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Screening of Novel Acidified Solvents for Maximal Antimicrobial Peptide Extraction from *Zophobas morio* fabricius.

Johan Ariff Mohtar, Faridah Yusof, Najala Mahmoud Hag Ali

Department of Biotechnology Engineering, Kulliyah of Engineering, International Islamic University Malaysia.

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ABSTRACT

A solvent screening study using four alcohols with decreasing polarity to select novel acidified extraction solvents for maximal antimicrobial peptide (AMPs) recovery from the whole body extracts of supermealworm *Zophobas morio* larvae was performed. 20 grams of final instar larvae were homogenized in four solvents at fixed concentration of 90% methanol, 90% ethanol, 90% isopropanol and 90% butanol; each acidified with trifluoroacetic acid (TFA). All samples were extracted at constant solid-solvent ratio (w/v), extraction time and temperature. The solvent extracts were tested on four bacteria in the antimicrobial susceptibility test to evaluate the inhibitory effects. Inhibition zones (mm) were recorded and the range of values was categorized as good, moderate, weak and very weak antibacterial activity. All extracts were found to exhibit significant inhibitory effects on the bacteria ($p < 0.001$). Isopropanol extract produced significantly ($p < 0.05$) the largest zone of inhibition in *Staphylococcus aureus* (10.67 ± 3.06 mm), *Escherichia coli* (8.67 ± 2.08 mm), *Klebsiella pneumonia* (8.00 ± 1.00 mm) and *Pseudomonas aeroginosa* (7.00 ± 2.65 mm) compared to other solvent extracts. The extract was considered a good antibacterial agent against *S. aureus, E. coli*, and *K. pneumonia* and as a moderate antibacterial agent against *P. aeroginosa*. *S. aureus* was shown to be the most susceptible to isopropanol extract followed by *E. coli*, *K. pneumonia* and *P. aeroginosa*. This finding has a significant implication to improve the extraction procedure over the use of conventional methanolic extraction for new AMP discovery from insect whole body extracts. It appears that acidified isopropanol extracted a maximum amount of AMPs compared to acidified methanol, hence, is selected as the best novel solvent in this study.

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INTRODUCTION

Antibiotic resistance has been a major problem in impeding the efficiency of commonly used antibiotics exhibited by a plethora of microbes as a result of the excessive use of the drugs in medical treatments and animal feed [1]. Due to the unpredictable degree of resistance, antibiotic research has now turned its direction to the manipulation of antimicrobial peptides (AMPs). AMPs are short bioactive molecules either cationic or non-cationic in nature with broad-spectrum antimicrobial effects against many pathogens [2]. They generally act in destabilizing the cell membrane permeability or interacting with the specific targets in cells which cause signaling pathway disruption [3]. Their rapid effects have shown a significant promise in the development of new generation of antibiotics which is able to contribute to the reduction of the antibiotic resistance problem. AMPs can potentially be developed as a 'stand alone antibiotic', synergistic drug and endotoxin neutralizer in a wide range of applications for parasitic infection, cancer and viral treatments [3,4,5,6]. Since the first antimicrobial peptide isolation from the giant silk moth *Hyalophora cecropia* in 1981, insects have become the important manipulatory source in which almost 50% of the characterized antimicrobial peptides were contributed by the insect orders [3]. Insects possess a unique innate immune system with specific pattern of AMP production in response to pathogenic infection as a defense. They are produced in all life stages of insects and the activity was found the highest in the final instar larva [7]. In insects, AMPs are constitutively expressed or highly inducible in response to bacterial infection by various epithelia of midgut and salivary glands, fat bodies and hemocytes in which they are secreted into the hemolymph. In holometabolous insects with complete metamorphosis, AMPs are produced by the fat bodies [8] whereas the hemocytes act as the vital synthesizer for AMP in heterometabolous insects with incomplete metamorphosis [9]. Many cationic AMPs of insect origin have been isolated from the bacterial challenged hemolymph or from the fat bodies and the hemocytes. Interestingly, insect whole body is also

Corresponding Author: Faridah Yusof, Department of Biotechnology Engineering, Kulliyah of Engineering, International Islamic University Malaysia.
E-mail: faridah@iium.edu.my

an alternative source for the discovery of a new class of small AMPs which may have a role in processes other than immunity. The AMP extraction from the whole body is governed by the following facts: (i) AMP production is not restricted to fat bodies and hemocytes, (ii) unknown physiological location of the molecules and (iii) small-sized body of insects [7].

