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In vitro hepatoprotective efficacy of extract of *Hedychium spicatum* rhizome in paracetamol induced toxicity in HepG2 cell line

GOVIND KUMAR CHOUDHARY[1] and SATYA PAL SINGH[2]

Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttrakhand 263 153 India

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Liver is the major organ of the body and is markedly involved in homeostasis of body in animal and human. In last decade, despite the advances in modern medicine, limited success was observed in cure for hepatic disorders further accompanied with severe side effects as aftermaths of the treatment (Pareek *et al.* 2013). HepG2 cells retain several specialized functions which are characteristic of normal hepatocytes and hence are used as a model for extensive toxicity studies of the liver (Knasmuller 1998).The various liver protectants used to ameliorate the liver ailment around the globe are not up to the mark. In the absence of effective modern drugs for liver disorders, today scientists face a serious challenge to explore the hepatoprotective potential of plants based on their traditional use. *Hedychium spicatum* commonly known as Gandhapalaashi have long been used in traditional medicine for the cure of different ailments. *Hedychium spicatum has* been used for the treatment of diarrhea and as a stimulant, expectorant, antidiabetic, anthelmintic, antipyretic and analgesic (Asolkar *et al.* 1992).

H. spicatum rhizome extracts have been reported to possess hepatoprotective potential *in vivo* but *in vitro* especially in HepG2 cell line has not been reported.

The present study was aimed at determining the hepatoprotective potential of methanolic, ethanolic and aqueous rhizome extracts of *Hedychium spicatum* against paracetamol (PCM) induced toxicity in HepG2 cell line.

The rhizomes of *Hedychium spicatum* were obtained and taxonomically authenticated from Medicinal and Aromatic Plant Research Development Centre (MRDC), of the University. The rhizomes were shade-dried and on complete drying were ground to make a fine powder. Methanolic, ethanolic and aqueous extract was prepared from dried rhizome powder. Ten gram of shade-dried powder was taken and added to 100 ml of ethyl alcohol, methyl alcohol and aqueous solution respectively and was homogenized. The supernatant was filtered using Whatman No. 1 filter paper and was dried by rotary-evaporator at 40°C and later freeze dried in a lyophilizer.

HepG2 cells (Human hepatocellular carcinoma cell line, passage no. 5), obtained from the National Centre for Cell Sciences, Pune, India were maintained in culture in 25 cm^2 polystyrene flasks (Nunc Thermo Scientific) with minimum essential medium (MEM) (Sigma, USA) containing 10% Foetal Bovine Serum (FBS) (HyClone, USA), 1% antibiotic–antimycotic solution, 1 mM sodium pyruvate and 1.5 g/l sodium bicarbonate under an atmosphere of 5% CO_2 at 37°C until confluent. Continuous cultures were maintained by subculturing every 6 days at 10^5 cells/25 cm^2 flask by trypsinization. HepG2 cells in exponential growth phase, i.e. after 24 h of growth post-subculturing in pre-confluent state were used for the experiments. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]-based cytotoxicity assay was used to determine the IC_{50} of PCM, ME of *H. spicatum* rhizome (MEHS), EE of *H. spicatum* rhizome (EEHS), aqueous extract of *H. spicatum* and silymarin (Freshney 2009). For prophylactic study, HepG2 cells were grown in six-well plates seeded at a concentration of 1×10^5 cells per ml (2 ml/well) for 24 h at 37°C. Cells were preconditioned with different extracts of plant (alone) and silymarin for 24 h followed by treatment with 15 mM PCM for 24 h. The experiments were carried out in the following groups:

Group 1 (Vehicle control) – cells + media; Group 2 (Negative control) – cells + 15 mM PCM; Group 3 (Positive control) – cells + 15 mM PCM + 10 µg/ml silymarin; Group 4 (ME of *H. spicatum)* – cells + 15 mM PCM + 150 µg/ml MEHS; Group 5 (EE of *H. spicatum)*– cells + 15 mM PCM + 100 µg/ml EEHS; Group 6 (AE of *H. spicatum)*– cells + 15 mM PCM + 200 µg/ml AEHS.

