Original Research

Microbial Growth Inhibitory Activities of Skin and Seed Extracts of *Pisum sativum*

Kiefer Lim Han Way, Hong-Wai Tham

Biopharmaceutical Research Unit, Biology Research Laboratory, Faculty of Pharmacy, SEGi University, Jalan Teknologi, Kota Damansara, 47810 Petaling Jaya, Selangor, Malaysia.

Abstract

Introduction: The improper use of antibiotics to treat various bacterial infections occurring in humans had caused the rise of antibiotic-resistant bacterial strains. These lead to an urgent need in developing novel antimicrobial compounds. **Objective:** The extracts of the skin and seeds of *Pisum sativum* were screened for antimicrobial activity against two different species of Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and one species of Gram-negative bacteria (*Escherichia coli*). **Materials and methods:** Methanolic crude extracts were prepared at different concentrations. Their antimicrobial activities were screened by disc diffusion assays and minimum inhibitory concentration (MIC) determination assays. **Results:** Antimicrobial inhibitory effects were observed in *B. subtilis*. However, further investigation is needed to identify the reasons of *S. aureus* and *E. coli* being resistant towards both types of phytochemical extracts. **Conclusions:** Our findings support the hypothesis that the skin and seeds of *P. sativum* possess antimicrobial properties. Subsequent studies should be driven towards the identification of key phytocompounds, which can be potentially developed into new plant-based antimicrobial agents.

Keywords: Antimicrobial activities; Methanolic extracts; *Pisum sativum*

Corresponding author:

Dr Hong-Wai Tham

Email: thamhongwai@outlook.my

Introduction

Antibiotics are medications that are widely used in the management of pathogenic infections. The increasing concerns on antibiotic-resistant bacterial strains have gathered research focus on phytochemicals which carry antimicrobial activity. As a valuable source of

bioactive compounds with potent antimicrobial activities, phytochemicals possess different chemical classes which could reverse the antibiotic resistance [1].

Pisum sativum (also known as garden pea or green pea) is a species originates from *Fabaceae* family, which usually consists of a pod where it contains several small spherical seeds. One of the first antimicrobial activities of *P. sativum* reported was the phenolic extracts of sprouted peas against *Helicobacter pylori*, a Gram-negative microorganism that leads to gastric ulcers [2]. The same study also reported that *P. sativum* exhibited a better antimicrobial property when its cotyledons were treated with aspirin.

Limited studies were conducted to investigate antimicrobial activities of *P. sativum* against bacteria. In 2014, the peel extracts of *P. sativum* exhibited antimicrobial activities against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonela enterica* [3]. In this study, the researchers showed that the methanolic extracts of *P. sativum* carried antimicrobial activities, while the aqueous extract did not. The findings were in accordance with some earlier studies reporting antimicrobial activities of *P. sativum* extracts derived from seeds and skins [4]. In another experiment, Nair *et al.* reported that the phytolectins isolated from the seeds of *P. sativum* exhibited antimicrobial activity against several bacterial species, including *P. aeruginosa* [5]. In recent years, the extracts of *P. sativum* have been used in green biosynthesis of silver nanoparticles in an effort to explore its antidiabetic, cytotoxicity, antioxidant, and antimicrobial activities against foodborne pathogens[6,7]. Its potential applications in nutraceutical formulations and food packaging have also been explored in some recent research articles [8,9]. Nevertheless, fundamental questions such as types of extraction and their specific antimicrobial abilities remained elusive.

Due to the limited information available on the antimicrobial properties of *P. sativum*, the methanolic extracts of its skin and seeds were of our interest in this study. The findings fill the existing research gaps in identifying novel modern antibiotics to treat microbial infections, especially those which caused by bacteria. Hence, this study aims to determine the level of antimicrobial activities in the extracts of skin and seeds of *P. sativum*.

Materials and methods

Procurement and preparation of samples

P. sativum was precured from a local grocery store located in Kota Damansara, Malaysia. The samples were opened using a sterile scalpel, and the seeds were removed from

the pods. The skin and seeds were placed separately in two sterile beakers and allowed to dry in an oven (50 ℃, 72 hours).

