

Anticancer peptides derived from supermeal worm (*Zophobas morio*) larvae

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Abstract

Cancer still presents enormous challenges in the medical world. Currently, the search for anticancer compounds has garnered a lot of interest, especially in finding them from the natural sources. In this study, by using Sulforhodamine B (SRB) colorimetric assay, compounds, extracted from supermeal worm (*Zophobas morio*) larvae using two types of acidified organic solvent (ethanol and isopropanol), were shown to inhibit the growth of a breast cancer line, MCF-7. A comparative study of the effect was carried out on a normal cell line, Vero. Results showed that, the two types of extracts inhibits growth of MCF-7 cell at varying degrees, on the other hand, have much less effect on Vero cell. Extracts analysed by UV-vis spectroscopy, showed peaks in the range of 260 to 280 nm, inferring the presence of aromatic amino acids, whereas the highest peak of 3.608 AU at 230 nm indicates the presence of peptide bonds. By Raman spectroscopy, peaks are observed at 1349 cm⁻¹, 944 cm⁻¹ and 841 cm⁻¹ indicating the presence of Tyr, Try and Gly, confirming the UV-vis analyses. All results of analyses implied that the anticancer compounds contain peptides.

Keywords

Anticancer peptides

Zophobas morio Fabricius

MCF-7 breast cancer cell line

Vero cell line

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Introduction

Human cancer remains a cause of high morbidity and mortality worldwide (Resnick *et al.*, 2008). Doctors and scientists around the world are working to find better ways to prevent, detect and treat cancer. In this modern era, cancer treatments have numerous pitfalls (Rodrigues *et al.*, 2008), such as the drugs are very unaffordable and expensive, toxic to healthy cells leading to many side-effects, ineffective in induction of cell death and drug resistance (Chowdhury *et al.*, 2016). To overcome these problems, they are constantly studying and discovering new drugs and also looking for new ways to use existing drugs. One of the ways is to find any potential anticancer compounds in natural sources (Bhutia and Maiti, 2008) such as from plants, animals, microbes and insects.

Antimicrobial peptides (AMPs) are part of the innate immune defence mechanism of many organisms, including insects (Diamond *et al.*, 2009). Although AMPs have been studied and developed as potential alternatives for fighting infectious diseases, their use as anticancer peptides (ACPs) need to be explored. There are reports regarding AMPs which are alternatively effective in treating cancer (Hoskin and Ramamoorthy 2008). Peptides extracted from *Calliphora vicina* is a good example of an AMP

presenting anticancer properties (Sherman *et al.*, 2000; Chernysh *et al.*, 2002).

Insects make up about 55% of total biodiversity and are important natural source of AMPs and ACPs (Faruck *et al.*, 2016). Bullet *et al.* (1991) has isolated three potent AMPs from *Zophobas atratus*; coleopteracin, peptide B and C isoforms from the haemolymph of immunized larvae. Coleopteracin is an 8.2 kDa peptide with bactericidal effect against gram-negative strains while the two isoforms are 4.8 kD peptides which is effective against gram-positive bacteria. Recently, the effectiveness of AMPs sourced from larval stage darkling beetle, *Zophobas morio*, also known as supermeal worm has been reported (Mohtar *et al.*, 2014; Faruck *et al.*, 2016). The cheaply reared larvae are mainly used commercially as animal pet feeder and previous work showed that it is rich in protein (Zhang *et al.*, 2011). Most of the peptides researches on *Zophobas morio* were carried out via whole body extraction of the non-immunized larvae by using acidified alcohol. In this study, the same extracts, which are, the acidified ethanolic and isopropanolic extract of *Zophobas morio* was investigated for their anti-cancer properties on MCF-7 cell line. For the control, Vero cell has been used. IC₅₀ values for the extracts exhibiting anticancer property was calculated. Positive identification of the constituents of the extract was carried out by UV-

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vis and Raman spectroscopies.

Materials and methods

Reagent

Human breast cancer cells, MCF-7 and normal cell line, Vero, were obtained from the Institute for Medical Research, Malaysia (IMR). All cell culture reagents including Foetal Bovine Serum (FBS), Dulbecco's Modified Eagle's Media (DMEM), phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO), sulforhodamine B (SRB) reagent and trichloroacetic acid (TCA) were procured from Sigma-Aldrich (St. Louis, MO, USA).

