PJHRD Philippine Journal of Health Research and Development

RESEARCH ARTICLE

Partial purification and characterization of antimicrobial peptide(s) from *Mimosa pudica* (Mimosaceae)

Johnalyn C. Go [1]*, Marilou G. Nicolas [2], Gracia Fe B. Yu[1]

*Corresponding author's email address: jcgo4@up.edu.ph

[1]*Department of Biochemistry and Molecular Biology, College of Medicine, University of the Philippines Manila, Philippines*
[2]*Department of Physical Sciences and Mathematics, College of Arts and Sciences, University of the Philippines Manila, Philippines*

ABSTRACT

Plants have been a major source of natural products for sustaining human health. The use of the different parts of the plant as infusions, decoctions, extracts, and powders are being employed in the treatment of different diseases in humans, plants, and animals. One property of great significance in terms of therapeutic treatments, especially with the emergence of multi-drug resistant microbes, is the antimicrobial activity. A new promising source of antimicrobials that demonstrate novel mechanisms of therapeutic strategies is low molecular weight peptides. In this study, the antimicrobial activities of *Mimosa pudica* crude and partially purified peptide extracts against Gram-negative *Enterobacter cloacae* ATCC 23355 and *Enterobacter aerogenes* ATCC 13048, and Gram-positive *Staphylococcus epidermidis* ATCC 12228 using resazurin colorimetric assay and tricine SDS-PAGE bioautography were reported. *M. pudica* crude and partially purified extracts exhibited antimicrobial activity against all the bacteria tested. Specifically, the peptide that was partially purified from *M. pudica* with a molecular weight of 5.14 kDa inhibited the growth of *Enterobacter cloacae*.

Keywords: *plant antimicrobial peptides, Mimosa pudica, resazurin colorimetric assay, tricine SDS-PAGE bioautography*

Introduction

Bacterial diseases are the main cause of morbidity and mortality among human beings both in developing and developed countries [1]. Healthcare professionals are becoming helpless in effectively treating bacterial infections due to improper use of antimicrobials that has resulted in the development of multi-drug resistant bacterial strains. The antimicrobial effects of current antibiotics are due to the compounds' ability to inhibit functions of bacterial cell components that are required for living such as cell wall synthesis, nucleic acid synthesis, protein synthesis, or a metabolic pathway [2]. The specificity of the antibiotic to its target molecule is the key that permits the bacteria to easily develop resistance. The most common mode of acquiring resistance is through mutation of the gene that encodes the target protein/enzyme. This causes a modification in the structure and conformation of the target molecule which greatly decreases its affinity to the antibiotic compound, hence, resisting the antimicrobial effect of the compound [3]. Acquisition of antimicrobial genes through the misuse of antimicrobials is another mode of antimicrobial resistance [4]. Therefore, there is an urgent need for new therapeutic agents with novel mechanism of actions for the treatment of infectious diseases caused by multi-drug resistant bacteria.

Recently, considerable interest by researchers in antimicrobial peptides as potential alternatives to conventional antibiotics has greatly increased due to their higher potency, higher activity, higher specificity, low toxicity, inability to bioaccumulate, few drug-drug interaction challenges, and biological and chemical diversity [5]. Antimicrobial peptides (AMPs) are amphipathic, cationic molecules rich in nitrogen-containing amino acids such as arginine, histidine, and lysine, and typically short with 5-100 amino acid residues. Some of these peptides also possess antibacterial properties that act differently than modern conventional antibiotics [6]. The positivity of the charge of these peptides plays an important role in the mechanism of disrupting the anionic lipid membrane of the pathogen, such

as lipopolysaccharides of gram-negative bacteria or lipoteichoic acids of gram-positive bacteria, and the presence of both hydrophilic and hydrophobic ends allow penetration through it [7]. Formation of transient pores that enables the efflux of essential ions and nutrients from the cell; targeting essential intracellular processes, such as nucleic acid synthesis, protein synthesis, cell wall synthesis, and protein folding; and detergent-like disruption of the cell membrane are some of the general modes of actions that can lead to bacterial cell death that can possibly reduce the risk of antimicrobial resistance development [7,8].

Plants respond to numerous environmental stresses such as pests and microbial pathogenic predation by evolutionarily developing several survival mechanisms to defend themselves such as the presence of physical barriers that limit the spread of pathogen and production of antimicrobial compounds including secondary metabolites and peptides [9]. *Mimosa pudica* (Mimosaceae), known as a sensitive plant and locally known as *makahiya* in the Philippines, is commonly found in open spaces and is propagated via seeds and vegetative methods [10]. The plant is believed to have come from South and Central America and is regarded as an invasive species in Tanzania, South Asia, Southeast Asia, and may Pacific Islands [11]. *Mimosa pudica* is one of the commonly used herbal medicinal plants [12-16] according to the List of Philippine Medicinal Herbal Plants [10]. This plant has been known to have various bioactivities [17-25] and may be a potential source of antimicrobial peptides. The aqueous and methanolic extracts of the different parts of the plant were shown to have antimicrobial activity against various bacteria such as *S. aureus* and *B. subtilis* [26,27]. However, there are still no studies conducted on antimicrobial peptides extracted from this plant.

