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In vitro study of cytotoxic activity of the different extracts of the tubers of *Pueraria tuberosa*

Trupti P. Durgawale[1*], Pratik P. Durgawale[2] and Chitra C. Khanwelkar[3]

[1]*Ph.D. candidate, Department of Pharmacology, Krishna Institute of Medical Sciences, Karad, Maharashtra, India*
[2]*Research Officer, Molecular and Genetic Laboratory, Krishna Institute of Medical Sciences, Karad, Maharashtra, India*
[3]*Professor and Head of Department, Department of Pharmacology, Krishna Institute of Medical Sciences, Karad, Maharashtra, India*

ABSTRACT

Different extraction techniques such as Soxhlet extraction, microwave-assisted extraction, and maceration were used to prepare aqueous, ethanolic, and methanolic extracts of Pueraria tuberosa tubers separately. For a comparative study of their cytotoxic activities in-vitro, brine shrimp lethality assay was used. The ethanolic extract of P. tuberosa tubers obtained using the microwave-assisted technique exhibited the highest cytotoxicity (LC_{50} = 1.56 µg/mL). Vincristine sulphate (LC_{50} = 0.367 µg/mL) was used as the positive control. Further phytochemical analysis and in-vivo studies need to be done to determine its potential as a source of cytotoxic drug candidates.

Keywords: *Pueraria tuberosa*, brine shrimp lethality assay.

INTRODUCTION

Interest in phytochemicals as a pharmacological tool has been increasing in the past decades. The importance of phytochemicals has been fortified by the research of polyphenols obtained from plants which exhibit activity against cardiovascular diseases, postmenopausal symptoms, and cancer [1-4]. *Pueraria tuberosa* is a perennial, woody climber belonging to family Fabaceae. *P. tuberosa* has been used in traditional Indian, Chinese, and Japanese medicines [5, 6]. The tubers of this plant are known to be a rich source of various isoflavanoids including puerarin, genistin, daidzein, and genistein [7]. Studies have proven an association of isoflavanoids and reduced risk of cancer in humans [8, 9]. Hence, in this study we attempted to study the *in-vitro* cytotoxic activity of different extracts of *P. tuberosa* tuber extracts. These extracts were separately prepared using Soxhlet apparatus, microwave-assisted extraction, and maceration techniques and their cytotoxicity was studied using the brine shrimp lethality assay.

MATERIALS AND METHODS

1. Collection and preparation of the plant materials – *P. tuberosa* tubers sample was provided as a gift by Mr. Vivek Gourbroom. After proper authentification, the tubers were dried and grinded into a coarse powder with a mortar-pestle and stored in a dry place away from direct sunlight.
2. Extraction- The extraction techniques used were as follows:
a) Soxhlet Extraction-

i. Ethanolic extract -100 gram (g) of powdered tubers was extracted with 95% ethanol in Soxhlet apparatus at 60 - 70°C for 18 hours (hrs). The extract obtained was concentrated to dryness in a heating mantle at 35 - 40°C.The thick paste of extract in the beaker was kept in freezer at temperatures below 4°C until required for experimental study.

ii. Butanolic extract- 100 g of powdered tubers sample was extracted with n-butanol in Soxhlet apparatus at 60 - 70°C for 18 hrs. The extract obtained was concentrated to dryness in heating mantle at 35 - 40°C.The thick paste of extract obtained was kept in freezer at temperatures below 4°C until required for experimental study.

b) Aqueous extract by maceration - 100 g of powdered tubers sample was taken in a round bottom flask and macerated with 500 milliliter (mL) distilled water for 24 hr with occasionally shaking every 1 hr and 10 ml of chloroform was added as a preservative. The marc was removed by filtering the extract using Whatman filter paper and was concentrated on water bath at 50°C. The extract was then stored in refrigerator until needed.

c) Microwave-assisted extraction:

i. Ethanolic extract - 5 g of powdered tubers of *P. tuberosa* was extracted in 25 mL ethanol as solvent in a microwave set at 560 watt for 10 minutes (min).

ii. Butanolic extract -5 g of powdered tubers of *P. tuberosa* was extracted in 25 mL ethanol as solvent in a microwave set at 490 watt for 10 min.

