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# ANTIMICROBIAL ACTIVITIES AND TOXICITY OF CRUDE EXTRACT OF THE *PSOPHOCARPUS TETRAGONOLOBUS* PODS

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## Abstract

The extract of the *Psophocarpus tetragonolobus* pods has been tested for antimicrobial activity in a disk diffusion assay on eight human pathogenic bacteria and two human pathogenic yeasts. The extracts of *P. tetragonolobus* possessed antimicrobial activity against all tested strains. The ethanolic extract of *P. tetragonolobus* pods was further tested for *in vivo* brine shrimp lethality test and *in vitro* sheep erythrocyte cytotoxic assay. The brine shrimp lethality test exhibited no significant toxicity ($LC_{50}$=1.88 mg/ml) against *Artemia salina*, whereas sheep erythrocyte test showed significant toxicity. The reason for haemolysis of erythrocyte was discussed. The *P. tetragonolobus* extract with high $LC_{50}$ value signified that this plant is not toxic to human. This result also suggested that the ethanolic extract of *P. tetragonolobus* pods is potential source for novel antimicrobial compounds.

**Keywords:** Antimicrobial activities; *Artemia salina*; Cytotoxicity assay; *P. tetragonolobus*

## Introduction

*Psophocarpus tetragonolobus* L. (Leguminosae) is found throughout Malaysia. Native people use the pods of *P. tetragonolobus* for food preparation. The *P. tetragonolobus* pods are used as traditional medicine for many years (Burkil, 1935). In New Guinea, the pods and the edible tubers are considered roborant (Stop, 1962). Leaves and seed are eaten to cure skin sores such as boils and ulcers (Perry, 1980). Not withstanding the widespread use of *P. tetragonolobus* in traditional medicine and despite the fact that many plants exhibit significant toxicity, same as enhancement of mutagenecity, carsinogenecity or embryotoxicity, no toxicological study has been undertaken on this species. Therefore, the aim of the present work was to study the toxicity the crude extract of *P. tetragonolobus* pods against *Artemia salina* and sheep erythrocytes, and antimicrobial activities of this extract.

The toxicity activity of brine shrimp (*Artemia salina*) assay was developed by Michael et al. (1956) and adapted by others (Meyer et al., 1982; Solý´s et al., 1993). It is a convenient preliminary toxicity test, since the brine shrimp is highly sensitive to a variety of chemical substances. The assay is considered a useful tool for preliminary toxicity assessment of plant extract (McLauglin et al., 1991; Solý´s et al., 1993).

## Materials and methods

### *P. tetragonolobus* Sample

Fresh *P. tetragonolobus* pods (with voucher number NP2305 ) were collected from Penang, Malaysia, and authenticated by the botanist of School of Biological Sciences at Universiti Sains Malaysia, where the herbarium was deposited. The plant materials were dried in an oven at 60 °C.

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**Preparation of crude extract**

The dried pods of *P. tetragonolobus* were cut into small pieces. The cut pods (100 g) were extracted in a soxhlet with 300 ml of 80% ethanol (v/v) for 4 h. The entire extract of *P. tetragonolobus* pods was evaporated to dryness under reduced pressure. The dried extract was then re-dissolved in 80% methanol (v/v) to yield solution containing 100 mg of extract per ml.

**Antimicrobial activity**
**Microbial strains**

Gram-positive bacteria *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, Gram-negative bacteria: *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Salmonella typhi*, and yeast *Candida albicans* and *Rhodotorula rubra* used as test organisms were obtained from the stock culture of laboratory, University Science of Malaysia. Stock cultures were maintained at 4 °C on slopes of Tryptic soy broth (BBL, Cockeysville, MD) amended with 5 g/l Yeast extract (Oxoid, Nepean, ON) and 15 g/l Agar agar (BDH, Toronto, ON). Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to flasks of Mueller–Hinton broth (MHB) (Oxoid) for bacteria and Sabouraud dextrose broth (SDB) for yeast that were incubated without agitation for 24 h at 37 and 25 °C. The cultures were diluted with fresh Mueller–Hinton and Sabouraud dextrose broth to achieve optical densities corresponding to $2.0\times10^6$ colony forming units (CFU/ml) for bacteria and $2.0\times10^5$ spore/ml for yeast strains.

