

IN VITRO HEPATOPROTECTIVE ACTIVITY OF ISOLATED DITERPENE FROM *HEDYCHIUM SPICATUM*

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Summary

Hedychium spicatum (Zinziberaceae) commonly known as Kapurkachari, is widely recognized in Indian system of medicine as anti-inflammatory and antistress agent. It is also reported to be used as tonic and stimulant in the liver disorders.

The present investigation evaluates in vitro hepatoprotective activity of the isolated diterpene from *H. spicatum*. The diterpene isolated from methanol extract of *H. spicatum* was subjected to in vitro hepatoprotective studies using paracetamol induced hepatotoxicity in primary rat hepatocytes. In vitro hepatoprotective activity was assessed by determining the change in hepatocytes viability and other parameters like glutamic transaminase, glutamic pyruvic transaminase and total protein. The isolated diterpene showed significant protective effect by restoring altered parameters in the selected in vitro model. The results justify the claims of *H. spicatum* in the folklore used as a hepatoprotective agent.

Key words: Diterpene, in vitro, hepatoprotective, *Hedychium spicatum*.

Introduction

Liver plays a major role in detoxification and excretion of many endogenous and exogenous compounds, any injury or impairment of its functions may lead to many implications on the health. Management of liver diseases is still a challenge to the modern medicine. Modern medicines have little to offer for alleviation of hepatic ailments, phytoconstituents become important representatives (1) *H. spicatum*

(Zingiberaceae) is reported to be a bitter tonic and stimulant (2). It is also used in treatment of inflammation, vomiting. It is also used as brain tonic. It is recommended to treat the liver disorders (3). The plant showed presence of sesquiterpene alcohols, furanoid diterpene like hedechnone (4) glycosides, carbohydrates, steroids etc (5)

Isolated hepatocytes have become a useful model for pharmacological, toxicological, metabolic and transport studies on xenobiotics since the development of techniques for high yield isolation of rat hepatocytes (6). The present study was undertaken to standardize an in vitro test system using primary cultured rat hepatocytes to detect the protective effect of extracts against paracetamol induced cellular damage, as in vitro model can offer a more detailed approach to understand the mechanism of toxic action thus hepatoprotective action of drugs being developed (7). In the present study, diterpene isolated from methanol extract of *H. spicatum* was examined against the hepatotoxin, in vitro using primary cultured rat hepatocytes. Silymarin was used as positive control.

Materials and methods

Plant material:

Authenticated rhizomes *H. spicatum* were obtained from Indian Herbs, Saharanpur. A voucher specimen is preserved in Pharmacy Department, Baroda (MS/PH/UJ/07).

Animals:

Albino rats of either sex (100-150 g) were obtained from M/S Zydu Research Center, Ahmedabad and were housed under standard environmental conditions with free access to food and water. The experiments were performed after the experimental protocols approved from Institutional Animal Ethics Committee, M. S. University, Baroda Vadodara, Gujrat, India (404/01/9/CPCSEA).

Isolation of diterpene

Dried and milled rhizomes of *H. spicatum* (1 kg) were extracted with 2.5 L of methanol in soxhelt apparatus. The extract was concentrated in a rotary vacuum evaporator and then placed in a desiccator. The Yield obtained was 7.39%. This concentrated extract was subjected to column chromatography using silica gel of 60- 120 mesh size and eluted with hexane-Benzene, benzene-ethyl acetate and ethyl acetate- chloroform. The diterpene was yielded from hexane-Benzene (1:1). The isolated compound was subjected to physicochemical tests, TLC and spectral studies to confirm the nature. The data was compared with that of standard.

In vitro hepatoprotective activity:

Hepatotoxins and test substances

Paracetamol 300 µg/ml was used to produce submaximal toxicity in isolated rat hepatocytes. The test solution was tested at dose level 100, 500 and 1000 µg/ml. Silymarine was used as positive control at dose level 100 µg/ml. All the solutions were dissolved in 30% DMSO.

