

# Research Article

## Cytotoxicity of different extracts of *Mucuna pruriens* seeds on hepatoma cells but not on normal hepatic cells

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### ABSTRACT

**Object:** Cancer has become leading cause of death in India as well as world. Synthetic anticancer agents have costly and highly toxic to human body. Therefore, it is necessary to discover new therapeutic agents from natural origin for this disease. **Materials and Methods:** *Mucuna pruriens* Linn. (MP) (family Fabaceae), is widely used in folk medicine and there is no any such report on antiproliferative effects of this plant. The first objective of this study is to investigate the anticancer properties using hepatoma cells of MP seeds. The first objective of this study is to investigate the anticancer properties of MP seeds by using/on hepatoma cells. Secondly, it is necessary to explore its hepatotoxicity potential in normal cells as well. Therefore, the other objective of this study is to investigate the toxicity using normal hepatic cells. To achieve this goal, hot extraction of MP seeds were performed using petroleum ether 60-80°C (PE), ethyl acetate (EA) and Methanol (ME) as a solvent. All the three extracts were subjected to screen for anti-proliferative action using human hepatoma cell line (Huh-7 cells) at different concentrations from 0.5 to 100 µg/ml, followed by apoptotic activity using caspase-3 enzymes. On the other hand, hepatoprotective activity was performed using human primary hepatic cells (THLE-2 cells). **Results:** Our result collectively suggested that both EA and ME extract possessed anti-proliferative action on Huh-7 cells and less cytotoxic action on normal cells. **Conclusion:** Among these two extracts, it is demonstrated for the first time that ME extracts of MP seeds have cytotoxic effect on hepatoma cells but not on normal hepatic cells.

**Key words:** *Mucuna pruriens* seeds, Huh-7 cells, THLE-2 cells, anti-proliferation action, hepatoprotective action.

### INTRODUCTION

Human beings have relied on natural products as a resource of drugs for thousands of years. Plant-based drugs have been used as the basis of traditional medicine systems in many countries such as Egypt, China and India.<sup>1</sup> It has been estimated by the World Health Organization that 80% of the population of the world relies mainly on traditional medicines for their primary health care.<sup>2</sup>

Cancer has become one of the ten leading cause of death in India. It is estimated that there are nearly 2-2.5 million cancer cases at any given point of time.<sup>3</sup> Over 7,00,000 new cases and 3,00,000 deaths occur annually due to cancer.<sup>3</sup> This is challenging situation for healthcare institutions as demand is increased for cancer. The anticancer drugs are toxic in nature and developed resistance during therapy. The molecular mechanisms of drug resistance may involve a variety of factors such as mutation of target genes and decreased drug concentrations in the cells due to renal toxicity.<sup>4,6</sup> As a result, it is necessary to discover new therapeutic agents to treat cancer. Plants are the better alternative for providing efficacious agents for cancer disease from ancient years as synthetic drugs are costly and highly toxic to human body. Natural resources have

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played an important role to provide natural drugs such as taxol, camptothecin, vincristine, and vinblastine.<sup>7</sup> on this contrary, it is necessary to explore some newer anticancer plants from natural origin for safer life against cancer.

*Mucuna pruriens* Linn. (MP) (family Fabaceae), commonly known as cowhage plant, is widely used in folk medicine and is one of the most useful herbs in Middle East Asia especially in India. It has multiple pharmacological actions which include antioxidant, hypoglycemic, antimicrobial, aphrodisiac, antiinflammatory and antiparkinsonism activities.<sup>8</sup> There is no report on anti-proliferative effects of this plant. The approach for selecting this plant is that the seed part of MP is rich with isoquinoline alkaloids and recent investigations have suggested that these alkaloids may have a dramatic role in anticancer therapy.<sup>9,11</sup> reported that the isoquinoline alkaloids, berberine and berberubine produced antiproliferative action via binding to non-specific deoxyribonucleic and inhibit topoisomerase II enzyme.<sup>10</sup> A recent review suggested that isoquinoline alkaloids have higher affinity to polymorphic nucleic acid structures (B-form, A-form, Z-form, HL-form, triple helical form, quadruplex form) during antiproliferation.<sup>11</sup> All of this information promoted us to study the antiproliferation action of MP seeds. Therefore, the objective of our study is to investigate the anticancer properties of MP seeds using hepatoma cells. Secondly, it is necessary to explore the hepatotoxicity potential of MP seeds in normal cells as well. Therefore, the other objective of this study is to investigate the toxicity using normal hepatic cells.

