol as solvent. The yield of multilevel extraction by UAE technique was significantly higher than maceration technique (Table 1). Hence, the length of the extraction time was also reduced until 98.61%. Evaluation of extraction techniques for leaves of *Mikania glomerata* Spreng. (Celeghini *et al.*, 2001), *Salvia officinalis* L. and *Salvia glutinosa* L. (Velickovic *et al.*, 2007) have also found that the extraction yields of UAE technique were higher than maceration. Furthermore, Aspe and Fernandez (2011) reported that samples which were extracted by UAE techniques resulted in an increase in yield and concentration of total phenol and tannin.

The superior performance of UAE technique over maceration in reducing the length of extraction period and the yields of extraction has been explained by different mechanism. The maceration is based on direct contact between solvent and materials, wherein the solvent will penetrate into matrix material through diffusion process. Treatment of agitation and or heat improves the extraction performance by increasing the diffusion of the solvent (Azmir *et al.*, 2013). Meanwhile, UAE is generally attributed

Table 1. Yields and the length of extraction process for each solvent by multilevel extration using UAE and maceration techniques

Extraction technique	Yields ± SD (%)			Extraction periods for each solvent
	Hexane extract	Ethyl acetate extract	Ethanol extract	
UAE	1.71*±0.08	1.92*±0.02	15.38*±0.41	20 min
Maceration	0.71±0.05	1.41±0.01	5.98±0.02	24 h

*significant (t-test, p < 0.05)

Table 2. Compounds of *P. minus* extracts by phytochemical analysis

Compounds	Hexane extract	Ethyl acetate extract	Ethanol extract
Alkaloids	-	-	+
Flavonoids	+	+	+
Phenols	+	+	+
Steroids	+	+	-
Triterpenoids	+	-	+
Tannins	-	+	+
Saponins	-	-	+

to mechanical, cavitation and thermal effects, which can causes disruption of cell walls, particle size reduction and intensification of mass transfer across cell membranes of the plant tissues (Shirsath *et al.*, 2012; Azmir *et al.*, 2013). Great improvements of the extraction by UAE technique that decrease the length of the extraction and increase the yield resulted in reducing energy use (Khan *et al.*, 2010; Mantegna *et al.*, 2012). Conventional extraction technique like maceration for natural compounds such as polyphenols, however, may damage some of the target molecules due to prolonged extraction time (Mantegna *et al.*, 2012).

Antibacterial activity of P. minus extracts

Phytochemical analysis of the crude extracts demonstrated the presence of different compounds, i.e. phenols, flavonoids, alkaloids, tannins, triterpenoids, saponins, and steroids (Table 2). Ethanolic extract of *P. minus* contains more phytoconstituens than the other extracts. Wibowo *et al.* (2009) reported that methanolic extract of *P. minus* contains phenolics, flavonoids, alkaloids, terpenoids and steroids compounds. Almey *et al.* (2010) and Qader *et al.* (2011) found that *P. minus* leaves contains high phenolic compounds. Moreover, the ethanolic extract of *P. minus* contains phenolic compounds such as gallic acid, rutin, coumaric acid (Qader *et al.*, 2012). Furthermore myricetin and quercetin was reported as major flavonoid of *P. minus* leaves (Miean and Mohamed, 2001).

During the screening step, three extracts of *P. minus* were evaluated for their antibacterial activity using disc diffusion method against *E. coli* and *S. aureus*. The antibacterial activities of three extracts of *P. minus* at various concentrations against *E. coli* and *S. aureus* are summarized in Figure 1. All extracts of *P. minus* showed inhibition on the growth of *E. coli* and *S. aureus*. The greatest inhibition zones were obtained from the polar ethanol extract against *E. coli* at concentration of 400 mg/mL, but it was not
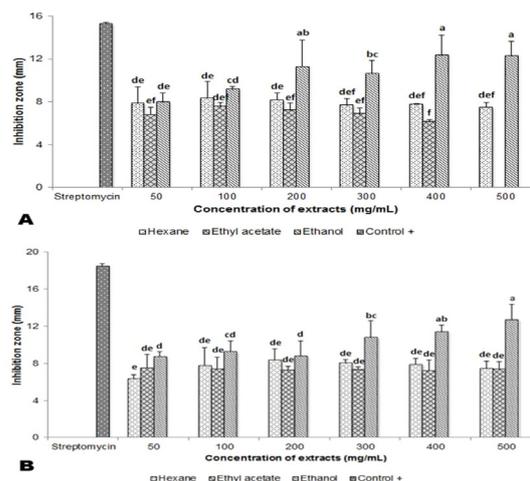