Methodology

Sample Collection and Preparation

The leaves of *Mimosa pudica* (Mimosaceae) were harvested from Sibao, Calabanga, Camarines Sur, a biodiverse area due to its constantly warm and humid equatorial climate throughout the year, during the dry season of year 2014. Samples were washed with sterilized distilled water upon collection and frozen overnight at -20°C prior to transportation in an ice-salt mixture. The samples were freeze-dried using Labconco Freezone® lyophilizer to deactivate proteases [28]. The freeze-dried samples were pulverized using liquid nitrogen and stored at -87°C until extraction proper. Peptide extraction, partial purification, and characterization were done at the Research Laboratory of the Department of Physical Sciences and Mathematics, College of Arts and Sciences, University of the Philippines Manila.

Peptide Extraction and Partial Purification

Extraction of crude and partially purified peptides was done according to Claeson *et al.* [28] with some modifications.

Dichloromethane-Ethanol Extraction

Pulverized plant leaves (20g) were sonicated for one hour in 300 mL dichloromethane (AR), then shaken for one hour each in the same amount of dichloromethane thrice. The plant residue was dried and then extracted exhaustively with 50% ethanol (EtOH, analytical grade). The 50% EtOH extract was rotary evaporated. An aliquot was lyophilized and stored at -87°C as a crude extract for further assays. The remaining supernatants were acidified by adding acetic acid (HOAc, analytical grade) to a final concentration of 2%.

Filtration through Polyamide Column Chromatography

Filtration through polyamide was done for the removal of tannins. Briefly, 15 g of pre-swollen polyamide 6S gel was rinsed with 50% EtOH / 2% HOAc and then with 2% HOAc. Then, the acidified concentrated ethanol extract was applied and eluted by gravity flow using 2% HOAc and 50% EtOH / 2% HOAc. The combined eluates were rotary evaporated and then lyophilized for the next purification step.

Size Exclusion Chromatography

Preswollen 50 g sephadex G-10 (molecular biology grade) in 50% EtOH / 2% HOAc / 0.2M sodium chloride (NaCl, analytical grade) mobile phase were packed in a glass column to give an effective size of 3 x 23 cm, which was subsequently washed with 1 M pyridine (analytical grade) in 50% EtOH and then equilibrated with several column volumes of the mobile phase. Blue Dextran 2000 (molecular biology grade) was used to determine the column void volume. Polyamide-filtered peptide extracts were dissolved in the mobile phase (1:10), centrifuged, and applied to the column. The high molecular weight fractions were collected and lyophilized for the next purification step.

Solid-phase Extraction

The high molecular weight fractions were dissolved in 50 mM ammonium bicarbonate (NH4HCO3, analytical grade)

buffer (1:40). An aliquot of 5 mL was applied to a 500 mg reversed-phase C18 (RP18) SPE column (Merck, Inc.) previously conditioned in 95% EtOH and preequilibrated in the same buffer. The column was washed with the buffer and the eluate discarded. Then, the column was eluted sequentially with 20%, 50%, and 80% EtOH in 50mM NH4HCO3 buffer. The combined eluates were rotary evaporated, lyophilized, and stored at -87°C as partially purified peptide extract. Crude extracts were dissolved in 1 mL of 1:1 dimethyl sulfoxide (DMSO, molecular biology grade) and water (H20, HPLC grade) while the partially purified peptide extracts with 0.4 mL of DMSO:H20 (1:1).

Peptide Quantification

Peptide concentrations were determined using Sigma-Aldrich® Bicinchoninic Acid Assay kit. Briefly, 0 to 1 mg/mL bovine serum albumin standards were prepared. BCA Working Reagent was mixed with standards and samples in 8:1 ratio and then incubated at 37°C for 30 min. Absorbance readings were measured at λmax using Shimadzu UV-1800®. Peptide concentration of the samples was determined by comparison of the absorbance to a standard calibration curve.

Peptide Characterization

Tricine SDS-PAGE

The molecular weight of the peptide extracts was measured according to the method of Schagger [29] with some modifications. Electrophoretic peptide profiling of the samples was done using Biorad Mini-Protean® 16% resolving polyacrylamide gel. Kaleidoscope Polypeptide Standards® (3.578 - 31.668 kDa) was used as the standard marker. Fifty micrograms of crude and partially purified extracts were applied at a constant volume of 10 µL. The voltage was set to 30V for sample migration in the stacking gel and subsequently increased to 100V after the samples have completely entered the resolving gel. Visualization of the bands was done using Calbiochem® Silver Staining kit. Density analysis of the peptide bands was done using Quantity One®.

Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)

The purity of the partially purified peptide extracts was assessed according to the method of Aguilar [30] with some modifications. Briefly, 10 µL pre-filtered peptide extracts (0.15 mg/mL) dissolved in water (HPLC grade) with 0.1% TFA (HPLC grade) were subjected to HPLC Thermo Scientific Accela® using Hypersil Gold™ RP18 column with a photodiode array detector and eluted by gradient elution using acetonitrile (can, HPLC grade) with 0.1% TFA and water with 0.1% TFA, at a flow rate of 0.5 mL / min for 60 min. Fractions were monitored at 215, 254, and 280 nanometers [31,32].