AMP extraction from insect whole body is a challenging step in peptide analysis because of their low concentration in specific tissues and the presence of proteases and other non-peptidic molecules. Without proper sample preparations, peptide analysis employing various procedures for isolation, purification, and characterization can be extremely time-consuming. Initial peptide recovery from insect whole body requires the sample to be first subjected to homogenization [7]. Homogenization with protease inhibitors in water or in organic acids for deproteinization aims to extract the hydrophilic peptides while the highly hydrophobics are often recovered by employing organic solvents.

Due to uncertain deproteinization abilities and inherent limitations, there is no single solvent that can extract a complete set of peptides. Nevertheless, the acidified methanol (methanol/water/organic acid: 90/9/1, v/v/v) extraction system was shown to fulfill the above criteria for many peptide recovery [10]. In fact, extraction of AMPs from the insect whole body has been mainly practiced via homogenization in 0.1% TFA-acidified methanol [7,11,12,13]. Yet, the ability of other acidified solvents in comparison to the acidified methanol to recover a maximum AMP amount from the whole body extract is still unknown.

Due to the fact that many solvents may exert different effects on each type of tissue, this study aims to screen for the best acidified solvent using four alcohols with decreasing polarity including methanol, ethanol, isopropanol and butanol in order to select novel solvents capable of extracting maximum AMP amount from the whole body extracts of unchallenged supermealworm *Zophobas morio* larvae.

MATERIALS AND METHODS

Insect:

Zophobas morio larvae at different larval stages were purchased from a local pet shop and reared in the laboratory at room temperature with 55% RH in a 12 h : 12 h (L:D) photoperiod. They were kept in six cylindrical plastic containers of 10 cm height with a diameter of 22.5 cm and maintained on substrate mixture of grind chicken bran and wheat bran (2:1; w/w/w) as the food source [14]. The substrate was substituted every three weeks upon cleaning. Holes were made on the lid of the container to allow air circulation and each container accommodated 150 larvae supplied with slices of fresh carrot as water source that were changed thrice a week.

Microorganisms for Bioassay:

Four bacterial test strains encompassed of one gram (+) bacteria *Staphylococcus aureus* and three gram (-) bacteria *Escherichia coli*, *Pseudomonas aeroginosa* and *Klebsiella pneumonia* were procured from the microbial stock culture at the Department of Biotechnology Engineering, Kulliyah of Engineering, IIUM for the use in antimicrobial assay. The bacteria were cultured on sterile Luria-Bertani (LB) rich agar medium (1% trypton, 0.5% yeast extract, 1% NaCl and 1.5% agar; w/v) (LAB, UK) at 37 ºC.

Solvent Screening Study:

In the screening study, acidified solvent extractions and sample preparations were carried out in accordance to the protocols by [15]. 20 grams of laboratory reared final instar larvae (equivalent to 24 larvae) with body weight ranged between 600 to 800 mg were used. The determination of the final instar was based on the body weight characteristic since there is no morphological study to distinguish between the larval stages. In general, the body weight of a final instar larva of *Z. morio* ranges between 600 to 800 mg [16]. For the ease of body weight measurement, larvae were initially frozen for 20 min in the chiller to restrain their movements. They were then washed three times with tap water to remove dirt, rinsed once with distilled water and disinfected with 70 % ethanol. The excess of ethanol was dried with paper towel and the larvae were succumbed to death in the freezer for 40 min. The larvae were homogenized in four precooled (4 ºC) acidified alcohols at constant concentrations: (i) methanol/water/TFA (90:9:1,v/v/v), (ii) ethanol/water/TFA (90:9:1,v/v/v), (iii) isopropanol/water/TFA (90:9:1,v/v/v) and (iv) butanol/water/TFA (90:9:1,v/v/v) complemented with 10 µg/ml of aprotinin by grinding in a 250 watt electric blender following a fixed solid-solvent ratio (1:3) for 1 min. The resulting homogenates were filtered using nylon cloth to remove large exoskeleton debris and subjected to centrifugation at 12,000 rpm for 40 min at 4 ºC. The supernatant was collected and the extraction solvents was evaporated to dryness under reduced pressure until residues emerged.

