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In Vitro Hepatoprotective Activity of *Limnanthemum Indicum* [Whole Plant] Against Paracetamol Induced Toxicity Using Brl-3a Cell Line

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ABSTRACT

Liver plays a major role in detoxification. Any injury to it or impairment of its function may lead to many implications on one's health. Management of liver diseases is still a challenge to modern medicine. The allopathic medicine has little to offer for the alleviation of hepatic ailments whereas the most important representatives are of phytoconstituents. The work presented in this paper is on plant *Limnanthemum indicum* which is reported that the whole plant is traditionally used as hepatoprotective. The study was aimed at evaluation of the hepatoprotective activity of the whole plant of *Limnanthemum indicum* on the BRL3A (Rat, Liver cell line) cell line. The various solvent extracts were tested for its inhibitory effect on BRL3A (Rat, Liver cell line) cell line. The percentage viability of the cell line was carried out. The cytotoxicity of *Limnanthemum indicum* on rat liver cell was evaluated by the MTT assay [(3-(4,5 dimethylthiazole –2 yl)-2,5 diphenyl tetrazolium bromide) assay]. The principle involved is the cleavage of tetrazolium salt MTT into a blue coloured derivative by living cells which contains mitochondrial enzyme succinate dehydrogenase However, the information available on the pharmacological activity of the plant is very limited. Hence, it was proposed to carry out a preliminary in vitro analysis of the hepato protective activity of the plant. Out of the various extracts evaluated, ethanolic extract of *Limnanthemum indicum* gave promising results.

Keywords: Hepatoprotective, Rat liver cell line, MTT, Mitochondrial

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INTRODUCTION

Liver disease is a worldwide problem. Liver is an organ of paramount importance as it plays an essential role in maintaining the biological equilibrium of vertebrates. The spectrum of functions include: metabolism and disposition of chemicals (xenobiotics) to which the organ is exposed directly or indirectly; metabolism of lipids, carbohydrates and proteins; blood coagulation and immunomodulation [1].

Treatment options for common liver diseases such as cirrhosis, fatty liver and chronic hepatitis are problematic. The conventional drugs used in the treatment of liver diseases viz., corticosteroids, antiviral and immunosuppresive agents are sometimes inadequate and may lead to serious adverse effects. Paradoxically, these may themselves cause hepatic damage. Eg: Cholestatic jaundice with azathioprine and elevation of serum transaminases by Interferon and Virazole[2]. It is therefore imperative to search alternative drugs for the treatment of liver disease to replace the currently used drugs of doubtful efficacy and safety [3].

In India, numerous medicinal plants are used for liver disorders in traditional systems of medicine. Some of these plants are evaluated for their hepatoprotective actions against hepatotoxins. However, the readily available hepatoprotective herbal drugs are not sufficiently active to effectively combat severe liver disorders. Therefore, there is a need to develop satisfactory hepatoprotective drugs.

Limnanthemum indicum is an aquatic floating herb commonly called as water snow flake belongs to the family Menyanthaceae[4]. It is traditionally used as bitter, febrifuge and antiscorbutic[5]. It is used as a substitute for *Swertia chirata* for the treatment of fever and jaundice [6]. It is reported that *Limnanthemum indicum* contains different sub types of flavonoids[7]. It is used as a substitute of Ayurvedic drug *Tagara* in the treatment of various diseases like epilepsy [8], anemia, jaundice, tuberculosis [9]. It is reported that the whole plant of *Limnanthemum indicum* is traditionally used as hepatoprotective[10]. *Limnanthemum indicum* has also been reported to show a good anti-proliferative activity [11].

After scrutiny of published literature showing its medicinal importance, the present protocol has been outlined regarding the in vitro hepatoprotective activity on this selected plant using different extracts. It is in view of this, that an attempt was made to evaluate the in vitro hepatoprotective activity of *Limnanthemum indicum*, using different plant extractions(Petroleum Ether, Chloroform, Ethyl Acetate, Ethyl Alcohol, Distilled Water) against paracetamol induced toxicity.

MATERIALS AND METHOD

Plant source:

The whole plant of *Limnanthemum indicum* is obtained from the local market, Tirunelveli, and identified by Prof. V.Chelladurai, Research Officer-Botany (Scientist-C), Central Council for Research in Ayurveda & Siddha, Govt.of India, Tirunelveli, Tamilnadu.