Preparation of microorganisms

Three types of microorganisms were used in this study. They were *Staphylococcus aureus* (cocci, Gram positive), *Bacillus subtilis* (rod-shaped, Gram positive) and *Escherichia coli* (rod-shaped, Gram negative). These microorganisms were cultured and maintained on Nutrient agars under aseptic environment.

Extraction of skin and seeds of *P. sativum*

The skin and seeds of *P. sativum* were dried (50 ℃, 72 hours) and ground into smooth powder form using a mortar and pestle. The weights of these powdered samples were recorded and placed in a cleaned conical flask. Methanol (100 mL) was added to cover the samples completely. Then, the conical flasks were sealed with parafilm, immersed partially into a beaker pre-filled with paraffin oil, which was placed on a hot plate set at 45 ℃. Using magnetic stirrers, the mixture was constantly mixed and heated at 45 ℃ for 72 hours.

Filtration of extracts

After extraction, the mixture was filtered using a filter funnel and a Whatman filter paper. The filtrate obtained was placed in a cleaned beaker, and incubated at 45 ℃ until it was completely dried. The weight of the dried extract was calculated by subtracting the total weight (beaker with the dried extract) with the original weight of empty beaker.

Reconstitution of extracts

The dried extracts were reconstituted with 2 mL of methanol. Mild sonication was applied to help dissolving the dried extracts. After the extracts have dissolved completely, the concentrations of methanolic extract were calculated and stored in -20 ℃ until used.

This procedure was repeated for determination of minimum inhibitory concentration (MIC), with the 2 mL of methanol substituted by 2 mL of sterile water.

Antimicrobial disc diffusion assays

Microbial broth cultures of all three microorganisms were prepared to 0.5 McFarland standard. Under aseptic conditions, 100 µL of each microorganism was transferred and spread

evenly onto Nutrient agars. All inoculated Nutrient agars were let dried before discs with extracts were placed.

Discs were prepared from Whatman filter paper using a paper puncher. The discs were autoclaved and dried before used. 10 µL of extracts were transferred onto each disc and allowed to dry in a biosafety cabinet. Dried discs were visually inspected before placed onto the surface of inoculated Nutrient agars. After that, all inoculated Nutrient agars with discs were incubated at 37 ℃ for 16 hours. Then, the diameters of inhibition zones were measured (in mm). All samples in this procedure were prepared in triplicates.

Determination of minimum inhibitory concentration (MIC)

MIC was conducted on *B. subtilis* as it showed promising results in antimicrobial disc diffusion assay.

Two sets of *P. sativum* skin and seed extracts with a series of different concentrations were prepared. Each sample was diluted by half using sterile Nutrient broth. After which, 10 µL of *B. subtilis* (prepared to 0.5 McFarland standard) were inoculated into each tube. These tubes were then incubated at 37 ℃ for 16 hours. Microbial growths were observed visually and on fresh Nutrient agar plates after that.

Statistical analyses

All statistical data were compiled and recorded in Microsoft Excel. Standard deviations and One-way ANOVA (reported in *P*-values) were conducted using the same software based on data obtained from samples prepared in biological triplicates.

Ethical clearance

Ethical clearance for this research project was granted by SEGi University Research Ethics Committee, with reference number SEGiEC/SR/FOP/12/2020-2021.

Results

Skin extract of *P. sativum*

The skin extracts of *P. sativum* were prepared in four independent assays. These extracts were reconstituted in methanol. The concentrations obtained were 0.12 g/mL, 0.28 g/mL, 1.03 g/mL, and 1.85 g/mL.

Seed extract of *P. sativum*

The seed extracts of *P. sativum* were prepared in three independent assays. These extracts were reconstituted in methanol, with concentrations recorded at 0.17 g/mL, 0.37 g/mL, and 0.51 g/mL.

Antimicrobial disc diffusion assays

For *P. sativum* skin extracts, our results indicated that only *B. subtilis* was susceptible to these samples, while *E. coli* and *S. aureus* had no visible inhibition zone was observed. Overall, the size of inhibition zones was in proportion to the concentrations of the extracts used in disc diffusion assays (Table 1). In addition, the concentration of skin extract was found to be significant in altering the antimicrobial activity of our samples ($P < 0.05$).