The residues of each solvent extract were dissolved in 10 ml of 0.1% TFA, pH 1.98 and further subjected to lipid extraction to remove fluid lipids which can interfere with the resin in chromatographic column. Each 5 ml of ethylacetate and *n*-hexane was sequentially added and the lipophilic layer formed was removed after vigorous shaking. Traces of ethylacetate and *n*-hexane were discarded under reduced pressure evaporation for 40 min and

the refined aqueous extracts were further concentrated under vacuum and used as crude samples. After the 2nd evaporation, 2 ml from the extractant was aliquoted into 2 ml Eppendorf tube and centrifuged at 10,000 rpm for 5 min at room temperature in order to percipitate the remaining debris.

Antimicrobial Susceptibility Bioassay:

In vitro antimicrobial tests were conducted to screen the antibacterial activity of the crude extractants against *Escherichia coli*, *Pseudomonas aeroginosa*, *Staphylococcus aureus* and *Klebsiella pneumonia* using the agar well diffusion method [17]. All test strains were grown 24 hours prior to performing the assay. Medium preparation incorporated the use of well mixed LB agar in 25 ml sterile Petri dishes (90 mm in diameter) in aseptic conditions. After solidification of the medium, each plate received 75 µl of the overnight cultured bacteria inoculum with adjusted concentration equivalent to 0.5 McFarland Standard which was evenly spread on the agar surface using a sterile glass rod and left to dry for 10 min. Wells were punched using sterile Eppendorf pipette tips (diameter 9 mm). 100 µl of the crude extractant was pipetted into the wells and tetracycline (final concentration: 10 µg/ml) was used as the positive control while 0.1 % TFA was employed as the negative control. In order to allow effective diffusion of the samples and controls into the agar, the plates were left at room temperature for one hour prior to incubation [18]. The plates were incubated for 18 hours at 37 ºC and the diameters of the clear zones (minus the well diameter) were recorded using a caliper. The range of the diameter values was ranked in accordance to the degree of antibacterial activity based on the method by Ali *et al.* [19]; ≥ 8 mm: good, 6 – 7 mm: moderate, 4 – 5 mm: weak and 2 – 3 mm: very weak. The experiments were conducted in triplicates.

Results:

Antibacterial Activity of the Solvent Extracts:

In the solvent screening study, the sample extracts of the four acidified solvents from the unchallenged larvae were tested against each of the bacterial strain in the plate growth inhibition assay. The diameters of the inhibition zones were recorded after 18 hr as illustrated in Table 1. ONE-WAY analysis of variance indicated that all tested bacteria were significantly inhibited by the extracts of the four solvents ($p < 0.001$) based on the microbial growth inhibition zone (mm). Due to this positive inhibitory effects on all bacteria tested, the solvent extracts are considered to possess a significant antibacterial activity.

Following the post-hoc analysis using Duncan Multiple Range test, isopropanolic extract was shown to display a strong antibacterial effect against all gram (-) and gram (+) positive bacteria by producing significantly the largest inhibition zone ($p < 0.05$) in *Staphylococcus aureus* (10.67 ± 3.06 mm), *Klebsiella pneumonia* (8.00 ± 1.00 mm), *Escherichia coli* (8.67 ± 2.08 mm) and *Pseudomonas aeroginosa* (7.00 ± 2.65 mm) compared to other solvent extracts (Table 1). However, the isopropanolic extract did not exert significant inhibitory effect against the four bacteria indicating an equal susceptibility towards the extract (Figure 1). In comparison to the diameter of inhibition zones of tetracycline, a greater inhibition zone was produced by the isopropanolic extract in *E. coli*. Although the diameter zone of the extract in *S. aureus* was larger than that of the tetracycline, it was not significantly different.