Antimicrobial Activity Assay

Resazurin Colorimetric Assay

Antimicrobial activity of the crude and partially purified extracts was measured using resazurin colorimetric assay [4,33-35] with some modifications. Cultures of bacterial test organisms were bought from the Department of Medical Microbiology, College of Public Health, UP Manila. Briefly, following overnight incubation of Mueller Hinton broth (MHB) cultures of Gram-negative *Enterobacter cloacae* ATCC 23355 and *Enterobacter aerogenes* ATCC 13048, and Gram-positive *Staphylococcus epidermidis* ATCC 12228 at 37°C, turbidity was adjusted according to 0.5 McFarland standard solution using sterile MHB. An absorbance range of 0.08 - 0.10, measured at 625 nm to represent an approximate bacterial cell count of 1.5 x 108 CFUs / mL was used. Two hundred microliter aliquots of extracts (0 - 200 µg of crude extracts and 50 µg of partially purified extracts) and controls were added to 800 µL of adjusted broth cultures. Samples were incubated for 6 hours at 37°C and 100 µL of 0.01% (w/v) resazurin was added. Absorbance at lambda max was then measured. Positive controls (200 µg) for gram-positive and gram-negative bacteria used were chloramphenicol and tetracycline, respectively. The antimicrobial activity exhibited by the samples was calculated using the formula: % Relative Inhibition = [(A − Ab)/As] × 100, where A = absorbance of sample; Ab = absorbance of blank; and As = absorbance of standard.

Tricine SDS-PAGE Bioautography

The antimicrobial activity of the partially purified peptide extracts was done according to the method of Tunney *et al.*, Valgas *et al.*, Cytrynska *et al.*, and Alhiamadi *et al.* [36-39] with some modifications. As prepared in the antimicrobial colorimetric assay, MHB cultures of Gram-negative *Enterobacter cloacae* and *Enterobacter aerogenes*, and Gram-positive *Staphylococcus epidermidis* were used. To localize the in situ antimicrobial activity, crude, and partially purified peptide extracts (50 µg) run through tricine SDS-PAGE gels were washed for 1 hour in each step, under agitation with: (1) fixing solution (20% EtOH/10% HOAc/sterile distilled H2O), (2)

sterile distilled H2O, (3) 1% Triton X-100 (analytical grade) in sterile distilled H2O, (4) twice with sterile distilled H2O, and (5) sterile MHB. Gels were placed on a sterile petri dish and overlaid with semisolid 0.7% MHA medium containing the adjusted bacterial broth and 0.5 mg/mL XTT - 50µM menadione as the staining reagent. Zones of bacterial growth inhibition were observed after incubation for 6, 12, and 24 hours at 37°C. Gels were analyzed using Fiji Image J® and Quantity One®.

Statistical Analysis

Results were presented as mean ± standard deviation, with n=3. Statistical analysis was determined using one-way ANOVA (analysis of variance), Tukey-Kramer's multiple comparison test [40], and Dunnett's test. The level of significant difference between mean values was set at p-value < 0.01. The post hoc analysis was done using the online Statistica calculator [41] employing the algorithm of Gleason [42].

Results and Discussion

Peptide Extraction and Partial Purification

Crude extraction involves the dichloromethane-ethanol exhaustive extraction of the pulverized lyophilized plant leaves samples. The fresh plant material was lyophilized to preserve it and prevent the enzymatic degradation of the peptides. Then, the lyophilized plant material was pulverized and extracted exhaustively with dichloromethane to remove the lipophilic compounds (e.g., chlorophyll, lipids, terpenoids, etc.). Subsequently, 50% ethanol was used to exhaustively extract the peptides since it was found that the solubility of peptides is better in this solvent than in pure water or ethanol; it also preserves the extract by inhibiting microbial growth and it does not extract polysaccharides and enzymes. [28]

For the partial purification of the crude peptide extracts, three additional chromatography steps were employed. The acidified concentrated ethanolic extract was filtered through polyamide column to remove the tannins which can interfere with subsequent bioassays. The binding of tannins to polyamide is highly pH-dependent, thus the pH of the crude peptide extracts was lowered. The polyamide-filtered extracts were then passed through Sephadex G-10 to collect the high molecular weight fractions (i.e. peptides with a molecular weight above 700 Da), and compounds with molecular weights lower than 700 Da (e.g. glycosides, alkaloids, etc.) were retarded on the column. EtOH (50%) / HOAc (2%) / NaCl (0.2M) was used as the mobile phase since nonspecific interactions between the gel and plant pigment were suppressed [28]. The resulting peptide fractions were then passed through RP18 solid-phase extraction column to remove the salts and polysaccharides and yield the partially purified peptides. The crude and partially purified peptides were stored at -87°C in lyophilized form to minimize peptide degradation by preventing bacterial degradation, oxidation, and formation of secondary structures. Prior to analysis, lyophilized extracts were dissolved in DMSO:H20 (1:1) and stored at -87°C in aliquots to limit the freeze-thaw cycle.

Peptide Quantification

The peptide concentration of the crude and partially purified extracts was determined by comparing the absorbance of the samples with the standards having a concentration of 0.0 mg/mL to 1.0 mg/mL, using the pre-determined lambda max of 558nm.

The result of the BCA assay showed that *M. pudica* crude extract had the highest peptide concentration. *M. pudica* crude and fraction 2 extracts also exhibited the highest percent peptide yield of 0.55% and 0.10%, respectively.

Peptide Characterization

The crude and partially purified peptide extracts were characterized using Tricine SDS-PAGE and RP-HPLC to determine the molecular weight and purity of the samples, respectively.