For curative study, cells were grown in six-well plates seeded at a concentration of 1×10^5 cells per ml (2 ml/well) for 24 h at 37°C. Damage was induced by treating the cells with 15 mM PCM for 24 h followed by treatment with different extracts of plant (alone) and silymarin for 24 h and in another plate, cells were damaged with 75 µg/ml indoxacarb for 24 h followed by treatment with different extracts of plant (alone) and silymarin. The experimental

Present address: [1]Subject Matter Specialist-Veterinary Sciences (drgovindvet@yahoo.co.in), ICAR-KrishiVigyan Kendra, ICAR- IIVR, Bhadohi. [2]Professor and Head (sppharma @rediffmail.com), Department of Pharmacology and Toxicology, College of Veterinary and Animal Sciences.

groups and doses used for rhizomes extracts of plant and silymarin were similar to the prophylactic study. For cytoprotection assay, HepG2 cells were grown in 96-well plates for 24 h at a concentration of 5×10^4 cells per ml (10^4 cells/well, 200 µl/well) at 37°C. Cell viability was determined for both prophylactic and curative studies using the MTT assay and results were expressed as % cytoprotection (Akanitapichat *et al.* 2010)

% Cytoprotection = % Viability of treatment group – % Viability of negative control.

Biomarker enzyme, viz. serum aspartate amino transaminase (AST) and serum alanine amino transaminase (ALT) were spectrophotometrically assessed in spent media as an indicator of cellular damage by kinetic method kits (Erba Mannheim, Germany).The cells were treated in triplicate with plant extracts and toxicants as described earlier. After treatment, the cells were trypsinized and the pellet was washed twice with PBS at 4°C. The cell pellet (1×10^7 treated cells/ml of lysis buffer) was then lysed in cell lysis buffer (50 mM HEPES buffer, pH 7.0, 150 mM NaCl, 1 mM Na_2EDTA, 1% Triton X-100) by repeated pipetting.The homogenate was then centrifuged (10,000 rpm, 4°C, 10 min) and the supernatant (cell extract) was used for further experiments. Total protein was estimated in the cell extract using Lowery method (1951). Morphology of the HepG2 cells was observed to evaluate the extent of degenerative changes and recovery for both prophylactic and curative treatment. Results are reported as mean±SEM. Total variation present in a group was determined by one-way analysis of variance (ANOVA), and Student's *t*-test was used to determine significance (Wilson 1987). The graphs were prepared using the software GraphPad Prism 5.

The percentage of yields of prepared ME, EE and aqueous extract for *H. spicatum* rhizome were 4.94%, 4.60% and 2.34% respectively. The IC_{50} values for silymarin, MEHS, EEHS and AEHS were 110, 282, 356 and 515 µg/ml respectively. HepG2 cells showed growth equivalent to normal untreated cells at a concentration of 10 µg/ml silymarin, 150 µg/ml MEHS, 100 µg/ml EEHS and 200 µg/ml AEHS. IC_{50} for PCM was 15 mM. PCM toxicity involves initial metabolism of paracetamol into a reactive metabolite NAPQI (*N*-acetyl-*p*-benzoquinone imine) followed by its binding to cellular proteins, especially to mitochondrial proteins. In later stages, this protein binding induces mitochondrial oxidative stress, which eventually leads to necrotic cell death (Jaeschke *et al.* 2011). In the prophylactic study, it was observed that methanolic, ethanolic and aqueous extracts gave significant cytoprotection (16%, 13% and 9% respectively) in comparison to 19% cytoprotection provided by silymarin (Fig. 1).The cytoprotection provided by plant extracts during pre-incubation may be due to their interference with the metabolic activation of paracetamol by Cyp450 enzymes; by interfering with the binding of NAPQI to cellular proteins at the initial steps of paracetamol toxicity, or because of their antioxidant properties which avoid mitochondrial oxidative stress in later stages.