Table 1: Antibacterial activity of the solvent extracts against the tested bacteria.

Solvent Extract	Inhibition Zone (mm)			
	Gram-Negative Bacteria			Gram-Positive Bacteria
	E. coli	*P. aeroginosa*	*K. pneumonia*	*S. aureus*
Methanol	3.33 ± 1.53^{ab}	2.00 ± 1.00^{ab}	3.67 ± 0.58^{ab}	4.00 ± 1.73^{ab}
Ethanol	4.00 ± 1.73^{b}	2.00 ± 0.58^{b}	3.00 ± 1.00^{b}	6.00 ± 2.65^{b}
Isopropanol	8.67 ± 2.08^{c}	7.00 ± 2.65^{ce}	8.00 ± 1.00^{ce}	10.67 ± 3.05^{ce}
Butanol	2.67 ± 2.52^{db}	3.00 ± 1.16^{db}	2.30 ± 1.53^{db}	3.67 ± 2.31^{db}
Positive Control				
Tetracycline	4.00 ± 2.65^{e}	9.33 ± 2.08^{ec}	8.67 ± 1.53^{e}	8.00 ± 2.00^{eb}
Negative Control				
0.1 % TFA	NZ	NZ	NZ	NZ

Values with identical superscript letters did not differ ($p > 0.05$) in each bacteria column (ANOVA, Duncan Multiple Range Test). Inhibition zones excluding the diameter of the well (9 mm) were presented as mean ± standard deviation. NZ - no zone of inhibition, Tetracycline - working concentration 10 µg/ml, *E.coli – Esherichia coli, P. aeroginosa – Pseudomonas aeroginosa, K. pneumonia – Klebsiella pneumonia, S. aureus – Staphylococcus aureus.*

Meanwhile, the inhibition zones of the isopropanolic extracts in *P. aeroginosa* and *K. pneumonia* were smaller than that of tetracycline. No inhibition zones were detected in the negative control. It was observed that *S. aureus* was the most susceptible towards isopropanolic extract followed by *E. coli*, *K. pneumonia* and *P. aeroginosa*. Based on the degree of antibacterial activity of the inhibition zones, isopropanolic extract can be

considered a good antibacterial agent against *S. aureus*, *E. coli* and *K. pneumonia* (≥ 8 mm) and as a moderate antibacterial agent against *P. aeroginosa* (6 – 7 mm).

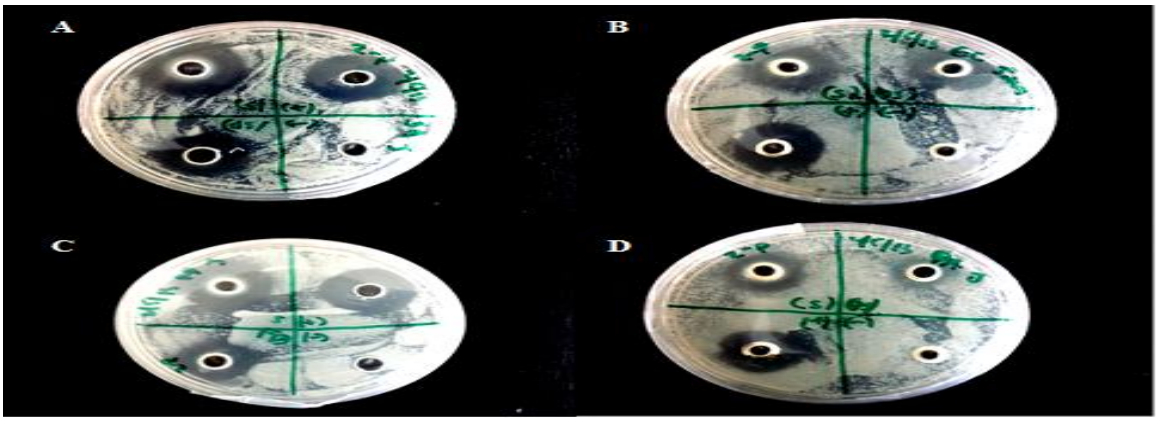

Fig. 1: Inhibition zones of the four bacteria in response to isopropanolic extract. A: *S. aureus*, B: *E. coli*, C: *K. pneumonia*, D: *P. aeroginosa*. The symbols (+) and (-) indicate the positive and negative controls respectively.