Mimosa pudica

Two low molecular weight peptide bands (6.32 and 5.14 kDa) were seen in the tricine SDS-PAGE profile of the *Mimosa pudica* fraction 2. This was validated after running

Table 1. *Concentration of Crude Peptide Extracts at 558nm and Corresponding % Yield.*

Samples	Concentration (mg/mL)	Percent Yield (%w/w)
Mp Crude	123.4850 ± 1.5500	0.55
Mp F1	0.000 ± 0.110	0.00
Mp F2	56.149 ± 3.398	0.10
Mp F3	13.063 ± 0.507	0.02

Mp = *Mimosa pudica*; F = Fraction; Values are expressed in mean ± SD, n = 3.

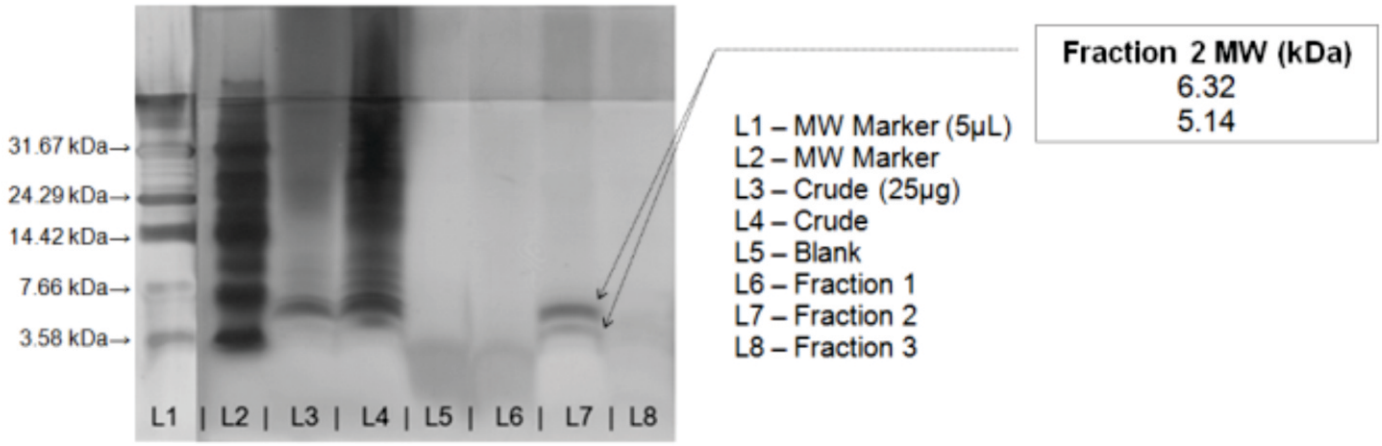

Figure 1. *Tricine SDS-PAGE profile of M. pudica crude and partially purified peptide extracts. Samples were applied at a constant volume of 10 µL. L3 contains 25 µg of crude extract which showed fewer bands when compared with L4 containing 50 µg of crude extract. Upon partial purification of the crude extract, fraction 1 (L6) and fraction 3 (L8) did not show any peptide bands, while fraction 2 (L7) showed 2 peptide bands with a molecular weight of 6.32 and 5.14 kDa. DMSO:H2O (1:1) and Kaleidoscope Polypeptide Standard® were used as solvent blank and MW marker, respectively.*

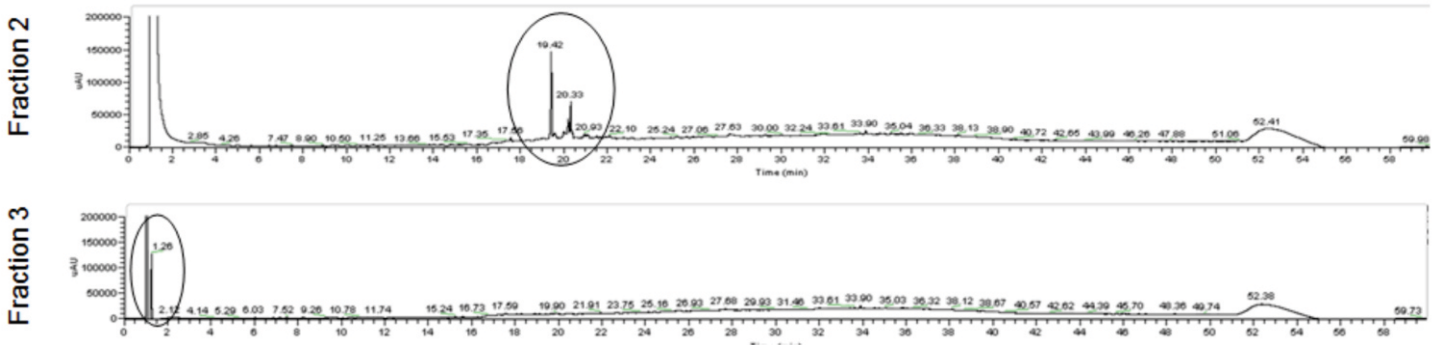

Figure 2. *RP-HPLC profile of M. pudica fraction 2 and 3 extracts monitored at 215, 254, and 280 nm. Fraction 2 showed two peaks with retention factors of 19.42 and 20.33 min., while fraction 3 showed a peak at 1.26min.*