To achieve this goal, hot extraction of MP seed was performed using petroleum ether 60-80°C (PE), ethyl acetate (EA) and methanol (ME). All three extracts screened for antiproliferative action using human hepatoma cell line (Huh-7 cells) at different concentrations (0.5 to 100 µg/ml). The apoptotic activity induced by caspase-3 enzymes was also investigated. On the other hand, hepatoprotective activity was examined using human primary hepatic cells (THLE-2 cells). It is demonstrated for the first time that ME extract of MP seed have cytotoxic effect on hepatoma cells but not on normal hepatic cells.

## MATERIALS AND METHODS

### Materials

Human plasma fibronectin, collagen-I, LHC-9 medium and caspase-3 colorimetric assay kit were purchased from Invitrogen Bioservices, India. PE, EA, ME, and dichloromethane (DCM) were purchased from Merck

(Merck, India). Dimethyl sulfoxide (DMSO, ACS grade), ethylenediaminetetraacetic acid disodium salt dihydrate (Na<sub>2</sub>EDTA.2H<sub>2</sub>O, 98% purity), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), Trichloroacetic acid (TCA), trypsin ethylenediaminetetraacetic acid (trypsin-EDTA) and guanidine hydrochloride were obtained from S.D. Fine Chemicals, India. THLE-2 and Huh-7 cell lines were purchased from ATCC (Manassas, VA, USA). Water was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other reagents were purchased from Himedia, India.

### Preparation of Extracts

The seeds were collected from costal area of West Bengal in the month of August-September, authenticated by Botanical Survey of India, Howrah, India and a voucher specimen no. 98/BSI/2013 was deposited for future references. The seeds crushed with mixer grinder (Bajaj Appliances, India) and powered accordingly. A mass of 150 g of seed powder was taken separately for Soxhlet extraction (three times). Extraction was performed with PE, EA and ME solvents separately for successive three days. The solvent was subsequently evaporated through rotary vacuum evaporation and % yields of extracts were 13% (PE), 16% (EA) and 18% (ME).

### Preparation of cells

THLE-2 cells were cultured in plates pre-coated with collagen-I (2.9 mg/ml), fibronectin (1 mg/ml) in LHC-9 medium while Huh-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM). All media were supplemented with 10% foetal bovine serum and 100 units/ml penicillin/streptomycin (Himedia, India). Cells were maintained in a humidified air with 5% carbon dioxide (CO<sub>2</sub>) at 37°C.

### MTT viability assay

MTT [3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole] viability assay was performed as described previously.<sup>12</sup> Briefly, THLE-2 cells were seeded at 1.0×10<sup>4</sup> cells/well and Huh-7 cells at 5.0×10<sup>3</sup> cells/well in 96-well plates. After 24 h, the medium was removed and 200 µL of varying concentrations of extracts (0.5 to 100 µg/ml) in media was added to each well and the samples were incubated in a humidified condition 5% CO<sub>2</sub> at 37 °C. After 72 h, MTT solution (1 mg/ml, 50 µl) was added to each well and incubated for another 4 h. After which, the media and excess MTT were removed and 150 µl of DMSO was added. The absorbance at 570 nm was recorded using the Infinite M200 microplate reader controlled by Magellan and i-control softwares (Tecan Group Ltd., Mannedorf, Switzerland). Samples containing media

with and without cells were also analyzed and labelled as 'control' and 'blank', respectively. All the experiments were performed in triplicates. The final organic solvent concentration was 0.3% (v/v). Cell survival (% of control) was calculated relative to untreated control cells (vehicle: 0.3% solvent). Data were analyzed by Graph Pad Prism 5 and represented as the mean  $\pm$  S.D. of triplicate experiments. Statistical differences were analyzed using paired *t*-test and  $P < 0.005$  represented the statistically significant data.

### Caspase-3 colorimetric assay

Apoptotic activity in Huh-7 cells was determined using Caspase-3 colorimetric assay kit. Briefly, cells were seeded at  $2 \times 10^4$  cells/well density in 96-well micro plates and divided into control (DMSO) group containing 100  $\mu$ l of solvent control (DMSO, 1%) and treatment group containing varying concentrations of the test compounds (0.5, 1.0, 10.0, 25.0, 50.0 and 100.0  $\mu$ g/ml) ( $n=3$ ). After 24 h treatment at 37 °C, 100  $\mu$ l of Caspase-3 reagent was added and the mixture further incubated for 2 h at room temperature. Relative luminescence (RLU) was recorded using the Infinite M200 micro plate reader controlled by i-control soft ware and results expressed as change versus control. Statistical differences were analyzed using one way-ANOVA and  $P < 0.001$  represented the statistically significant data.