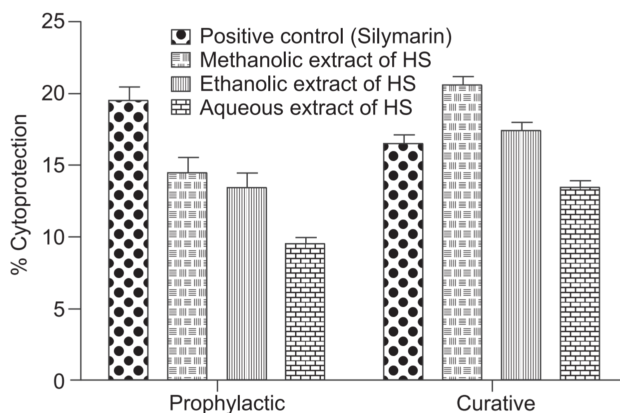

Fig. 1. Cytoprotective effects of preconditioning and post-treatment of methanolic, ethanolic and aqueous extract of *Hedychium spicatum* against paracetamol induced cytotoxicity in HepG2 cells for 24 h. Each value represents mean±SEM (n=3). *P* value versus negative control (PCM): * < 0.05.

Akanitapichat *et al.* (2010) have shown that pre-incubation with 50 and 100 µg/ml of eggplant (*Solanum melongena*) extracts was effectively able to protect HepG2 cells against the cytotoxicity caused by 300 µM of tert-butyl hydroperoxide (t-BuOOH). They demonstrated that phenolic antioxidants present in the eggplant extracts are responsible for their hepatoprotective effect against t-BuOOH-induced toxicity.

Post-treatment of cells with a combination of plant extracts after challenging them with paracetamol and ethanol for 24 h, showed a significant cytoprotection (29.2%) compared to 16.3% for treatment with silymarin. Curative study ensures that cytoprotection by plant extracts occurs as a result of their antioxidant potential to quench reactive oxygen species (ROS), thus avoiding mitochondrial oxidative stress and eventually cell death. It has already been established that delayed treatment with antioxidants which scavenge ROS inhibit sparacetamol-induced toxicity without relevant effect on metabolic activation and protein binding of NAPQI (James *et al.* 2003). Curative treatment with plant extracts was more potent than the prophylactic treatment, as is evident from Fig. 1. The cells on treatment with PCM showed asignificant (P<0.05) increase in the levels of AST and ALT leakage in the culture medium for both prophylactic and curative studies in comparison to the untreated normal cells. The marked increase in the levels of AST and ALT signifies damage to the structural integrity of hepatocellular plasma membrane, thus leading to their leakage from cytoplasm into culture medium (Jain *et al.* 2011). During the curative study, post-treatment of HepG2 cells with silymarin, MEHS, EEHS and AEHS for 24 h was able to significantly (P<0.05) restore the increased levels of AST and ALT to near normalcy compared to PCM treated cells (Figs 2 a and b). The AST and ALT level was significantly (P<0.05) elevated by 182.22% and 91.72% in PCM treated group as compared to the normal control in curative studies whereas this elevated level of enzymes were significantly (P<0.05) reduced by the treatment of silymarin,