Discussion:

The discovery of different types of antimicrobial peptides which partly play a pivotal role in insect innate immune system against microbial infections had prompted a vast number of studies on the application of AMPs towards combating human infections and diseases. Studies on the isolation and purification of inducible insect AMPs by the incorporation of endogenous bacteria as a medium to search for new antimicrobial peptides from the hemolymph had been long conducted. Since then, a majority of the insect AMPs were extracted directly from the hemolymph in response to bacterial immunization. Several inducible AMPs including cecropins, sarcotoxins, defensins, thanatin, droscosin and coleoptericins had been well characterized in terms of the structures and mechanisms [20,21,22,23]. Parallel to the increasing number of studies on AMP purification from immunized hemolymph, attempts had been done in exploring the use of insect whole body as the alternative for obtaining new class of AMPs without immunization. Giving credits to the AMP production by various organs in insect body and the nature of AMP itself, the technique relies upon the use of the solvent extraction system to recover different types of AMPs. Methanol has been commonly used in the extraction procedure. Several non-cationic peptides such as β-alanyl-tyrosine, p-hydroxycinnamaldehyde and N-β-alanyl-5-S-glutathionyl-3-4dihydroxyphenylalanine had been extracted from the insect whole body using solvents including methanol and water acidified with TFA [7,24,25]. These constitutively expressed peptides exhibited strong inhibitory effects against many bacteria and fungi. In the solvent screening study, *Zophobas morio* larvae were used as the source of antimicrobial peptides as Bulet *et al.* [21] had previously identified three inducible AMPs, a coleoptericin and two isoform defensins from the induced larval hemolymph.

Our data shows that the acidic solvent extracts of methanol, ethanol, isopropanol and butanol from the whole body of the unchallenged final instar larvae were found to posses antibacterial activity against all tested bacteria. This observation was supported by other studies. Gundappa *et al.* [12] reported that the methanolic extracts of the whole body of sixteen individual dung beetles exhibited a strong antibacterial activity against tested bacteria. In addition, injection of endogenous bacteria in the naive (unchallenged) beetles resulted in the increase of the antimicrobial activity of the extract as judged from the diameter of the inhibition zones in the assay of the same study. Dang *et al*. [26] also observed that the crude extractant from the last instar larvae of oriental fruit fly, *Bactrocera dorsalis* Hendel displayed antibacterial effect despite the use of ammonium acetate as solvent. The presence of the antibacterial activity of the solvent extracts in this study strongly corroborates the existence of the constitutively expressed antimicrobial compounds in the whole body of many naïve insects as reported in the literature [9,27]. These compounds can be constitutive peptides or some rarely small non-peptidergic molecules with antimicrobial properties since the use of mild acids such as TFA in the extraction solvent precipitates high molecular mass proteins [28]. Constitutive peptides occur naturally in low concentration however, they may also exist in parallel to the induced antimicrobial peptides displaying a greater level of antimicrobial activity. For instance, the longest cecropin-like peptide stomoxyn, from the stable fly *Stomoxyns calcitrans* and spinigerin from the fungus growing termite *Pseudacanthotermes spiniger* were reported to be not induced by microbial infection but constitutively present in the secretion of female reproductive glands, in the anterior midgut and in the hemocytes [29]. Unlike the production of inducible AMPs which occurs in fat bodies and hemocytes, the synthesis of constitutive antimicrobial peptides has been observed in the epithelia of midgut and salivary glands and also in the hemocytes [9,30]. Meylears *et al.* [7] suggested that insects fight microbial infection with respect to

antimicrobial peptides in two ways: (i) the infection-induced transcription of genes coding for AMP synthesis in fat bodies and (ii) the synthesis and storage of constitutive AMPs in granular cells.