3. Experimental Procedure:

Brine shrimp lethality bioassay is widely used in the *in-vitro* cytotoxicity study of bioactive compounds. In this study, cytotoxicity screening of the samples was carried out against a simple zoological organism, brine shrimp nauplii. *Artemia salina* Leach (brine shrimp eggs) were placed in a small tank containing 3.8 per cent (%) non-iodized sodium chloride solution (sea water) for two days for the eggs to hatch and mature as nauplii. 4 milligram (mg) of each fraction was dissolved in 800 microlitre (µL) of dimethyl sulfoxide (DMSO). Then 100 µL of solution was added to a test tube containing 4.9 mL of sea water and 10 shrimp nauplii. Thus, final concentration of the first test tube solution was 100 microgram per milliliter (µg/mL). Then a series of solutions of varying concentrations was prepared from the stock solution by serial dilution. Thus, the concentrations of the obtained solution in individual test tubes were 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.125 µg/mL, 1.5625 µg/mL, and 0.78125 µg/mL respectively. 0.2 mg of Vincristine sulphate, as the positive control, was dissolved in DMSO to get an initial concentration of 20 µg/mL from which serial dilutions were made using DMSO to get 10 µg/mL, 5 µg/mL, 2.5µg/mL, 1.25 µg/mL, 0.625 µg/mL, 0.3125 µg/mL, 0.15625 µg/mL, 0.078125 µg/mL, and 0.0390 µg/mL solutions respectively. The positive control solutions also contained 10 living brine shrimp nauplii each in 5 mL sea water. For negative control, 100 µL of DMSO was added to the premarked test tubes containing 4.9 mL of sea water and 10 shrimp nauplii. After 24 hours of incubation, the test tubes were inspected using a magnifying glass and the number of survivors were counted. The concentration-mortality data were analyzed statistically using probit analysis for the determination of half maximal lethal concentration (LC_{50}) values and linear regression for the fractions.

RESULTS AND DISCUSSION

The isoflavanoids derived from different plant sources are known to have anti-oxidant and anti-proliferative properties [10]. The brine shrimp lethality assay has been used for a long time to study the *in-vitro* cytotoxic effects of active plant ingredients at a preliminary stage in drug development [11-15]. In this assay, a fixed number of brine shrimp nauplii are added separately to brine solution containing known concentration of the test compound. After incubation, the number of live nauplii are counted and compared to a control containing the solvent instead of the test compound. In this study, vincristin sulphate was used as a positive control to compare the LC_{50} values of the test compounds. Following the procedure, the lethality of the ethanolic, butanolic, and aqueous extract of *P. tuberosa* tubers using brine shrimp was evaluated and summarized in *Table 1* and *Figure 1* below.

Table 1: Brine shrimp lethality assay results after 24 hr exposure

Sample	LC_{50} (µg/ml)
VS *	0.367
EPT	6.25
EMPT	1.56
BPT	25
BMPT	12.5
APT	3.125

VS - Vincristin sulphate ('' stands for 'positive control').*
EPT - Ethanolic extract of P. tuberosa using Soxhlet apparatus.
EMPT- Microwave-assisted ethanol extract of P. tuberosa.
BPT- Butanolic extract of P. tuberosa using Soxhlet apparatus.
BMPT- Microwave-assisted butanolic extract of P. tuberosa.
APT- Aqueous extract of P. tuberosa using maceration.

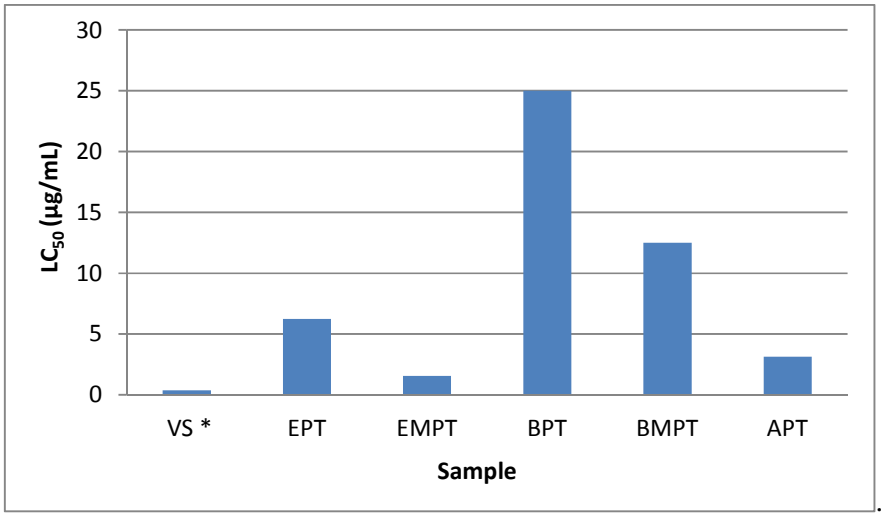

Figure 1: Representation of the LC_{50} values of different extracts and positive control

The negative control tubes did not show any brine shrimp lethality. In this bioassay, microwave-assisted ethanolic extract and aqueous extract of *P. tuberosa* tubers revealed prominent cytotoxicity with LC_{50} values of 1.56 µg/mL and 3.12 µg/mL respectively. Soxhlet apparatus-extracted ethanolic extract showed moderate cytotoxicity having LC_{50} value of 6.25 µg/mL; whereas, poor cytotoxicity was exhibited by Soxhlet apparatus-extracted butanolic extract and microwave-assisted butanolic extract having the LC_{50} values 25 µg/ml ,12.5 µg/ml respectively.

CONCLUSION

The ethanolic extract of *P. tuberosa* tubers obtained using microwave-assisted technique exhibited the most potent *in-vitro* cytotoxic activity. Further identification of phytochemicals using gas chromatography- mass spectrometry and *in-vivo* pharmacological studies of this sample could help determine the its active constituents and their cytotoxicity potential.

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