Peptide extraction from *Zophobas morio*

Non immunized late instar larvae of *Zophobas morio* from the stock culture was subjected to whole body extraction following the method of Mohtar *et al.* (2014). Only larvae with a body weight of around 0.70 g to 0.80 g were selected for the study. Initially, the larvae were frozen for half an hour to restrict their movement. Then, the larvae were washed with distilled water twice and with 70% ethanol once, to remove contaminants, and then dried with tissue paper to remove excess ethanol. They are kept for 1 hour in -20°C freezer to kill, followed by blending in the electric blender for 5 min with acidified (trifluoroacetic acid) alcohol (ethanol and isopropanol). The exoskeleton debris was removed by first filtering using a nylon cloth, followed with centrifugation at 12000 rpm for 40 min at 4°C to collect the supernatant. The supernatant was left to evaporate to dryness and dissolved in tri sodium phosphate buffer (pH 7.0). To remove any lipid in the sample, ethyl acetate and n-hexane was added and vigorously shaken (Leem *et al.*, 1996). A lipophilic layer formed on the surface was removed followed by evaporation to remove excess organic solvent yielding an aqueous clear extract. Extracts were freeze-dried and kept in -20°C until further use.

Cell culture

Human breast cancer cell, MCF-7 and Vero cell were maintained in Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% Foetal Bovine Serum (FBS) and 50 mg/mL gentamicin (Thermo Fisher Scientific Inc. USA) and incubated in a 5% CO₂ incubator (RS Biotech, Malaysia) at 37°C humidified atmosphere. All the experiments were conducted with cell passages of less than 10 for MCF-7 and Vero cell.

Cytotoxicity assay

The Sulforhodamine B (SRB) assay was carried out as described previously (Vichai and Kirtikara, 2006). In brief, before drug exposure, 1×10^3 cells in 200 µl of media were seeded in 96-well plates and left for attachment in the incubator. Six-fold serial dilutions were done for varying drug concentrations in DMEM, where initial concentration was 4 mg/mL and 100 µl were added to the monolayer during 80% exponential phase. Only DMEM were used as vehicle control. The cultures were assayed after 72 hours of incubation in 5% CO₂ at 37°C humidified atmosphere by adding 100 µl ice cold 10% (w/v) TCA and incubated at 4°C for 1 hour to fix the cells in the monolayer. The TCA containing media was then aspirated using five times washing with distilled water and allowed to dry in air. Then 100 µl of 0.057% (w/v) SRB solution was added to each well and after staining at room temperature for 30 min, the plates were rinsed four times with 1% (v/v) acetic acid to remove unbound dye and left for air dry. Before determining the absorbance on a Multiskan™ GO Microplate Spectrophotometer at 492 nm, the bound SRB were solubilized by adding 200µl of 10 mM tris base solution (pH 10.5). The cell viability percentage was calculated as follows:

$$\text{Cell viability \%} = (\text{Mean OD of treated cell}) / (\text{mean OD of vehicle treated cells (negative control)}) \times 100 \quad (1)$$

The IC₅₀ values were calculated using a dose response plot analysis and individual drug concentrations. All analytical conformation was performed in triplicates. Data of different parameters were expressed as the Mean ± SEM (standard error mean) and statistical evaluation was performed using the one way ANOVA. Statistically significant data were considered from p values of less than 0.05.

Identification of anticancer peptide

For the Raman measurement, the freeze-dried crude sample was used. The Raman microscopy measurement was done in a closed and vapour saturated chamber so that the sample evaporation during spectra acquisition is minimum. For spectra acquisition A, 100X water immersion lens were used. This spectra links with sampling spot of 3 µm X 30 µm in the renishaw and 1200 lines/mm grating was used in the Raman microscopy. The similarly freeze-dried sample was submitted for UV-vis analysis. The dried sample was dissolved in distilled water. A volume of 200 µl of the sample was transferred to a 96 well microtiter plate and absorbance was taken at 200 nm wavelength to 300 nm wavelength.