Results from the antimicrobial assay indicated that acidified isopropanolic extracts can significantly inhibit the growth of the tested bacteria as judged from the production of the largest inhibition zones in the antimicrobial assay compared to other solvent extracts (Table 1). Unfortunately, the nature of the bioactive compounds present in the extract was not yet identified but it is most likely a peptide-based molecule since the extraction protocol used had incorporated the addition of TFA that precipitates large proteins and polypeptides leaving the low to medium molecular weight peptides behind. Nevertheless, further investigation in protease assay is needed to confirm the peptidic nature of the compound. In addition to that, the mode of action of the compounds in the extract either bacteriostatic or bactericidal in action was unknown. At present, no literature reviews have reported the use of isopropanol as an extraction solvent for AMPs from insect whole body.

In practice, methanol acidified with acetic acid or TFA has been commonly employed for extracting antimicrobial peptides from insect whole body. However, studies on the use of other solvents that have the ability to recover a maximum amount of antimicrobial peptides in comparison to methanol have not been conducted. This study was the first to elucidate that isopropanol could extract a maximum amount of AMPs compared to methanol as based on the diameter of the inhibition zone. This potential is probably due to the degree of amphipathicity of isopropanol which might be compatible with the amphipathic nature of the AMP by increasing the solubility of the peptides in the solvent. In contrast, the potentials of ethanol and butanol are most likely equivalent to that of methanol as judged by the overall similarities in the diameter of inhibition zones produced. Many solvents including acetone, acetonitrile and benzene may have more potential in extracting antimicrobial peptides from insect thus it is recommended to conduct more screening studies in order to identify potential solvents of low toxicity and environmentally-friendly. Since isopropanolic extract retained its antibacterial activity during the assay, it is inferred that isopropanol did not have deleterious effect on the antimicrobial compounds and is relatively non-toxic. Although the concentration of isopropanol at 90% was lethal to bacteria as preliminarily tested, it was discarded completely in the study. This result supports the two criteria of extraction solvents for antimicrobial peptides by Dang *et al*. [25]: (i) the solvent can maintain the antibacterial activity of AMP and (ii) the solvent has no effects against bacteria.

As shown in this report, acidic isopropanolic extract produced significantly the largest inhibition zone compared to that of other solvent extracts. Since the selection of the best solvent is made on the basis of the production of the largest inhibition zone in the antimicrobial assay, isopropanol has fulfilled the above criteria, hence, is selected as the best extraction solvent that can be used as a novel solvent for the extraction of a maximum AMP amount from insect whole body. As part of the ongoing project on the purification of insect antimicrobial peptides, the data indicated that we have successfully achieved the first objective of the research to isolate antimicrobial peptides from the non-immunized final instar larvae of *Z. morio* via the solvent screening study. This finding has a significant implication to improve the extraction procedure over the use of the conventional methanolic extraction for new AMP discovery from insect whole body.

Conclusion:

In conclusion, we have successfully selected isopropanol as the best solvent that can be used for maximal extraction of AMPs via the solvent screening study. The extract displayed a strong antibacterial activity against all tested bacteria compared to other solvent extracts with a greater effect on the gram (+) bacteria *S. aureus*. We presume that the extract contains constitutive antimicrobial peptides which can be potentially developed into peptide-based drug due to its inhibitory effect on the bacteria which is relatively equivalent to that of the commercial tetracycline. In addition, the employment of the solvent system in the AMP extraction of insect whole body is highly advantageous from the view point of biotechnology engineering. This is because a number of parameters such as pH, temperature and concentration of a solvent can be likely optimized to increase the mass recovery rates of peptides for scale-up purposes. Finally, further studies including purification and characterization of the constitutive antimicrobial peptides in the isopropanolic extract from this study may contribute to the discovery of a new class of antimicrobial peptides in *Z. morio*.

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