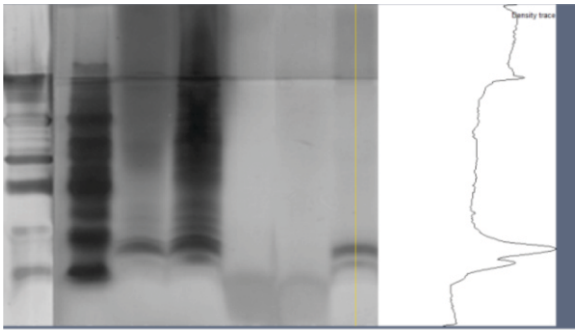

Figure 3. *Density analysis of M. pudica fraction 2 tricine SDS-PAGE profile. The peptide band with a molecular weight of 6.32 kDa gave higher band intensity than the 5.14 kDa peptide band.*

the partially purified peptide extract fraction 2 in RP-HPLC which showed two peaks with retention factors of 19.42 and 20.33 min. Upon intensity correlation of the tricine SDS-PAGE peptide bands (figure 3) and RP-HPLC peaks (figure 2) of fraction 2, it appears that the peaks at 19.42 min. and 20.33 min. correspond to peptide bands 6.32 kDa and 5.14 kDa, respectively. Fraction 3 showed an RP-HPLC peak at 1.26 min accounting for its peptide concentration of 13.063 mg/mL; this was not resolved by tricine SDS-PAGE indicating a very low molecular weight hydrophilic peptide.

Antimicrobial Activity

The crude and partially purified peptide extracts were tested in vitro for antimicrobial activity against different bacterial strains using colorimetric assay and tricine SDS-PAGE bioautography.

Enterobacter cloacae

It has been shown that upon partial purification of the crude peptide extracts, the percent relative inhibition of the *Enterobacter cloacae* growth increased. For the partially purified peptide extracts, fraction 3 followed by fraction 2 exhibited the highest percent relative inhibitions. Statistical analysis using Tukey-Kramer multiple comparison and Dunnett tests evaluated the level of significance of the antimicrobial activity among the crude and partially purified extracts for each plant with a p-value <0.01 and it was determined that the differences among the antimicrobial activity values obtained wee statistically significant.

Table 3. *Percent Relative Inhibition of Extracts against E. cloacae.*

Samples	% Relative Inhibition
Negative Control	0.00
Tetracycline 200 µg	94.90
Mp 50 µg	2.82
Mp 100 µg	4.10
Mp 150 µg	7.03
Mp 200 µg	10.52
Mp F1	0.00
Mp F2	7.94
Mp F3	9.80

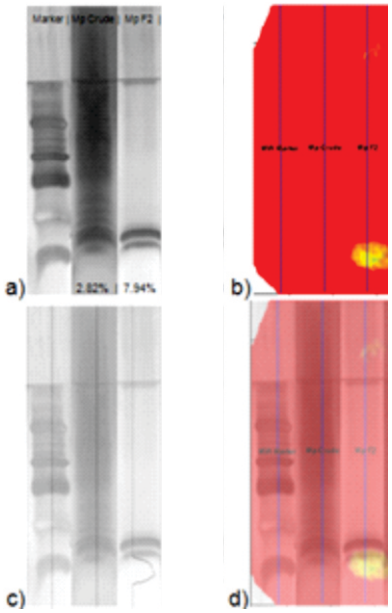

Figure 4. *Tricine SDS-PAGE bioautography against Enterobacter cloacae. a) tricine SDS-PAGE profile of 50µg crude and partially purified peptide fraction 2 extracts with indicated percent relative inhibition obtained in colorimetric assay; b) surface plot spectrum of tricine SDS-PAGE bioautography; c) tricine SDS-PAGE profile of 50µg crude and partially purified peptide fraction 2 extracts superimposed with density analysis of tricine SDS-PAGE bioautography; d) surface plot spectrum of tricine SDS-PAGE bioautography superimposed with tricine SDS-PAGE profile of crude and partially purified peptide extracts.*

Even though *Mimosa pudica* fraction 3 exhibited the highest percent relative inhibition in the antimicrobial colorimetric assay, fraction 2 was used for the tricine SDS-PAGE bioautography instead since peptide bands were present in its tricine SDS-PAGE profile. Fraction 2 exhibited *Enterobacter cloacae* growth inhibition in the tricine SDS-PAGE bioautography. Hence, it can be deduced that the antimicrobial activity of the extracts is due to the antimicrobial peptides – the fraction 2 of *Mimosa pudica* (7.94% relative inhibition) showing E. cloacae growth inhibition at 5.14 kDa peptide band. The loss of antimicrobial activity of *Mimosa pudica* crude extract (50µg) in the tricine SDS-PAGE bioautography could be due to (1) lower antimicrobial peptide band concentration upon resolving of the peptides in the tricine SDS-PAGE; (2) structure-activity relationship – loss of antimicrobial activity due to structure modification upon resolving of the peptides in tricine SDS-PAGE; and (3) synergistic or additive effect of the different compounds present in the crude extract.