Isolation of rat hepatocytes:

Hepatocytes were isolated from rat liver as per the reported method by Sarkar and Sil with some modifications (8). The liver was isolated under aseptic conditions and placed in chilled HEPES (N-2-hydroxyethylpiperazine-N- 2 ethane sulphonic acid) buffer containing HEPES (0.01M), NaCl (0.142M) and KCl (0.0067M), pH7.4. The liver pieces then incubated in a second buffer containing HEPES (0.01M), NaCl (0.142M) and KCl (0.0067M) and collagenase type IV, at pH 7.6 for about 45 min at 37°C. Hepatocytes were obtained after filtration through muslin cloth and cold centrifugation (4°C, 200rpm/min for 2 min three times) and resuspended in 4-5 ml HEPES buffer I. The viability of the hepatocytes was assessed by trypan blue exclusion method (9)

Primary cultures of rat hepatocytes

The method of Tinstorm and Obrink (10) with significant modification was used for this purpose. The freshly isolated viable hepatocytes were suspended in the culture medium RPMI- 1640 supplemented with calf serum (10%), HEPES and Gentamycin (1µg/ml). These cells approximately 1.2×10^6 /ml were seeded into culture bottles and incubated at 37°C in atmosphere of 5% CO₂. The hepatocytes formed a monolayer upon incubation for 24hrs. The newly formed cells were round and mostly appeared as individual cells. These cells were 96-97% viable as confirmed by trypan blue.

Hepatic cytotoxic testing

The isolated diterpene was tested for hepatic toxicity at 10, 50 and 100 µg/ml on isolated rat hepatocytes. After 24 hrs incubation at 37° C in CO₂ incubator, percentage viability of hepatocytes was tested by Trypan blue exclusion method and by estimation of total protein content.

Hepatoprotective activity

24 hrs after the establishment of the monolayers of the hepatocytes, the medium was decanted and the culture was washed with culture was washed with HEPES buffer I and finally the hepatocytes were suspended in 5ml of HEPES buffer I. The

hepatic toxicity was induced with paracetamol 300µg/ml. test substances including Sylimarine were dissolved in 30% DMSO. Hepatocytes suspension (0.1ml) in triplicate were distributed into various culture plates labeled as control, toxicant, standard (Syilmarine+ toxicants) and test (test samples+ toxicants). The control group received 0.1ml of vehicle (30% DMSO) and toxicant group received 0.01ml of respective test solutions (100,200 and 500µg/ml of isolated diterpene dissolved in 30% DMSO) followed by 0.1ml of Syilmarine solution (100µg/ml) followed by respective hepatotoxin. The content of all the tubes were made up to 1ml with HEPES buffer I. The contents of all the plates were mixed well and incubated for 24hrs at 37°C. In test and standard groups of hepatocytes were incubated with respective solution for 30 min and then exposed to hepatotoxin. After incubation hepatocytes suspensions were collected to assess cell damage by trypan blue exclusion method. Hepatocytes suspensions were centrifuged at 200rpm. The leakage of enzymes GOT, GPT and Total proteins secreted outside the cells were determined from the supernatant by using standard kits for enzyme estimation (11).

Assessment of hepatoprotective activity

The effect of different extracts in the liver protection was determined by measuring an increase in the percentage of viable cells in the treatment group compared with the control and the toxicant groups. Reversal of toxin induced elevation in the levels of enzymes and toxin induced reduction in the levels of proteins were considered to be the important criterion of hepatoprotective activity. The UV kinetic method based on the reference method of International federation of Clinical Chemistry was followed in which both SGPT and SGOT were assessed on the basis of enzyme coupled system where keto acid formed by the aminotransferase reacts in a system using NADH (12). The coenzyme is oxidized to NAD and the decrease in absorbance at 340 nm is measured. For SGOT malate dehydrogenate is used to reduce oxaloacetate to malate where as in SGPT the pyruvate formed in the reaction is converted to lactate by lactate dehydrogenase was followed for the assessment of activity of glutamic oxaloacetic transaminase and glutamic pyruvate transaminase in the cells. Total protein was estimated by Biuret test where protein produces a violet color complex with copper ions in the alkaline solution. The absorbance of the color complex is directly proportional to the protein in the sample.

Statistical analysis

The mean value \pm were calculated for each parameter. Percentage reduction against the hepatotoxin by the test sample was calculated by considering the difference in the enzyme level between the hepatotoxin treated group and control group as 100% reduction. Each parameter was analyzed by one way ANOVA followed by Dunnet's test.