**Determination of antibacterial activity by the disc diffusion method**

The extract was tested for antibacterial activity by the disc diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS, 2001) using 100 µl of suspension of the tested microorganisms; containing $2.0\times10^6$ CFU/ml for bacteria and $2.0\times10^5$ CFU/ml spore for fungal strains. Mueller–Hinton agar (MHA) (Oxoid) and Sabouraud dextrose agar (SDA) sterilized in a flask and cooled to 45–50 °C were distributed to sterilized Petri dishes with a diameter of 9 cm (15 ml). The filter paper discs (6 mm in diameter) were individually impregnated with 10 and 20 µl of the crude extract and then placed onto the agar plates previously inoculated with the tested microorganisms. The Petri dishes were kept at 4 °C for 2 h. The plates were incubated at 37 °C for 24 h for bacteria and at 30 °C for 48 h for the yeast. The diameters of the inhibition zones were measured in millimetres. All the tests were performed in duplicate. Vancomycin (30 µg), tetracycline (30 µg) and nystatin (30 µg) served as positive controls.

**Toxicity testing against the brine shrimp**
**Hatching shrimp**

Brine shrimp eggs, *Artemia salina* were hatched in artificial seawater prepared by dissolving 38g of sea salt in 1L of distilled water. After 24-h incubation at room temperature ($22\ ^0C$ - $29\ ^0C$), the larvae were attracted to one side of the vessel with a light source and collected with pipette. Larvae were separated from eggs by aliquoting them three times in small beakers containing seawater.

**Brine shrimp assay**

Bioactivity of the extract was monitored by the brine shrimp lethality test (Meyer et al., 1982). Samples were dissolved in 10% dimethylsulphoxide (DMSO) (v/v) and diluted with artificial seawater. Two ml of seawater was placed in all the bijoux bottles. A two-fold dilution was carried out to obtain the concentration from 10 mg/ml to 0.1525 mg/ml. The last bottle was left with sea salt water and 10% DMSO (v/v) only, serving as the drug free control. Hundred micro liters of suspension of larvae containing about 10 -15 larvae was added into each bottle and incubated for 24 h. The bottles were then examined and the number of dead larvae in each bottle was counted. The total number of shrimp in each bottle was counted and recorded. The mean percentage mortality was plotted against

the logarithm of concentrations. The concentration ($LC_{50}$), at which 50% of the larvae were killed, was determined from the graph.

**Data analysis**

The mean results of brine shrimp mortality against the logarithms of concentrations was plotted using the Microsoft Excel computer program, which also gives the regression equations. The regression equations were used to calculate $LC_{50}$ value. Extracts giving $LC_{50}$ values greater than 20µg/ml was considered non-toxic (Geran et al., 1972).

**Determination of cytotoxicity of sheep erythrocyte**

Ten-fold dilution of the extract was made in phosphate-buffered saline. A total volume of 0.8 ml for each dilution was place in an Eppendorf tube. A negative control tube (Containing saline only) and a positive control tube (containing tap water) were also included in the analysis. Fresh sheep erythrocytes were added to each tube, ($2.5\times10^5$ cells per tube) to give a final volume of 1 ml. Solution was incubated at 37°C for 30 min and then all tubes were centrifuged for 5 min, The degree of haemolysis was determined by reading the optical density of the supernatant with a spectrophotometer (GAT UV-9100) at 405 nm.

**Table 1:** Antimicrobial activity of the crude extract of *P. tetragonolobus* pods by the disc diffusion method

| **Microorganisms** | **Crude extract zone inhibition (mm)** | | **Antimicrobial agents zone inhibition (mm)** | | |
|---|---|---|---|---|---|
| | **10 µl/disc** | **20 µl/disc** | **Vancomicin 30 µg/disc** | **Tetracycline 30 µg/disc** | **Nystatin 30 µg/disc** |
| Gram positive | | | | | |
| *Bacillus cereus* | 14 | 23 | 12 | 28 | nd* |
| *B. subtilis* | 17 | 21 | 15 | 24 | nd |
| *Staphylococcus aureus* | 28 | 34 | 16 | 26 | nd |
| Gram negative | | | | | |
| *Escherichia coli* | 18 | 21 | 22 | 28 | nd |
| *Klebsiella pneumoniae* | 14 | 17 | 11 | 26 | nd |
| *Pseudomonas aeruginosa* | 7 | 12 | 8 | 11 | nd |
| *Proteus mirabilis* | 22 | 24 | 20 | 24 | nd |
| *Salmonella typhi* | 16 | 19 | 19 | 22 | nd |
| Yeast | | | | | |
| *Candida albicans* | 23 | 32 | nd | Nd | 25 |
| *Rhodotorula rubra* | 17 | 20 | nd | Nd | 25 |