Table 1: Average diameters of inhibition zone (mm) of four *P. sativum* skin extracts against the test microorganisms.

Bacteria	Average diameters of inhibition zone (mm) / Concentrations of skin extracts (g/mL)			
	0.122 (g/mL)	0.277 (g/mL)	1.03 (g/mL)	1.85 (g/mL)
E. coli	0	0	0	0
S. aureus	0	0	0	0
B. subtilis	7 ± 0	10.7 ± 1.15	9.7 ± 1.5	10.7 ± 0.58

For *P. sativum* seed extracts, no visible inhibition zone was observed for *E. coli* and *S. aureus*. In addition, the concentration of extract at 0.16 g/mL did not show any antimicrobial inhibitory effect against *B. subtilis* (Table 2). However, as the extract concentrations increased to 0.37 g/mL and 0.51 g/mL, the growth of *B. subtilis* was inhibited (Table 2).

Table 2: Average diameters of inhibition zone (mm) of three *P. sativum* seed extracts against the test microorganisms.

Bacteria	Average diameters of zone of inhibition (mm) / Concentrations of seed extracts (g/mL)		
	0.16 (g/mL)	0.37 (g/mL)	0.51 (g/mL)
E. coli	0	0	0
S. aureus	0	0	0

B. subtilis	0	10.7 ± 0.58	12.3 ± 0.58

Determination of minimum inhibitory concentration (MIC)

Table 3 indicates the possible range of MIC where *P. sativum* skin or seed extract may be able to inhibit the growth of *B. subtilis*. For *P. sativum* skin extract, the range of concentration fell between 0.06 - 0.12 g/mL, whereas for the seed extract, it fell between 0.03 – 0.06 g/mL.

Table 3: Microbial inhibitory concentration (MIC) determination for *P. sativum* skin and seed extracts.

***P. sativum* skin extract**		***P. sativum* seed extract**	
Concentration (g/mL)	Presence of *B. subtilis* colonies on Nutrient agar	Concentration (g/mL)	Presence of *B. subtilis* colonies on Nutrient agar
1.85	No	0.51	No
0.93	No	0.26	No
0.46	No	0.13	No
0.23	No	0.06	No
0.12	No	0.03	**Yes**
0.06	**Yes**	0.02	**Yes**
0.00	**Yes**	0.00	**Yes**

Discussion

The methanolic skin extracts of *P. sativum* exhibited its antimicrobial effect against *B. subtilis*, while the same effect was not detected on *S. aureus* and *E. coli*. These findings were consistent with several previous studies although the strengths of inhibitions varied [3,4]. In our opition, such observations drew a clear distinction between spore-forming (*B. subtilis*) and non-spore forming (*S. aureus* and *E. coli*) bacterial species. Although additional purification processes and tests are required for our *P. sativum* methanolic skin extracts, at this stage, it is our speculation that the product extracted contained sporicidal compounds which have been previously reported by other researchers [10,11]. In addition, our results indicated that the concentration of *P. sativum* skin extract carries a statistically significant role in exhibiting its antimicrobial properties ($P < 0.05$). These findings highlighted the needs in identifying the key

phytochemical compounds in the skin of *P. sativum* that inhibit the growth of both Gram-positive and Gram-negative bacterial cells. In addition, it will be of researchers' interest in understanding the biomolecular interactions between these compounds and *B. subtilis*, which was once known as the causing agent of several serious infections [6], with a recent detailed study on its virulence potential which may be lethal to vertebrate animals [7].

Although the methanolic seed extract of *P. sativum* exhibited a similar trend towards the three tested microorganisms – *E. coli*, *S. aureus*, and *B. subtilis*, the strength of microbial inhibition of seed extract was reported higher than that of skin extract. This conclusion was made based on the minimum inhibitory concentration (MIC) assays, where the possible range of MIC recorded for skin and seed extracts were 0.06 – 0.12 g/mL and 0.03 – 0.06 g/mL, respectively.

It is worth highlighting that a previous study by Nair *et al.* has presented the antimicrobial effect of phytolectin extracted from the seeds of *P. sativum*. In the study, researchers extracted and purified pure phytolectin which were later reported to possess inhibitory effect against *E. coli*, *S. aureus* and *B. subtilis* [5].