Enterobacter aerogenes

It has been shown that upon partial purification of the crude extracts, the percent relative inhibition of the *Enterobacter aerogenes* growth increased. Hence, this means that the antimicrobial activity is due to the peptides that were partially purified and not to the other compounds that were present in the crude extract. *Mimosa pudica* partially purified peptide fraction 2 extract exhibited the highest percent (10.58%) relative inhibition. Statistical analysis using Tukey-Kramer multiple comparison and Dunnett tests evaluated the level of significance of the antimicrobial activity among the crude and partially purified extracts for each plant with a p-value < 0.01 and it was determined that the differences among the antimicrobial activity values obtained were statistically significant.

Mimosa pudica crude (50µg) and partially purified peptide fraction 2 extracts exhibited *Enterobacter aerogenes*

Table 4. *Percent Relative Inhibition of Extracts against Enterobacter aerogenes.*

Samples	% Relative Inhibition
Negative Control	0.00
Tetracycline 200 µg	58.38
Mp 50 µg	2.94
Mp 100 µg	5.27
Mp 150 µg	5.76
Mp 200 µg	8.18
Mp F1	0.00
Mp F2	10.58
Mp F3	0.00

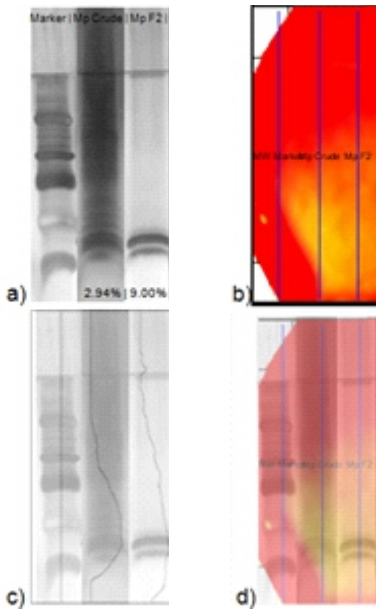

Figure 5. *Tricine SDS-PAGE bioautography against Enterobacter aerogenes. a) tricine SDS-PAGE profile of crude (50µg) and partially purified peptide fraction 2 extracts with indicated percent relative inhibition obtained in colorimetric assay; b) surface plot spectrum of tricine SDS-PAGE bioautography; c) tricine SDS-PAGE profile of crude (50µg) and partially purified peptide fraction 2 extracts superimposed with density analysis of tricine SDS-PAGE bioautography; d) surface plot spectrum of tricine SDS-PAGE bioautography superimposed with tricine SDS-PAGE profile of crude and partially purified peptide extracts.*

growth inhibition in the tricine SDS-PAGE bioautography in accordance with the results obtained in colorimetric assay. Hence, it can be deduced that the antimicrobial activity of the aforementioned extracts is due to the antimicrobial peptides.

Staphylococcus epidermidis

It has been shown that upon partial purification of the crude extracts, the percent relative inhibition of the *Staphylococcus epidermidis* growth increased. Among the partially purified extracts, *Mimosa pudica* fraction 3 exhibited the highest percent relative inhibition. Statistical analysis using Tukey-Kramer multiple comparison and Dunnett tests evaluated the level of significance of the antimicrobial activity among the crude and partially purified extracts for each plant with a p-value < 0.01 and it was determined that the differences among the antimicrobial activity values obtained were statistically significant.

Mimosa pudica crude and partially purified peptide fraction 2 extracts exhibited *Staphylococcus epidermidis* growth inhibition in the tricine SDS-PAGE bioautography, as supported by the colorimetric assay result.

Table 5. *Percent Relative Inhibition of Extracts against Staphylococcus epidermidis.*

Samples	% Relative Inhibition
Negative Control	0.00
Tetracycline 200 µg	63.52
Mp 50 µg	2.59
Mp 100 µg	3.39
Mp 150 µg	6.30
Mp 200 µg	8.25
Mp F1	0.00
Mp F2	8.59
Mp F3	15.56

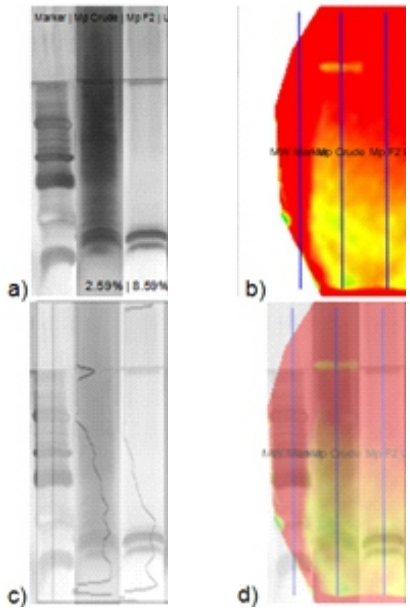

Figure 6. *Tricine SDS-PAGE bioautography against Staphylococcus epidermidis. a) tricine SDS-PAGE profile of crude (50µg) and partially purified peptide fraction 2 extracts with indicated percent relative inhibition obtained in colorimetric assay; b) surface plot spectrum of tricine SDS-PAGE bioautography; c) tricine SDS-PAGE profile of (50µg) and partially purified peptide fraction 2 extracts superimposed with density analysis of tricine SDS-PAGE bioautography; d) surface plot spectrum of tricine SDS-PAGE bioautography superimposed with tricine SDS-PAGE profile of crude and partially purified peptide extracts.*