Figure 1. Antibacterial activity of *P. minus* extracts at various concentrations against (A) *E. coli* and (B) *S. aureus*. Vertical lines above each bar indicates the standard deviation and letters on blocks of data shows the comparison between the mean concentration of the extract by Duncan's Multiple Range Test (p < 0.05).

significantly different compared to that of 200 mg/mL. Similarly, the largest inhibition zone against *S. aureus* was observed at concentration of 500 mg/mL, but it was not significantly different compared to that of 400 mg/mL. These results are consistent with the results obtained by related literature, i.e. methanol and ethanol extract of *P. minus* had antibacterial activity against *B. subtilis* (Jamal *et al.*, 2011) and *E. coli* (Wibowo, 2007).

Generally, preliminary screening showed that the ethanolic extract of *P. minus* demonstrated the largest zone of inhibition against *E. coli* and *S. aureus*. At concentration of 200 mg/mL, the ethanolic extract of *P. minus* showed inhibition to *E. coli* with diameter of 11.28 mm. This inhibition zone were relatively higher in comparison to the results of Jamal *et al.* (2011), whereby methanolic and ethanolic extracts of *P. minus* at the same concentration was not inhibit *E. coli*, but inhibit *B. subtilis* with inhibition zone of 9 mm, determined by agar well diffusion method. This antibacterial activity thought to be related with the presence of active compounds such as phenols, since *P. minus* has been reported having high total phenolic content (Huda-Faujan *et al.*, 2007; Maizura *et al.*, 2011; Qader *et al.*, 2011).

Ethanolic extract of *P. minus* was selected to be used for determination of the MIC and MBC by using the macro-dilution technique. The MIC of the ethanolic extract of *P. minus* against *E. coli* was found at a concentration of 25 mg/mL and against *S. aureus* at a concentration of 30 mg/mL. These concentrations were higher in comparison to the finding of Shan *et al.* (2008). In that study they found that the MIC of methanolic extract of *Polygonum cuspidatum* roots
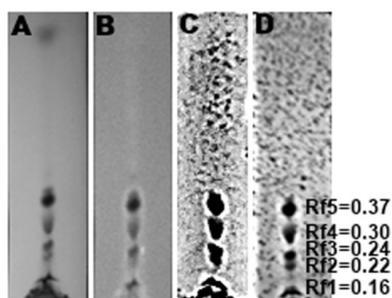
Figure 2. Chromatogram ethanolic extract of *P. minus* visualized by UV light at (A) 254 nm and (B) 366 nm; and bioautogram against (C) *E. coli* and (D) *S. aureus*

against *E. coli* was at a concentration of >2.5 mg/ mL and *S. aureus* at a concentration of 312.5 µg/ mL. The MBC of the ethanolic extract of *P. minus* against *E. coli* was found at a concentration of 100 mg/mL and against *S. aureus* at a concentration of 50 mg/mL. The high MIC value of the crude extract found in this study may not be practicable as food preservative. However, these results indicated that the crude ethanolic extract contains active compounds which were potential to be explored further. Active compounds of crude extract can be separated by chromatography and subsequently isolated, purified and identified.

Fraction of ethanolic extract of P. minus by TLC-bioautography

The ethanolic extract of *P. minus* was separated into six fractions by TLC technique that were visible under UV at 254 nm and into five six fractions when it was observed visible under UV at 366 nm (Figure 2). After the TLC chromatogram was covered with a thin agar layer that contains the test bacteria, the inhibition zone (clear zone) can be noticeable observed by spraying tetrazolium salts (TTC) on the bioautogram after incubation (Figure 2). In this case, the TTC was converted by the dehydrogenases of living microorganisms to intensely coloured, formazan (Choma, 2005). The presence of clear zone around the spot indicated existing antibacterial activity of the active fractions. The fractions that exhibited the clear zones were corresponding to $Rf_1 = 0.16$; $Rf_2 = 0.22$; $Rf_3 = 0.24$; $Rf_4 = 0.30$ and $Rf_5 = 0.37$. The fraction with $Rf_4 = 0.30$ and $Rf_5 = 0.37$ were found as the most noticeable fractions that exhibited antibacterial activity against *S. aureus* as well as *E. coli*. *S. aureus* were less sensitive to the bioactive fractions of the ethanolic extract of *P. minus* leaves, whereas *E. coli* was more sensitive. These results were in contrast with the results reported by Kannan *et al.* (2012) that found *S. aureus* were more sensitive towards active fractions of aqueous methanolic extract of *H. pinifolia* by TLC-bioautography method.
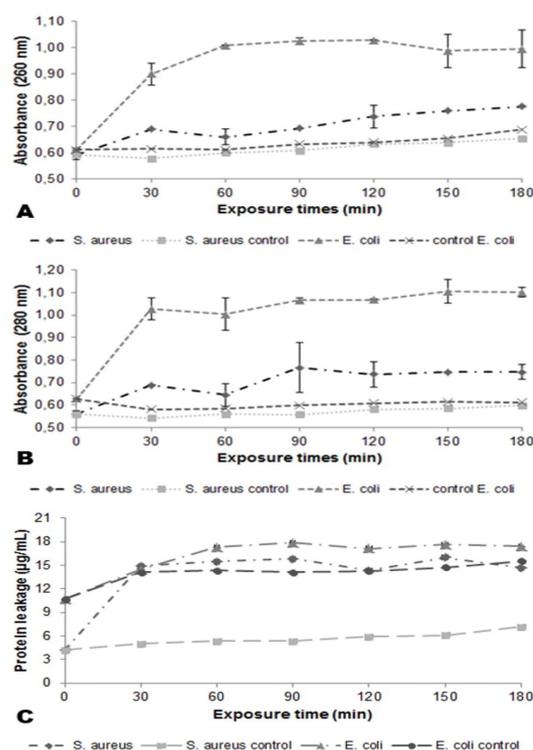