Results and discussion

Toxicity effect of extract on cancerous cell

The toxic nature of peptides extracted from *Zophobas morio* were examined by their membrane disturbance activity on cancerous cell MCF-7. The membrane disturbance of MCF-7 was characterized by measuring cell viability percentage of MCF-7 for both ethanolic and isopropanolic extracted peptides derived from *Z. morio*. The results in Figure 1 show that both crude samples resulted in a dose dependent inhibition of cell viability percentage of MCF-7, where, data are represented as Mean values \pm SEM of MCF-7 performed in triplicates. Using ethanolic solvent from the fitted curve, it is observed that sample concentration at 1.7 mg/ml is able to kill 50% of the MCF-7 cells. Similarly, using the isopropanolic solvent, 50% cells are killed at 0.7 mg/ml sample concentration. As shown in Figure 2, the MCF-7 cell lines were more sensitive to isopropanolic extracted peptide compared to the ethanolic extracted peptide. 'a' represents statistically significant difference with respect to ethanolic and isopropanolic solvents at $p < 0.05$. 'b' and 'c' represent non-significant differences between ethanolic and isopropanolic solvents at $p < 0.1$ and $p > 0.1$ respectively. 'ce' and 'cd' represent statistically significant and non-significant differences between sample treated MCF-7 and control at $p < 0.05$. However, in both extractions, the peptide showed a similar trend. This could be attributed to the fact that several concentrations of both samples were expressed differentially with MCF-7. Figures 1 and 2 show that more than 50% of the cancerous cells treated with 1 mg/ml ethanolic and 0.5 mg/ml isopropanolic extracted peptides were killed. Results showed that isopropanolic extract was more potent than the ethanolic extract in terms of their anticancer properties. This can be explained by the differences in their polarity index of both organic solvents (PI(isopropanol) = 3.92 and PI(ethanol) = 5.2). Isopropanol, being more hydrophobic, may have been more efficient in extracting the more hydrophobic anticancer compounds compared to ethanol. These findings were in agreement with previous studies on *Salacia chinensis* L (Ngo et al., 2017), *Limnophila aromatic* (Do et al., 2014) and *Phoenix dactylifera* L. (Kchaou et al., 2013), whereby the variation can be explained by the difference in solubility of different compounds in the sample.

Estimation of Toxicity on Normal Cell

In order to ascertain the toxicity of *Zophobas morio* extracted crude sample on normal cells, the effect of both crude samples including the ethanolic

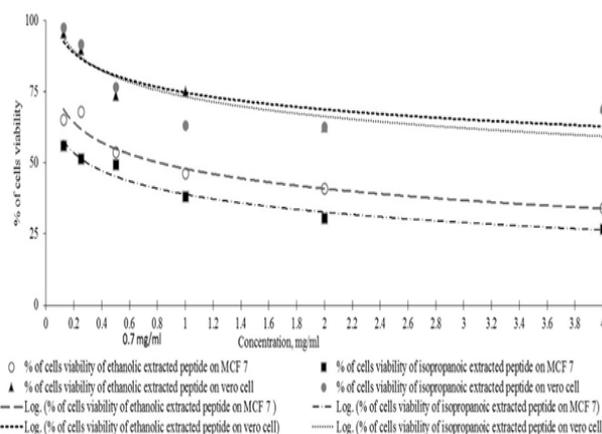


Figure 1. Dose response plot of cell viability percentage effect on MCF-7 and Vero cell for *Zophobas morio* extracted crude sample with ethanolic and isopropanolic solvents. Data are performed in triplicates and results given as Mean Values \pm SEM (Standard Error Mean).

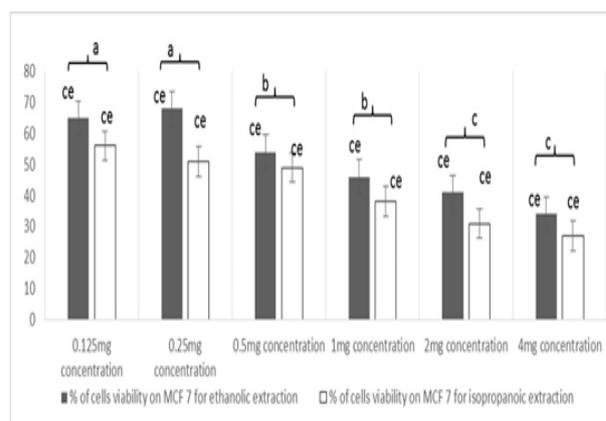


Figure 2. Effect of various concentrations of *Zophobas morio* extracted crude sample on MCF-7 using ethanol and isopropanol as solvents.

and isopropanolic solvent-based extractions were investigated. The toxicity on normal cells was measured using cell viability percentage with different concentrations of both samples. Here, Vero cells represent normal cells. Cell viability of Vero cells treated with 4 mg/ml ethanolic and isopropanolic extracted peptide concentration stands at 54.0% and 80.0%, respectively as shown in Figure 1. From Figure 1, it can be clearly seen that the viability of Vero cells increased with the decrease in concentration and the cell viability percentage were higher than the MCF-7 cells. Therefore, this indicates that the ethanolic and isopropanolic extracted peptide from *Zophobas morio* have significant anticancer activity, killing cancer cells without killing normal healthy cells, like Vero.