*nd : not detected

**Table 2:** Toxicity of crude extract of *Psophocarpus tetragonolobus* pods against brine shrimp larvae (*Artemia salina*)

| Concentration (mg/ml) | $Log_{10}$Concentration | Number dead | Number survived | Percent mortality (%) |
|---|---|---|---|---|
| Control | - | 0.00 | 15.00 | 0 |
| 10.00 | 1.000 | 13.00 | 3.00 | 86.67 |
| 5.00 | 0.699 | 12.00 | 3.00 | 80.00 |
| 2.50 | 0.398 | 9.00 | 6.00 | 60.00 |
| 1.25 | 0.097 | 7.00 | 8.00 | 46.67 |
| 0.63 | -0.204 | 3.00 | 12.00 | 20.00 |
| 0.31 | -0.505 | 1.00 | 14.00 | 6.67 |
| 0.16 | -0.903 | 0.00 | 15.00 | 0.00 |

The $LC_{50}$[a] was obtained by linear regression equations. $LC_{50}$ value lower than 20µg/ml was considered non-toxic.
[a] $LC_{50}$ value of crude extract of *Psophocarpus tetragonolobus* pods was 1.88 mg/ml.

## Results and discussion

### Antimicrobial assay

As shown in Table 1, the extract of *P. tetragonolobus* pods had great *in vitro* potential of antimicrobial activities against all 8 bacteria and 2 yeast species tested. In this study, the antimicrobial activities of extract at two different concentrations of 10 and 20 µl/discs are compared with those of positive control such as vancomicin, tetracycline and nystatin. The data obtained from the disc diffusion method (Table 1) indicated that the extract displayed a variable degree of antimicrobial activity on different tested strains. The inhibitory effect increased with increase of the extract concentration from 10 to 20 µl. Gram-positive *S. aureus* was the most sensitive strain with the strongest inhibition zones (28 – 34 mm). The crude extract also exhibited high antimicrobial activity against *B. subtilis* and *B. cereus*. Among these, Gram-negative strains also displayed variable degree of susceptibility to investigated extract. Maximum activity was observed against *P. mirabilis* (22–24 mm), followed by *E. coli* (18–21 mm), *Salmonella typhi* (16-19 mm), and *Klebsiella pneumoniae* (14-17 mm). Gram-negative bacteria, *P. aeruginosa* exhibited weak inhibition zones (7–12 mm), which is in accordance with the fact that it has high level of intrinsic resistance to virtually all known antimicrobials and antibiotics due to a combination of a very restrictive outer membrane barrier, and it is highly resistant even to synthetic drugs. The antimicrobial activity of this extract was also observed on the yeasts *C. albicans* (24–36 mm) and *Rhodotorula rubra* (17-20 mm).

### Toxicity studies

As shown in Table 2, the extract showed no significant toxicity against brine shrimp ($LC_{50}$ = 1.88 mg/ml). The results on brine shrimps assay indicate that the extract has $LC_{50}$ value greater than 20µg/ml; the recommended cutoff point for detecting cytotoxic activity (Geran et al., 1972). This signified that *P. tetragonolobus* pod might not be toxic to human.

Haemolysis of erythrocyte was observed at all dilution of the crude extract from 1:1 to 1:1000 to a similar degree as in a positive control of tap water, whereas the negative control containing only saline exhibited no haemolysis. It is not surprising that the crude extract of *P. tetragonolobus* pod demonstrates haemolysis of sheep erythrocytes. This assay of cytotoxic activity is extremely sensitive to a wide range of compounds and may be due to any number of the phytochemicals within the crude preparation (He, et al., 1994). Reevaluation of the active antimicrobial principle in the crude extract will be necessary to determine if the haemolysis is due to the compound itself or some other chemical constituent.

**Conclusion**

The extracts of *P. tetragonolobus* pods may be useful as an alternative antimicrobial agent as natural medicine for the treatment of many infectious diseases because of its potency.

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