Figure 3. Absorbance of supernatant at (A) 260 nm, (B) 280 nm and (C) protein content of supernatant from *E. coli* and *S. aureu*s suspensions without (control) and after exposure to the ethanolic extract of *P. minus* at concentration of 4x MIC, measured at different exposure times.

Cellular leakage

An important characteristic of plant extracts is their hydrophobicity which enables them to attack the lipids of the bacterial cell membrane, disturbing the structures and inducing more permeability (Burt, 2004; Oonmetta-aree *et al.*, 2006; Joshi *et al.*, 2011). Leakage of the cytoplasmic membrane has been analysed by determination of the absorbance of suspension containing cell materials including proteins, nucleotides, metabolites and ions that were absorbed at 260 nm (Liu *et al.*, 2004; Oonmetta-aree *et al.*, 2006; Xing *et al.*, 2009) and 280 nm (Henie *et al.*, 2009). The absorbance at 260 nm of supernatant of *E. coli* and *S. aureus* suspensions increased obviously after 30 min exposure to the ethanolic extract of the *P. minus* leaves (Figure 3). These results indicated that cell materials were released outside the cells. Similarly, the release of cell materials into the supernatant, indicated by the increasing absorbance at 280 nm, from *E. coli* and *S. aureus* suspensions was also observed after 30 min exposure (Figure 3). Hence, the absorbance values of *E. coli* suspensions were higher than that of *S. aureus* suspensions at the same exposure time, for all treatments. These results indicated that treatment with ethanolic extract of *P. minus* resulted in disturbing

of membrane permeability which induced leakage of the cytoplasmic membrane. It occurred due to severe and irreversible damage of cytoplasmic membrane (Carson *et al.*, 2002). The cytoplasmic membrane may become damaged and functionally disabled when bacterial suspensions are exposed to antibacterial agents. Various vital intracellular materials including small ions such as K^+ and PO_4^{3-} tend to leach out, followed by leaching of large molecules such as DNA, RNA and other materials (Shan *et al.*, 2008; Xing *et al.*, 2009). Considering that the crude extracts of *P. minus* contain some potential active compounds, the action of extract as antibacterial agent possibly affect various targets upon the bacterial cell. Phenolic compounds as the major compounds of the extract have been reported can disturb the cytoplasmic membrane, disrupt the proton motive force, electron flow and active transport (Burt, 2004).

Furthermore, the protein content of supernatant of *E. coli* and *S. aureus* after exposure to the ethanolic extract of *P. minus* increased gradually over time by Bradford test (Figure 3). Similar results were obtained by Klotz *et al.* (2010) that demonstrating disruption of membrane integrity of *E. coli* resulted in leakage of protein with a low molecular weight before losing their cell viability.

Conclusion

This study highlighted that UAE provided higher extraction yields and shortened the extraction time, in comparison to the conventional maceration technique. The TLC-bioautography method was satisfactory to be used for screening natural antimicrobial compounds. Further procedures, however, are needed to identify the potential compounds detected. The ethanolic extract of *P. minus* leaves were potential as source of antibacterial agent. When the bacterial cells were exposed to this extract the release of cells materials were observed as a result of disruption of cytoplasmic membrane integrity for both *E. coli* and *S. aureus*.

Acknowledgements

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