### Glutathione (GSH) depletion assay

GSH depletion assay was performed as described previously.<sup>13</sup> A concentration of 50 mM stock solution of GSH (Himedia, India) was prepared freshly in water and its calibrated concentrations were prepared via serial dilutions of this stock solution using water. A volume of 0.2 ml of stock solution was taken in a tube and 1.8 ml of distilled water was added to it. A volume of 3.0 ml of precipitating solution (1.67 g of glacial meta phosphoric acid, 0.2 g  $\text{Na}_2\text{EDTA}$ ,  $2\text{H}_2\text{O}$  and 30 g of sodium chloride in 100 ml distilled water) was added to the above mixture. The mixture was then allowed to stand for approximately 5 min and then filtered. A volume of 2.0 ml of the filtrate was added to 8.0 ml of the phosphate solution (0.3 M). A volume of 1.0 ml 0.4% DTNB was added, vortex-mixed and centrifuged at 13,000 X g for 1 min. A blank solution was prepared in the above mentioned procedure where GSH was absent. The Optical density (OD) was measured at 412 nm. The concentrations of the GSH-DTNB calibrants were 0.01, 0.05, 0.1, 0.5, 1.0 and 10.0  $\mu$ M. Triplicate samples were prepared at each concentration.

The data was calculated by the following procedure,

$\text{GSH } (\mu\text{M}/\mu\text{g of protein}) = 310.4 \times E_i \times \text{O.D.} / \text{mg of protein}$ , where O.D. at 412 nm and  $E_i$  is correction factor (0.542).

THLE-2 cells were seeded into 24-well plate ( $2.0 \times 10^4$  cells/well). After 24 h, 1 ml of varying concentrations of extracts in LHC-9 media (0.5, 1.0, 10.0, 25.0, 50.0 and 100.0  $\mu$ g/ml) were added to each well and incubated in a humidified condition 5%  $\text{CO}_2$  at 37 °C. After 72 h incubation, cells were washed with phosphate buffer saline (PBS) and trypsinized using 0.05% trypsin-EDTA. Subsequently, the excess trypsin was inactivated with LHC-9 media. The suspended cells were then transferred into clean tubes, centrifuged at 13,000 X g for 2 min and the supernatant was removed. After which, 0.2 ml of water was added to the cell samples, mixed for 1 min, and 1.8 ml water was then added. The rest of the treatment was as previously described method. Total protein content of cell suspension was measured at 72 h using the Pierce Micro BCA™ (Bicinchoninic acid) Protein Assay Kit. Statistical differences were analyzed using paired *t*-test and  $P < 0.005$  represented the statistically significant data.

### Protein Carbonyl (PC) assay

PC assay was performed as described previously.<sup>14</sup> In brief, THLE-2 cells were seeded at  $5.0 \times 10^4$  cells/well into 6-well plate which was previously coated with the fibronectin-collagen medium. After 24 h, 2 ml of varying concentrations of extracts in LHC-9 (0.5 to 100  $\mu$ g/ml) were added to each well and incubated at 37 °C in a humidified condition at 5%  $\text{CO}_2$ . After 48 h, each medium was removed and the cells were washed with PBS. The cells were removed from the surface of the plates using 0.05% trypsin-EDTA and the excess trypsin was deactivated by adding LHC-9 medium. Media containing cells were transferred into clean tubes and centrifuged at 13,000 g for 2 min. The supernatant was removed and the cell pellet was reconstituted with 300  $\mu$ l of water and vortexed for 1 min. A volume of 150  $\mu$ l of cell suspension was aliquoted into a clean tube and precipitated using 500  $\mu$ l of 10% TCA solution. The tubes were centrifuged subsequently at 13,000 g for 2 min and the supernatant was removed. Each cell pellet was incubated with 500  $\mu$ l of 0.2% diphenyl picryl hydrazine for 1 h with constant vortexing at every 5 min interval. 50  $\mu$ l of 100% TCA solution was added, vortexed and centrifuged at 13,000 X g for 5 min. All the supernatant was removed and the cell pellet was washed with 500  $\mu$ l of ethanol: EA (1:1 v/v) mixture three times. Finally, the cell pellet was dissolved in 600  $\mu$ l of 6 M guanidine hydrochloride and 300  $\mu$ l was transferred to a 96-well plate and absorbance was measured at 360 nm using the Infinite M200 microplate reader. The

remaining 150  $\mu$ l of cell suspension was used for measuring total protein content using the Pierce Micro BCA™ (Bicinchoninic acid) Protein Assay Kit. PC content ( $\mu$ M) per  $\mu$ g of protein was calculated at varying concentration of the test compounds. All data were analyzed by Graph Pad Prism 5. Statistical differences were analyzed using paired t-test.  $P < 0.005$  represented statistical significance between groups.