Extraction:

The dried material *Limnanthemum indicum* was used for extraction, initially 150gms of material was packed into the thimble and 2.5 liters of solvent used for extraction was poured into flask (Round Bottom flask). The soxhlet extraction was performed for 18- 24 hours until the siphon tube collected solvent appears to be clear. Later the extracted solvent was evaporated under reduced pressure to get dried extract. The Yield of the extraction was obtained by using the relation,

% of Yield = Weight of dried extract/ Weight of fresh material X 100

Cell lines and Culture medium [12]

BRL3A (Rat, Liver cell line) cell culture was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells of BRL3A were cultured in DMEM (Dulbecco's modified eagles medium) supplemented with 10% inactivated Foetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with TPVG(Trypsin-phosphate-versene-glucose) solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

Preparation of Test Solutions

For cytotoxicity studies, each weighed test drugs were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

Determination of cell viability by MTT Assay [13]

Principle: The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of tetrazolium salt 3-(4, 5 dimethyl

thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazan production by the cells used [14].

Procedure:

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using DMEM medium containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37^o C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 48 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37^o C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

% Growth Inhibition = 100 – Mean OD of control group / Mean OD of individual test group X 100

Determination Hepatoprotective activity [15]

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using DMEM medium containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium. 50 µl of DMEM with nontoxic concentration (4000 µg/ml in final volume) of toxicant and 50 µl of different non-toxic test concentrations of test drugs were added. The plates were then incubated at 37^o C for 24 h in 5% CO_2 atmosphere. After 24 h, the cell supernatants were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37^{o}C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage

cell viability was determined, based on which the percentage protection offered by test and standard drugs was calculated over the ethanol control.

Statistical analysis

Data are expressed as means ± SEM. Mean difference between groups were analyzed by Student's' test. P value < 0.001 was considered to be statistically significant.

RESULTS AND DISCUSSION

The longevity of an organism clearly depends on its individual parts and their effective organization. The screening for hepatoprotective activity was carried out on various solvent extracts of *Limnanthemum indicum* whole plant and shows a fairly good potential for use for the same. The tetrazolium salt (3-(4, 5 dimethylthiazole –2 yl)-2,5 diphenyl tetrazolium bromide) is taken up into the cells and reduced in a mitochondria dependent reaction to yield a blue coloured formazan product. The product accumulates within the cell, due to the fact that it cannot pass through the plasma membrane. On solubilization of the cells, the product is librated and can be readily detected and quantified by a simple colorimetric method. The ability of cells to reduce MTT provides an indication of mitochondrial integrity and activity which in turn may be interpreted as a measure of viability and /or cell number. The assay has therefore been adopted for use with cultures of exponentially growing cells[16]. Determination of their ability to reduce MTT to the formazan derivative after exposure to test compounds compared to the control situation, enables the relative protection of test chemicals to be assessed.

Table 1: Percentage of yield of *Limnanthemum indicum* with given solvents

Solvent	Appearance	% of Yield
Petroleum Ether	Brown	0.7%
Chloroform	Brown	1.3%
Ethyl Acetate	Brown	1.15%
Ethyl Alcohol	Brown	9.2%
Distilled Water	Brown	5.5%

Table 2: Cytotoxic property of test drug *Limnanthemum indicum* in BRL3A cell line by MTT assay.

Test drug(Extract)	CTC_{50} in µg/ml
Pet ether	360.00±1.3
Ethyl acetate	320.00±1.8
Chloroform	276.67±1.1
Alcohol	340.00±1.0
Aqueous	690.00±0.9

From the above results it is confirmed that cells which are exposed only with toxicant Paracetamol showed a percentage viability of 0% while cells which are pretreated with extract showed an

increase in percentage viability and the results were highly significant (P < 0.001, when compared to Paracetamol intoxicated cells). The percentage viability ranged from 7 to 26 % cell viability, when compared to standard drug Silymarin which showed 41% viability.

Table 3: Hepatoprotective study of *Limnanthemum indicum* in BRL3A cell line

Solvent Extracts *Limnanthemum indicum*	Concentration In µg/ml	% Protection offered over toxicant control*
Control(Untreated cells)	100±0	0±0[a]
Paracetamol control	4000	0±0[a]
Pet ether	150	0±0[a]
Chloroform	150	0±0[a]
Ethyl Acetate	250	26.65±3.34 [a b]
Alcohol	200	07.39±2.71 [a]
Aqueous	400	[b]41.35±0.36[a b]
Sylimarin	200	

*All values are mean ± S.E.M. n=6, [a]P < 0.001 when compared to untreated cells. [b] P < 0.001 when compared to Paracetamol intoxicated cells.

CONCLUSION

The various solvent extracts exhibited moderate toxicity against BRL-3A cell line and CTC_{50} value for these drugs was found to be in range of 250 to 700 µg/ml. The non-toxic concentrations tested against toxicant induced toxicity in BRL-3A offered protection to cells. Among the various solvent extracts of *Limnanthemum indicum*, alcoholic extract showed better protection when compared with other solvent extracts. Standard drug, Silymarin showed significant activity with 41.35±0.36 percent cell protection.

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