Conclusions

This is the first study that reported the antimicrobial activities of *Mimosa pudica* crude and partially purified peptide extracts against Gram-negative *Enterobacter cloacae* ATCC 23355 and Enterobacter aerogenes ATCC 13048, and Gram-positive *Staphylococcus epidermidis* ATCC 12228 using colorimetric and tricine SDS-PAGE bioautography assays. *M. pudica* crude and partially purified extracts exhibited potential antimicrobial activity against all the bacteria tested. Specifically, the peptide that was partially purified from M.

pudica with a molecular weight of 5.14 kDa inhibited the growth of E. cloacae. Antimicrobial colorimetric assay of the crude and partially purified peptide extracts is best supplemented with tricine SDS-PAGE bioautography, albeit the number of samples that can be loaded in it is limited since the phenomenon of synergistic effects and peptide structure-activity relationship has been observed in the antimicrobial activity of the different plant crude extracts and its partially purified fractions. With the increasing demand for antimicrobial compounds, these peptides might serve as templates for novel antimicrobial agents. Thus, sequencing and structure elucidation of the peptides that were partially purified are recommended for future studies. Furthermore, studies such as hemolytic activity against human erythrocytes and toxicity assays on mammalian and plant cells, together with resistance to heat treatment, pH, high salt concentrations, and putative resistance to proteases must be conducted to make the peptides extracted pharmacologically valuable.

Acknowledgments

The authors would like to thank Prof. Marcelina B. Lirazan, Prof. Francisco M. Heralde, and Dr. Jose B. Nevado for giving us guidance in preparing the manuscript.

Funding

This study was part of a research project funded by the Philippine Council for Health Research and Development entitled “Herbal Extracts from Mount Isarog for Bioactivity and ADMETox Assays”.

Conflicts of Interest

The authors would like to confirm that there are no known conflicts of interest associated with this publication.

References

1. Christensen KLY, Holman RC, Steiner CA, Sejvar JJ, Stoll BJ, Schonberger LB. (2009) Infectious disease hospitalizations in the United States. Clin Infect Dis. 49(7): 1025-1035.
2. Maroti G, Kereszt A, Kondorosi E, Mergaert P. (2011) Natural Roles of Antimicrobial Peptides in Microbes, Plants, and Animals. Research in Microbiology.162 (4): 363-374.
3. Sosa A de J, Byarugaba DK, Amabile C, Hsueh P-R, Kariuki S, Okeke IN. (2010) Antimicrobial Resistance in Developing Countries. Springer. XXIII, 554. Retrieved from http://www.springer.com/978-0-387-89369-3.
4. Chuah EL, Zakaria ZA, Suhaili Z, Abu Bakar S, Desa MNM. (2014) Antimicrobial Activities of Plant Extracts against Methicillin-Susceptible and Methicillin-Resistant Staphylococcus aureus. Journal of Microbiology Research. 4(1): 6-13.
5. Rehman S and Khanum A. (2011) Isolation and characterization of peptide(s) from Pisum sativum having antimicrobial activity against various bacteria. Pak J Bot. 43(6):2971-2978.
6. Monroc S, *et al.* (2006) De novo Designed Cyclic Cationic Peptides as Inhibitors of Plant Pathogenic Bacteria. Peptides. 27: 2567-2574.
7. Jenssen H, Hamill P, Hancock REW. (2006) Peptide Antimicrobial Agents. Clinical Microbiology Reviews. 19 (3): 491-511.
8. Peters BM, Shirtliff ME, Jabra-Rizk MA. (2010) Antimicrobial Peptides: Primeval Molecules or Future Drug? Plos Pathogens. 6(10).
9. Tavares LS, Santos MdO, Viccini LF, Moreira J, Miller R, Franco O. (2008) Biotechnological Potential of Antimicrobial Peptides from Flowers. Peptides. 29:1842-1851.
10. Ahmad H, Sehgal S, Mishra A, Gupta R. (2012) *Mimosa pudica L*. (Laajvanti): An overview. Pharmacogn Rev. 6 (12): 115-124.
11. Waterhouse DF. (1993) The Major Arthropod Pests and Weeds of Agriculture in Southeast Asia. ACIAR Monograph No. 21. Canberra, Australia: Australian Centre for International Agricultural Research. 141.
12. Behera SK, Panda A, Behera SK, Misra MK. (2006) Medicinal Plants Used by the Kandhas of Kandhamal District of Orissa. Indian J Traditi Knowl. 5: 519–28.
13. Chatterjee A and Pakrashi SC. (2006) The Treatise on Indian Medicinal Plants. New Delhi: National Institute of Science Commission and Information Resources. pp. 65–6.
14. Joseph B, George J, Mohan J. (2013) Pharmacology and Traditional Uses of *Mimosa pudica*. Int'l Journal of Pharmaceutical Sciences and Drug Research. 5(2): 41-44.
15. Khare CP. (2004) Encyclopedia of Indian Medicinal Plants. Germany: Springer. pp. 313–4.
16. Prajapati ND, Purohit SS, Sharma AK, Kumar T. (2003) A Complete Source Book. Jodhpur: Agrobios India: A Handbook of Medicinal Plants. p. 271.
17. Aarthi N, Murugan K. (2011) Antimalarial activity and phytochemical screening of ethanolic leaf extract of Phyllanthus niruri and *Mimosa pudica*.