In our study, we are confident that the phytocompounds that inhibit the growth of *B. subtilis* were found in the methanolic crude extracts of skin and seed of *P. sativum*. This again highlights the needs of purifying individual phytocompounds for subsequent detailed studies. The discovery of biomolecular interactions between these candidates and microscopic pathogens can be of great contributions towards the discovery and development of antimicrobial biopharmaceuticals.

Conclusion

In conclusion, the extracts of the skin and seed of *P. sativum* exhibited their antimicrobial properties against *B. subtilis*. However, based on our findings, the same samples did not exert any detectable antimicrobial activity against *E. coli* and *S. aureus*. This report serves as one of the first research findings focusing on antimicrobial properties of *P. sativum*, with parallel screenings on Gram-positive and Gram-negative bacteria in a single experimental design. Nevertheless, further investigations using various methods of phytocompound extraction should be conducted in near future.

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References

1. Khameneh, B., Iranshahy, M., Soheili, V. & Fazly Bazzaz, B. S. Review on plant antimicrobials: a mechanistic viewpoint. Antimicrob. Resist. Infect. Control 8, 118 (2019).

2. Ho, C.-Y., Lin, Y.-T., Labbe, R. G. & Shetty, K. Inhibition of Helicobacter Pylori by Phenolic Extracts of Sprouted Peas (pisum Sativum L.). J. Food Biochem. 30, 21–34 (2006).

3. Hadrich, F., Arbi, M. E., Boukhris, M., Sayadi, S. & Cherif, S. Valorization of the peel of pea: Pisum sativum by evaluation of its antioxidant and antimicrobial activities. J. Oleo Sci. 63, 1177–1183 (2014).

4. Saeed, S. & Tariq, P. Antibacterial activites of Mentha piperita, Pisum sativum and Momordica charantia. Pak. J. Bot. 37, 997–1001 (2005).

5. Nair, S. S., Madembil, N. C., Nair, P., Raman, S. & Veerabadrappa, S. B. Comparative analysis of the antibacterial activity of some phytolectins. Int. Curr. Pharm. J. 2, 18–22 (2013).

6. Patra, J. K., Das, G. & Shin, H.-S. Facile green biosynthesis of silver nanoparticles using Pisum sativum L. outer peel aqueous extract and its antidiabetic, cytotoxicity, antioxidant, and antibacterial activity. Int. J. Nanomedicine 14, 6679–6690 (2019).

7. Alarjani, K. M., Huessien, D., Rasheed, R. A. & Kalaiyarasi, M. Green synthesis of silver nanoparticles by Pisum sativum L. (pea) pod against multidrug resistant foodborne pathogens. J. King Saud Univ. - Sci. 34, 101897 (2022).

8. Castaldo, L. et al. Chemical Composition of Green Pea (Pisum sativum L.) Pods Extracts and Their Potential Exploitation as Ingredients in Nutraceutical Formulations. Antioxidants 11, 105 (2022).

9. Elsebaie, E. M. et al. Chitosan-Based Green Pea (Pisum sativum L.) Pod Extract Gel Film: Characterization and Application in Food Packaging. Gels 9, 77 (2023).

10. Abdelgani, A. M., Rukayadi, Y., Shaari, K. & Safinar, I. Antibacterial and Sporicidal Activities of Methanolic Sygygium polyanthum L. Leaf Extract against Vegetative Cells and Spores of Bacillus pumilus and Bacillus megaterium. J. Pure Appl. Microbiol. 12, 1047–1053 (2018).

11. Kusuma, S. A. F., Septyadi, R. & Sofian, F. F. Inhibition of bacillus spores germination by cinnamon bark, fingerroot, and moringa leaves extract. J. Adv. Pharm. Technol. Res. 13, 7–10 (2022).

12. R, S., S, R. & Dm, S. Serious infections caused by Bacillus species. Medicine (Baltimore) 66, (1987).

13. Gu, H.-J., Sun, Q.-L., Luo, J.-C., Zhang, J. & Sun, L. A First Study of the Virulence Potential of a Bacillus subtilis Isolate From Deep-Sea Hydrothermal Vent. Front. Cell. Infect. Microbiol. 9, 183 (2019).