Results

Methanol extract was subjected to phyrochemical screening. The extract showed presence of flavonoids, steroids, terpenoids and diterpenes. The presence of these constituents was confirmed by qualitative tests and TLC. Presence of furanoid diterpenes was confirmed by Libermann Burchard color test and orange color with acetone and sulfuric acid. Hepatic cytotoxic testing was performed by treating normal cells with the isolated diterpene. When the normal hepatocytes were treated with the sample under test, there were no alteration in the value of % viable cells and TPTN content as compared to control at the dose level up to 1000 μ g/ml indicating that the extracts were not toxic to the cells. In the present study, it was observed that there was reduction in cell viability due to injury to plasma membrane. The enzyme level was increased due to leakage which was restored with the treatment with isolated diterpene. Incubation of hepatocytes with diterpene increased cell viability as well as altered biochemical parameters induced by hepatotoxin. There was 81.62% restoration when the cells were treated with Sylimarine. When treated with HS 10 μ g/ml dose the restoration was found to be 27.11%. Dose dependent activity was observed at 50 and 100 μ g/ml ($p < 0.5$ and $p < 0.01$). The GPT level was increased in the toxicant group. The enzyme level was restored by 71.08% with Sylimarine. HS at 50 and 100 μ g/ml showed significant activity with 49.67 and 52.78 % respectively. Similar results were observed with GOT and total protein.

Table 1: Effect of isolated diterpene from *H. spicatum* against paracetamol induced toxicity in rats

Group	Viable Cells (%)	GOT (IU/L)	GPT (IU/L)	TPTN (g/dl)
Control	97.03±0.26	20.35±0.53	24.63±0.27	4.16±0.12
Paracetamol (100µg/ml)	27.65±1.43	46.32±0.35	55.62±0.69	2.09±0.51
Sylimarine (100µg/ml)	84.23±1.26* (81.62)	27.86±0.81* (71.08)	29.06±0.48* (85.70)	3.68±0.26* (76.81)
HS (10µg/ml)	46.46±0.65 (27.11)	44.56±1.12 (31.78)	45.26±0.39 (33.43)	2.08±0.24 (4.8)
HS (50 µg/ml)	51.21±1.09* (41.21)	38.26±0.45* (49.67)	39.26±0.92* (50.75)	3.10±0.37* (48.79)
HS (100 µg/ml)	66.29±0.95** (55.69)	33.39±0.85* (52.78)	36.56±0.69** (61.50)	3.28±0.58* (57.48)

n=3. All the groups were compared against control. The values in the parentheses represent % restoration compared to toxicant.

*p<0.5, ** p<0.01. Statistical analysis was done by one way ANOVA followed by Dunnet's test

Discussion

The in vivo studies require a large number of animals (n=6) and needs up to 3-5 days of drug administration for significant effect to be produced. It needs large amount of drug. On the other hand the in vitro model is rapid and requires fewer amounts of test substances. Bioactive fractions obtained from the plant extracts are usually available in the small quantities. Therefore, in vitro models can be more useful in assessment of activity.

Isolated hepatocytes have become a powerful model for pharmacological, toxicological, metabolic and transport studies of xenobiotics since the development of techniques for high yield isolation of rat hepatocytes (13). Freshly isolated rat hepatocytes are also very useful and common tool for study of cytotoxicity and metabolic studies in this area as they keep enzymatic activity similar to in vivo for several hours. Various hepatotoxins viz carbon tetrachloride, paracetamol and thioacetamide have been shown to result in reduction of viability of hepatocytes and leakage of enzymes which are considered to be the markers of cellular injury

(14). Similar changes in the present study confirm the satisfactory standardization of our isolation and culture procedures. Paracetamol is metabolized by microsomal cytochrome P₄₅₀. The hepatotoxicity of paracetamol is due to formation of toxic and highly reactive metabolite N acetyl p benzoquinoneamine. This highly toxic substance starts a chain of free radicals which attack membrane lipids and proteins thereby causing destruction of microsomes and liver cells leading to cell lysis. Leakages of cytosolic enzymes out of the cells thus occur due to increase in cell permeability, membrane damage and cell necrosis (15, 16). Deng and Choudhary (17, 18) have reported the hepatoprotective activity of andrographaloid, a known diterpene isolated from hepatoprotective drug *A. paniculata*. It was observed that andrographaloide showed significant activity in paracetamol and galactosamine induced toxicity (19, 20). Results of our findings are comparable to that of andrographaloide.

Conclusion

In conclusion, the study confirms the therapeutic potential of *H. spicatum* as a good hepatoprotective agent.

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