Identification of extract by UV-vis spectroscopy

To identify the constituents of the extract, isopropanolic sample was chosen since it gave higher activities compared to the ethanolic sample. Extract

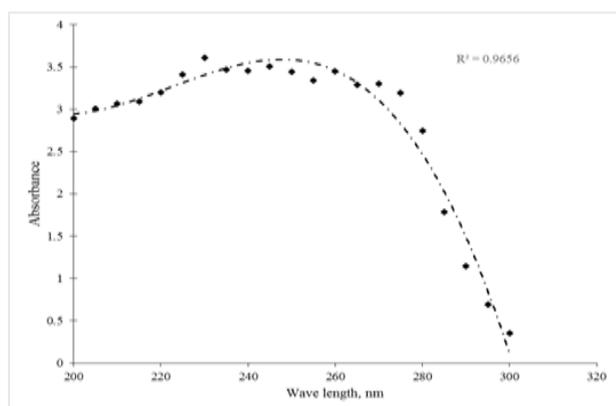


Figure 3. UV analysis of crude acidified isopropanolic extract from *Zophobas morio* (0.5 mg/ml).

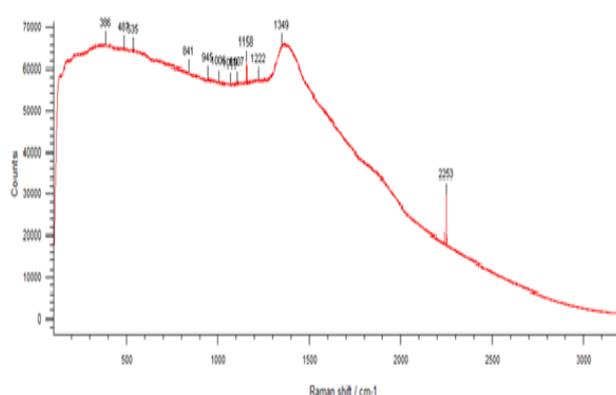


Figure 4. Raman spectrum of crude acidified isopropanolic extract of *Zophobas morio* with selected band assignments.

was subjected for an absorbency scanning from 200 to 300 nm as depicted by Figure 3. Result shows that in general absorbance are high at 200 nm, increased as the wavelength increases, but decreased sharply from 280 nm as it approaches 300 nm. It is observed that there are high peaks from 220-230 nm and 260-280 nm, inferring the presence of peptide bonds and aromatic groups, respectively. So it is fair to deduce that the extract contains amino acids and some of these are the aromatic ones such as tyrosine (Tyr), tryptophan (Trp) and phenylalanine (Phe) because of the presence of benzene, phenol and indole groups, respectively as their side chains. Aromatic amino acids containing peptides usually absorbed light with two distinct peaks. The peak centered on 280 nm is the result of absorbance by the aromatic ring portion whereas the peaks at the lower wavelength such as at 220-230 nm is caused by the absorbance of peptide bonds and carboxylic acid moieties in the compounds. Among the three aromatic amino acids, Tyr and Trp absorb more than Phe; Trp is responsible for most of the absorbance at 280 nm. Aromatic amino acids are relatively nonpolar, and Tyr is the only one with ionizable side chains. The aromatic side chains are electron-rich and they are bulky

compared to the side chains of other amino acids, and they can be involved in many interaction such as hydrophobic interaction, hydrogen bonding, cation- π and many other interactions with the nearby residues or exogenous components (Takeuchi, 2011). The presence of aromatic amino acid sometimes plays critical roles in peptide functions. The planarity of the aromatic ring limits the conformational freedom of the side chain, and the orientation of the ring plane sometimes plays an important role in the interaction. These unique properties of aromatic amino acids are fully utilized in the structural formation and the functional performance of proteins (Takeuchi, 2011). Thus the presence of these aromatic amino acids in the isopropanolic extract, which inhibit the viability of MCF-7 cancer cell line, is predictable and very probable. It can be suggested here that the aromatic amino acids could be the active component of the anticancer property in the isopropanolic extract.

