EVALUATION OF IN VITRO HEPATIC TOXICITY OF LEAVES OF PTEROSPERMUM ACERIFOLIUM (L.) WILLD.

RANA DATTA¹, SANKHADIP BOSE², SUDIP KUMAR MANDAL³§

¹Department of Pharmacology, Gupta College of Technological Sciences, Asansol, West Bengal, India. ²Department of Pharmacognosy, Bengal School of Technology, Hooghly, West Bengal, India. ³Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Dr. B. C. Roy College of Pharmacy and Allied Health Sciences, Bidhan Nagar, West Bengal, India. Email: gotosudip79@gmail.com

Received: 31 January 2020, Revised and Accepted: 18 March 2020

INTRODUCTION

The demand for herbal medicine has increased tremendously in recent years; however, there are many issues regarding their safety. Very less (<10%) marketed herbal products are actually standardized. Furthermore, quality control measures are not followed on many occasions [1]. Even in the literature, very little toxicological information is available. Since some plants produce toxic constituents for defense purposes, it becomes absolutely necessary to explore the safety profile of herbal drugs. The plant *Pterospermum acerifolium* (L.) Willd., belonging to the family Sterculiaceae, is used by the tribes of Chotamagar, Konkan, and Arunachal Pradesh region of India, for the treatment of different diseases such as wound healing and hemostatic activities [2,3].

However, no toxicological data are available for the plant. Preliminary phytochemical studies showed the presence of alkaloids, flavonoids, and glycosides in the methanolic extract of *P. acerifolium* (L.) Willd. (MEPA). Conventionally, the presence of alkaloids has been implicated to cytotoxicity; thus, it is worth to elucidate the toxicological profile of MEPA both from therapeutic and toxicological standpoint. Herbal medicines are normally considered safe, but chronic administration may lead to cumulative toxicity. Therefore, it is equally important to evaluate the cytotoxicity and reduction in optical density were also observed with incremental MEPA administration.

**METHODS**

**Preparation of extracts**

The leaves of *P. acerifolium* were collected from Asansol, West Bengal, in the month of September 2013 and 2014 at 11 a.m. The plant was identified and authenticated as *P. acerifolium* (L.) Willd. by the Director, Acharya Jagadish Chandra Bose Indian Botanic Garden, Shibpur, Howrah, India. The leaves of *P. acerifolium* were dried in the shade of about 30°C and crushed into a coarse powder.

**Cell culture**

Hepatocellular carcinoma cell line HepG2 was purchased from National Facility for Animal tissue and cell culture, Pune, India, and supplied from Indian Institute of Chemical Biology for in vitro studies. Hepatoma cells were subcultured after every 2 days at an initial concentration of 1×10⁵ cells/ml and maintained in sterile Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum [5]. The culture was maintained at 37°C in a humidified atmosphere containing 5% CO₂ in the air [6]. During subculturing or during the use of HepG2 in experiments, this adherent property has been diminished by adding 1× Trypsin solution in the cell [7]. In all the experiments, untreated hepatic cells were used as a control group.

**Preparation and extraction of the tested plant sample**

Leaves of *P. acerifolium* (L.) Willd. were air-dried in the shade. The dried leaves were extracted with methanol, were crushed, and then extracted by continuous hot extraction process for 72 h in Soxhlet apparatus, using a reflux condenser. Then, the solvent was removed by filtration. Fresh solvent was added and further extracted for 3 h. The extract was concentrated by vacuum under reduced pressure. Thereafter, the extract was lyophilized for 4 h to produce methanol free extract. It was kept in a container, sealed with parafilm and stored at 4°C in an airtight container, and was designated as MEPA. Stock solution was prepared as 1 mg/ml in phosphate buffer saline (PBS) from which desired doses were tested.
Preliminary phytochemical studies
Preliminary phytochemical studies showed the presence of alkaloids, flavonoids, and glycosides in the MEPA.

Cytotoxicity study by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay
Cytotoxicity studies after trypsinization of HepG2 cells (1×10⁴), 100 µl cell suspension per well in a log phase were seeded in 96 well tissue culture plates. They were treated with MEPA freshly prepared 1 mg/ml stock solution in a concentration of 25 µg, 50 µg, 100 µg, 200 µg, and 300 µg for 24, 48, and 72 h at 37°C in a humidified atmosphere containing 5% CO₂ in the air. Untreated cells served as control. Sorafenib tosylate was used as a standard [8]. At the end of treatment, in the respective time, the media from the upper layer have been removed; then again, 100 µl DMEM media was added. Then, 2.0 µl of MTT (5 mg/ml in PBS) as a stock solution was added to each well and incubated for another 4 h [9]. The MTT assay is a colorimetric assay for measuring the activity of enzymes that reduce MTT to formazan dyes, giving a purple color [4]. A solubilization solution dimethyl sulfoxide 100 µl was added to dissolve the insoluble formazan product into a colored solution [10,11]. The absorbance was taken at 570 nm by microplate manager (Reader type: Model 680 XR Bio-Rad Laboratories Inc.) inhibitory concentration 50 (IC₅₀) value for HepG2 cell line was determined after 24 h. The IC₅₀ value was determined (for the cell lines) and the cytotoxicity study was again performed using ½ IC₅₀, IC₅₀, and 2 IC₅₀ doses.