IJPRD 3(3)24: 198 – 205.
18. Bendgude RD, Maniyar MG, Kondawar MS, Patil SB, Hirave RV. (2012) Antihelmintic Activity of Leaves of *Mimosa pudica*. Intl Journal of Institutional Pharmacy and Life Sciences. 2(1).
19. Chandrashekar DK and Manthale DM. (2012) Invention of Analgesic and Anti-inflammatory Activity of Ethanolic Extract of *Mimosa Pudica L*. Leaves. J Biomed Pharma. 1(1):36-28.
20. Elango V, Carolin O, Raghu PS. (2012) Antiulcer activity of the Leaf ethanolic extract of *Mimosa pudica* in Rats. Hygeia. J. D. Med. 4(1): 34-40.
21. Mali PR, Patil CD, Rahila S, Mali PR, Karigar A. (2011) Studies on Antiasthmatic Activity of Aqueous Extract of Roots of *Mimosa pudica Linn*. IRJP. 2(1): 104-110.
22. Muthukumaran P, Shanmuganathan P, Malathi C. (2011) In Vitro Antioxidant Evaluation of *Mimosa pudica*. Asian Journal of Pharmaceutical Research. 1(2): 44-46.
23. Rajendran R, Hemalatha S, Akasakalai K, MadhuKrishna CH, Vittal BS, Sundaram RM. (2009) Hepatoprotective activity of *Mimosa pudica* leaves against Carbon tetrachloride induced toxicity. Journal of Natural Products. Vol. 2: 116-122.
24. Saifiddin Khalid MD, Kumar JS, Suresh DK, Kumar R. (2011) Evaluation of an anti-diarrhoeal potential of ethanolic extract of *mimosa pudica* leaves. Int J Green Pharma. 5(1): 75-78.
25. Zhang J, Yuan K, Zhou W, Zhou J, Yang P. (2011) Studies on the active components and antioxidant activities of the extracts of *Mimosa pudica Linn*. from southern China. Pharmacognosy Magazine. 7(25): 35-39. doi:10.4103/0973-1296.75899.
26. Gandhiraja N, Sriram S, Meena V, Srilakshmi K, Sasikumar C, Rajeshwari R. (2009) Phytochemical Screening and Antimicrobial Activity of the Plant Extracts of *Mimosa pudica L*. Against Selected Microbes. Ethnobotanical Leaflets. 13: 618-24.
27. Pawaskar SM and Kale KU. (2006) Antibacterial Activity of Successive Extracts of *Mimosa pudica*. Indian Drugs. 43: 476-480.
28. Claeson P, Goransson U, Johanssin S, Luijendijk T, Bohlin L. (1998) Fractionation Protocol for the Isolation of Polypeptides from Plant Biomass. J. Nat. Prod. 61, 77-81.
29. Schagger H. (2006) Tricine-SDS-PAGE. Nature Protocols. 1(1): 16-23.
30. Aguilar MI. (2004) Proteolytic Peptide Mapping. HPLC of Peptides and Proteins: Methods and Protocols. Humana Press. Totowa, NJ. 251: 227-244.
31. Schmid FX. (2201) Biological Macromolecules: UV-visible Spectrophotometry. Encyclopedia of Life Sciences. Macmillan Publishers Ltd, Nature Publishing Group.
32. Aitken A and Learmonth M. (2002) Protein Determination by UV Absorption. The Protein Protocols Handbook 2nd edition. Humana Press Totowa, NJ. 3-6.
33. Karuppusamy S and Rajasekaran KM. (2009) High Throughput Antibacterial Screening of Plant Extracts by Resazurin Redox with Special Reference to Medicinal Plants of Western Ghats. Global Journal of Pharmacology. 3 (2): 63-68.
34. Coban AY. (2012) Rapid Determination of Methicillin Resistance among Staphylococcus aureus Clinical Isolates by Colorimetric Methods. J. Clin. Microbiology. 50(7):2191–2193.
35. Sener S, Acuner IC, Bek Y, Durupinar B. (2011) Colorimetric-Plate Method for Rapid Disk Diffusion Susceptibility Testing of Escherichia coli. J. of Clin. Microbio. 49(3): 1124–1127.
36. Tunney MM, Ramage G, Field TR, Moriarty TF, Storey DG. (2004) Rapid Colorimetric Assay for Antimicrobial Testing of Pseudomonas aeruginosa. Antimicrobial Agents and Chemotherapy. 48(5):1879-1881.
37. Valgas C, de Souza SM, Smania EFA, Smania Artur. (2007) Screening Methods to Determine Antimicrobial Activity of Natural Products. Brazilian J Microbio. 38: 369-380.
38. Cytranska M, Mak P, Zdybicka-Barabas A, *et al.* (2007) Purification and Characterization of Eight Peptides from Galleria mellonella immune hemolymph. Peptides. 28: 533-546.
39. Aliahmadi A, Roghanian R, Emtiazi G, Ghassempour A. (2011) A simple method for primary screening of antibacterial peptides in plant seeds. Iranian J Microbiology. 3(2):104-108.
40. Chia YY and Yap WS. (2011) In vitro Antimicrobial Activity of Hexane:Petroleum Ether Extracts from Fruits of Momordica charantia L. Int'l J Pharm Bio Archives. 2(3):868-873.
41. Vasavada N. (2014) http://statictica.mooo.com/.
42. Gleason JR. (1999) An accurate, non-iterative approximation for studentized range quantiles. Computational Statistics and Data Analysis. 31: 147-158.