Identification of extract by Raman spectroscopy

In this work, isopropanolic extract was further subjected to Raman spectroscopy to confirm its identity. Raman spectra can provide a structural fingerprint by which molecules can be identified. According to Takuechi (2011), Raman spectroscopy can be used to observe molecular vibrations by using the inelastic light scattering. The energy difference between the incident and scattered photons correspond to the energy of vibration. In the Raman spectrum, the intensity of scattered light is plotted against the wavenumber. The wavelength of the light used to excite a molecule for Raman scattering observation is called the excitation wavelength. Figure 4 shows the spectrum of isopropanolic extract upon submission to Raman spectroscopy. There is a prominent, although quite a broad band, registered at around 1349 cm^{-1} wavelength, which is usually assigned to the presence of Amide III bands. According to Overman and Thomas (1998), in characterization of peptides, a frequently utilized peak is the Amide III band, signaled around 1230 to 1350 cm^{-1} , which involves a combination of peptide N-H bending and C-N stretching motions. In fact, all the Amide bands are shown to be present in the spectrum such as Amide I at around 1650 cm^{-1} (80% C=O stretch), Amide II near 1550 cm^{-1} (60% N-H bend and 40% C-N stretch) and Amide III near 1300 cm^{-1} (40% C-N stretch, 30% N-H bend) (Rygula *et al.*, 2013). Other prominent bands are at 844, 944 and 1349 cm^{-1} and they can be assigned to Tyr, Gly and Try bands, respectively. Nonetheless, in general the bands are all poorly resolved, because only crude extract are used in the analysis. But most importantly

this result confirmed the earlier inferences, that the extract contains amino acids and some of them are the aromatic ones.

Discussion

Although a growing number of AMPs from natural sources have been developed over the last few years, only few were tested against cancer cells for their features of multi-function, high sensitivity, and constancy. In this study, the crude extract of *Zophobas morio*, which was previously shown to be antimicrobial, has strongly shown to inhibit the growth of the MCF-7 cell line at low dosages and exhibited potential damage toward cell morphology. Both the ethanolic and isopropanolic extracted crude sample presented higher activities against cancer cells compared to normal cells.

Apparently, the anticancer activity of *Zophobas morio* had a direct effect on MCF-7. *Zophobas morio* was toxic *in vitro* to the MCF-7 cell line at low concentrations for both the ethanolic and isopropanolic extracted crude sample. These values are statistically significant compared to the vehicle cell. Nevertheless, the stronger exhibition of toxic activity was found in the isopropanolic extracted crude sample compared to the ethanolic extracted crude sample with an IC₅₀ value of 0.8 mg/ml and 0.3 mg/ml respectively.

In the present world, many anticancer agents with mode of action have been invented due to the increase in resistance of the cancer cell towards current anticancer drugs. Many researches show that a growing number of anti-microbial peptides are toxic for bacterial cells but not toxic for normal mammalian cell lines (Hoskin and Ramamoorthy, 2008). These antimicrobial sources can be natural sources or synthesized peptides. A growing number of researches showed Tyr (Esnouf, 1997) and Try (Zhang *et al.*, 2012) containing peptides can inhibit the growth of cancer cell line with no effect on normal cellular process, by assuming a critical role against the development and progression of MCF-7 cell line and many other type of cancer cell line. The positive charged Tyr and Try played a major role for selective disruption and strong binding with negatively charged component of cancer cell membrane by electrostatic attraction (Zwick *et al.*, 2001). However, it is difficult to understand why some host defense peptides are capable of killing cancer cells while others do not.

This investigation has confirmed that peptides containing compounds from *Zophobas morio* can kill tumour cells, but the details of the killing mechanism are yet to be understood and this should be further

investigated in future studies.

Conclusion

In summary, acidified organic solvent extracts of *Zophobas morio* were observed to cause a higher significant toxicity on cancer cells as compared to non-cancer cells. Most importantly, the results of this study demonstrated a novel and important uses of *Zophobas morio*, a darkling beetle larvae, as a source for anticancer peptides. It is believed that the anticancer activity of *Zophobas morio* proposed in this study presents a new and promising opportunity to be used as a therapeutic treatment for cancer. Further, initial identification confirms that crude sample contains Try and Tyr, implying that the compound contains aromatic amino acids which make the peptides.

Conflict of Interest

All authors don't have any conflict of interest.

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