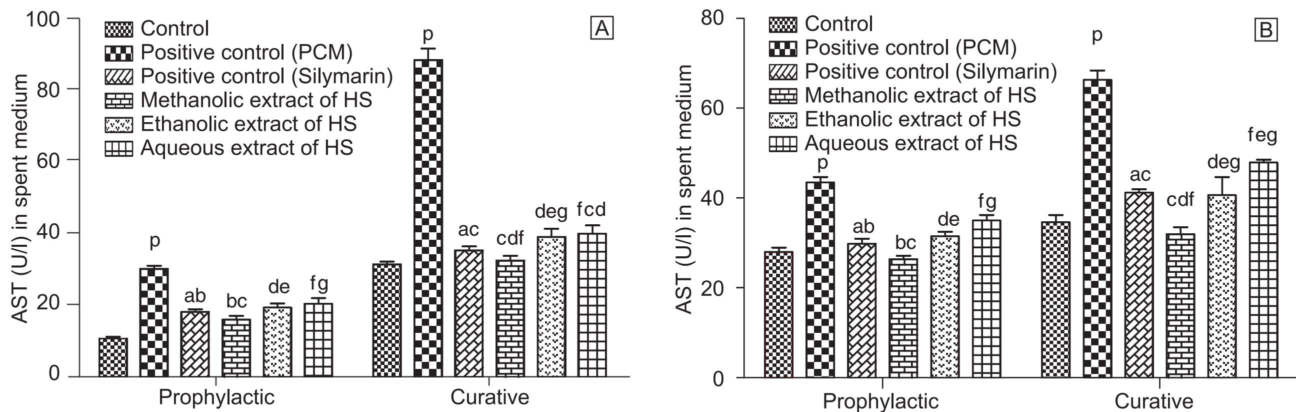

Fig. 2. Effect of silymarin and rhizome extracts, i.e. methanolic, ethanolic and aqueous of *H. spicatum* on biochemical and antioxidant parameters against paracetamol (PCM) induced toxicity (prophylactic and curative study). a. Representation of serum glutamate oxaloacetate transaminase. b. Serum glutamate pyruvate transaminase. All values represent mean±SEM (n = 3). P<0.05.

methanolic, ethanolic and aqueous rhizome extracts of *H. spicatum* by 60.15%, 63.38%, 55.9% and 54.85% respectively in AST level and 37.92%, 51.88%, 38.71% and 27.73% respectively for ALT level toward the normal and restore the enzyme levels. In prophylactic treatment, the AST and ALT levels significantly (P<0.05) increased by 182.53% and 112% in PCM treated group as compared to normal control in pretreatment studies. However, the elevated enzyme level were significantly (P<0.01) reduced by the treatment, with the silymarin, methanolic, ethanolic and aqueous rhizome extracts of *H. spicatum* by 39.27%, 46.99%, 35.52% and 32.08% respectively for AST level and 31.46%, 39.52%, 27.67% and 19.47% respectively in ALT level toward the normal.

Shah *et al.* (2015) also reported that PCM and ethanol induced cytoxicity produced in HepG2 cell line was ameliorated by treatment with alcoholic and water extract of *M. koenigii* leaves, alcoholic and water extract of *P. niruri* leaves and also reduced the AST and ALT activity in spent media. Preincubation with plant extracts and silymarin was able to significantly (P<0.05) ameliorate the increase in the levels of AST and ALT (Figs 2 a,b). In curative studies, the methanolic rhizome extract of *H. spicatum* treatment showed maximum percentage decrease in the elevated levels of AST (63.38%) and ALT (51.88%) in spent medium. In both the studies, the curative and prophylactic treatment possessed significant (P<0.05) hepatoprotective effect, emphasizing its membrane-stabilizing property. The plant extracts, i.e. MEHS, EEHS and AEHS possessed significant antioxidant potential during DPPH radical scavenging assay (Choudhary and Singh 2018). Previous work has established the presence of polyphenolic present in rhizome extracts of *H. spicatum*, which have been known for their rich antioxidant and hepatoprotective properties (Thapliyal *et al.* 2014).The hydroalcoholic and aqueous rhizome extracts of *Hedychium spicatum* produced cytotoxic effect on HepG2 cell (Choudhary and Singh 2017) and its rhizome powder produces ameliorative effect in indoxacarb intoxicated White LegHorn cockerels (Choudhary and Singh 2016) and also produces antioxidant effect in indoxacarb intoxicated cockerels (Choudhary and Singh 2017). The curative effect of methanolic rhizome extract of *H. spicatum* was more potent in comparison to the prophylactic effect. The better curative effect can be presumed have potentiated from the efficient replenishment of GSH and antioxidant nature of rhizome extract of *H. spicatum* in comparison to the prophylactic study where early exposure to plant extracts interferes with the initial steps of metabolism of PCM to NAPQI by Cyp 450. The hepatoprotective potential of plant extracts and silymarin was further substantiated by evaluating the morphology of HepG2 cells. Normal cells (Fig. 3a) after treatment with PCM (15 mM) showed granulation and detachment as is visible in Fig. 3b. During the prophylactic study, preconditioning with rhizome extracts was able to protect the cells against PCM induced cytotoxicity. The cells showed normal morphology with intact cell membranes (Fig. 3c). Curative treatment with plant extracts was able to show effective cytoprotection against paracetamol induced cell damage (Fig. 3d). Thus, the morphology study is supportive of effective hepatoprotective potential of extracts of *H. spicatum*. The plant extracts were able to restore normal cell morphology similar to untreated cells, showing no ballooning as is visible in ethanol-exposed cells (Sharma *et al.* 2011).