Statistical analysis
Statistical analysis was done by Student’s t-test. p<0.05 was considered as statistically significant.

The percentage cell inhibition was calculated by the following formula:

\[
\% \text{Cell inhibition} = \left[\frac{\text{OD of control} - \text{OD of treated}}{\text{OD of control}}\right] \times 100
\]

OD = Optical density

The percentage cell viability was calculated by the formula:

\[
\text{Viable cells} (%) = \left(\frac{\text{Total number of viable cells per ml}}{\text{Total number of cells per ml}}\right) \times 100
\]

RESULTS
MEPA at concentrations of 100 µg, 200 µg, and 300 µg significantly (p<0.05) inhibited the growth of HepG2 cells compared with that of the control cells after 24, 48, and 72 h of treatment in a concentration-dependent manner (Fig. 1). In the MTT assay, there was a significant (p<0.05) concentration-dependent reduction in the OD values after treating the HepG2 cells with the same concentrations of MEPA for 24, 48, and 72 h compared to the control cells (Fig. 2). These observations provided proof for cytotoxic nature of MEPA. Cytotoxicity of sorafenib tosylate was, however, much greater than MEPA [IC₅₀ of 2.09 µg/ml] [12-14]. The IC₅₀ as calculated after MTT assay was 150.42 µg/ml for HepG2 cells. Another graph of MTT assay taking ½ IC₅₀, IC₅₀, and 2 IC₅₀ doses for 24 h showed a dose-dependent decrease in OD at 570 nm (Fig. 2).

DISCUSSION
Preliminary phytochemical studies showed the presence of an ample amount of alkaloids. The observed cytotoxicity may be due to these phytoconstituents; alkaloids have historically been found to possess such properties. Therapeutically such cytotoxicity may be a constraint in the usage of MEPA for various in vivo ailments such as diabetes and wound healing activity. The dosage regimen and the duration of therapy are important parameters that may guide the researchers to balance between its beneficial and potential toxic properties. Since the study was carried out using whole MEPA, further isolation and characterization of the bioactive principles may provide potent molecules for targeting cancerous cells. Furthermore, from toxicological point of view, identification and separation of such principles may enhance the benefit: Risk ratio of the herbal drug.
REFERENCES

1. Pelkonen O, Xu Q, Fan TP. Why is research on herbal medicinal products important and how can we improve its quality? J Tradit Complement Med 2004;4:1-7.
2. Chatterjee P, Chakraborty B, Dwivedi A, Datta R. Pterospermum acerifolium (L.) Willd: A comprehensive review with significant pharmacological activities. Int J Pharm Life Sci 2012;3:1153-8.
3. Basu KR, Basu BD. Indian Medicinal Plants. 2nd ed. New Delhi: Bishen Singh Mahendra Pal Singh; 1987.
4. Van Meerloo J, Kaspers GJ, Cloos J. Cell sensitivity assays: The MTT assay. Methods Mol Biol 2011;731:237-45.
5. Wilkening S, Stahl F, Bader A. Comparison of primary human hepatocytes and hepatoma cell line HepG2 with regard to their biotransformation properties. Drug Metab Dispos 2003;31:1035-42.
6. Hewitt NJ, Lechon MJ, Houston JB, Halifax D, Brown HS, Maurel P, et al. Primary hepatocytes: Current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. Drug Metab Rev 2007;39:159-234.
7. Sinz M, Kim S. Stem cells, immortalized cells and primary cells in ADMET assays. Drug Discov Today Technol 2006;3:79-85.
8. Soldatow VY, Le Cluyse EL, Griffith LG, Rusynet I. In vitro models for liver toxicity testing. Toxicol Res 2013;2:23-39.
9. Rodrigues RM, De Kock J, Branson S, Vinken M, Meganathan K, Chaudhari U, et al. Human skin-derived stem cells as a novel cell source for in vitro hepatotoxicity screening of pharmaceuticals. Stem Cells Dev 2014;23:44-55.
10. Brecht C. Pathogenesis of hepatitis B virus-related hepatocellular carcinoma: Old and new paradigms. Gastroenterology 2004;127:S56-61.
11. Yu MC, Yuan J. Environmental factors and risk for hepatocellular carcinoma. Gastroenterology 2004;127:S72-8.
12. Block TM, Mehta AS, Fimmel CJ, Jordan R. Molecular viral oncology of hepatocellular carcinoma. Oncogene 2003;22:5093-107.
13. Dey S, Roy S, Deb N, Sen KK, Besra SE, Anti-carcinogenic activity of Rauvillia tuberosa L. (Acanthaceae) leaf extract on hepatoma cell line and increased superoxide dismutase activity on macrophage cell lysate. Int J Pharm Pharm Sci 2013;5:854-61.
14. Carter CA, Chen C, Brink C, Vincent P, Maxuitenko YY, Gilbert KS, et al. Sorafenib is efficacious and tolerated in combination with cytotoxic or cytostatic agents in preclinical models of human non-small cell lung carcinoma. Cancer Chemoth Pharm 2007;59:183-95.