This study signifies the hepatoprotective potential of rhizome extracts of *H. spicatum* during both prophylactic and curative studies. The antioxidant-rich plant extracts were able to show significant (P<0.05) per cent cytoprotection, reduction in leakage of AST and ALT in comparison to PCM treated cells. The plant extracts showed effective protection against radical damage accompanied with membrane stabilization. The rhizome extracts showed immense potential as a hepatoprotectant with curative treatment showing better results in comparison to prophylactic treatment. Many studies have established antioxidant (Sravani and Paarakh 2012, Choudhary and Singh 2017), antimicrobial and hepatoprotective

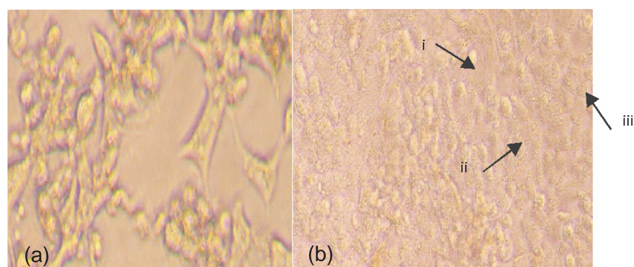

Fig. 3. Effect of different treatments on HepG2 cell morphology. a. Normal untreated cells showing regular morphology. b. Cells treated with PCM (15 mM) showing (i) excessive granulation, (ii) clumping and (iii) detachment.

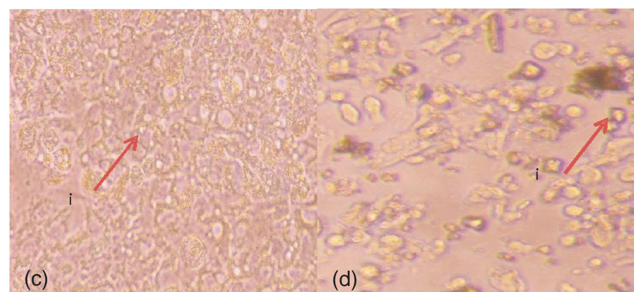

Fig. 3. Effect of different treatments on HepG2 cell morphology. c. Curative treatment of cells with rhizome extract of *Hedychium spicatum* shows repair by restoring morphology similar to normal untreated cells (i). d. Prophylactic treatment with rhizome extract of *Hedychium spicatum* protects the cells from damage induced by PCM as is evident with restoration of cell structure (i).

(Thapliayal *et al.* 2014), and cytotoxic (Choudhary and Singh 2017) potential of rhizome extracts of *H. spicatum.* Phytochemical studies have revealed the presence of organic constituents such as phenolic, flavonoid, carbohydrates, protein, steroids, triterpenoids, cardiac glycosides, tannin, saponin and essential oils in rhizome extract of *H. spicatum* (Singh and Bagh 2013).

Thus, it is concluded from the present finding that the extract of *Hedychium spicatum* may be used as a promising drug against the liver diseases. Further studies at the biochemical and molecular level are required to determine the extent of effect of phytoconstituents at the cellular level.

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