## RESULTS

### Effects on anti-proliferation of Huh-7

Three extracts (PE, EA, ME) was used to investigate antiproliferative action with Huh-7 cells as an *in vitro* model of hepatocellular carcinoma. Using MTT viability assay, it was observed that PE extract had no effect on anti-proliferation whereas EA and ME extracts demonstrated this action at and above 1.0  $\mu$ g/ml concentration (Figure 1). The ME extract showed higher inhibitory action on hepatoma cells than EA extract with 90% to 30% inhibition for EA extract and 90% to 10% inhibition for ME extract. Both the extracts (EA and ME) had  $EC_{50}$  value in between 1 to 10  $\mu$ g/ml concentration (Figure 1). Statistically significant differences were observed between PE and EA extracts (paired t-test,  $P < 0.05$

at 25, 50 and 100  $\mu$ g/ml concentrations. Statistically significant differences were observed between EA and ME extracts (paired t-test,  $P < 0.05$ ) at and above 1.0  $\mu$ g/ml concentration.

### Caspase-3 activity assay

Caspase-3 assay was performed to measure levels of apoptotic activity in treated cells as a phenotype of anti-cancer potential. Various concentrations of EA and ME extracts of MP seed (0.5 to 100  $\mu$ g/ml) were administered on Huh-7 cells for 24 h before analysis of caspase-3 activity. It was shown that both extracts demonstrated dose dependent increase in apoptosis in Huh-7 cells with respect to control (Figure 2A and 2B). It is apparent from the graph that ME extract showed double apoptotic action than EA extract. Statistical differences were demonstrated between control and Test groups for both extracts (one-way ANOVA,  $P < 0.001$ ) at all concentrations.

### Cytotoxicity on THLE-2 cell

To determine toxicological profile of the extracts, a series of toxicity assays were performed to compare the toxicity potential of EA and ME extracts. THLE-2 cells were treated with both these extracts from 0.5 to 100  $\mu$ g/ml concentration for 72 h. Here, the EA extract was found to be more toxic than the ME extract (Figure 3). Statistically significant differences were observed between EA

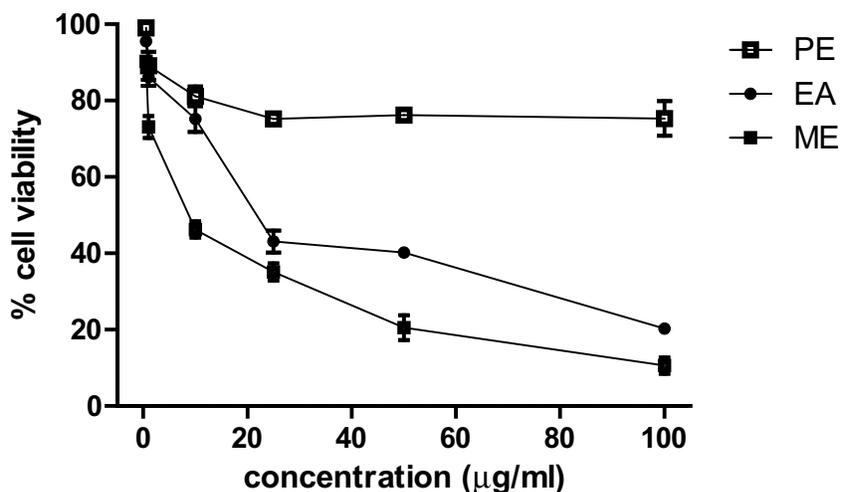


Figure 1: Viability of Huh-7 cells after treatment with PE, EA and ME extracts of MP seed (0.5, 1, 10, 25, 50 and 100  $\mu$ g/ml) for 72 h before being subjected to MTT assay to determine potential anti-cancer effects. All samples were prepared in triplicate ( $n=3$ ). Viability is expressed as a percentage of vehicle-treated control and data was expressed as mean  $\pm$  S.D. Statistically significant differences were observed between PE and EA extracts (paired t-test,  $P < 0.05$ ) at 25, 50 and 100  $\mu$ g/ml concentrations. Statistically significant differences were observed between EA and ME extracts (paired t-test,  $P < 0.05$ ) at and above 1.0  $\mu$ g/ml concentration.

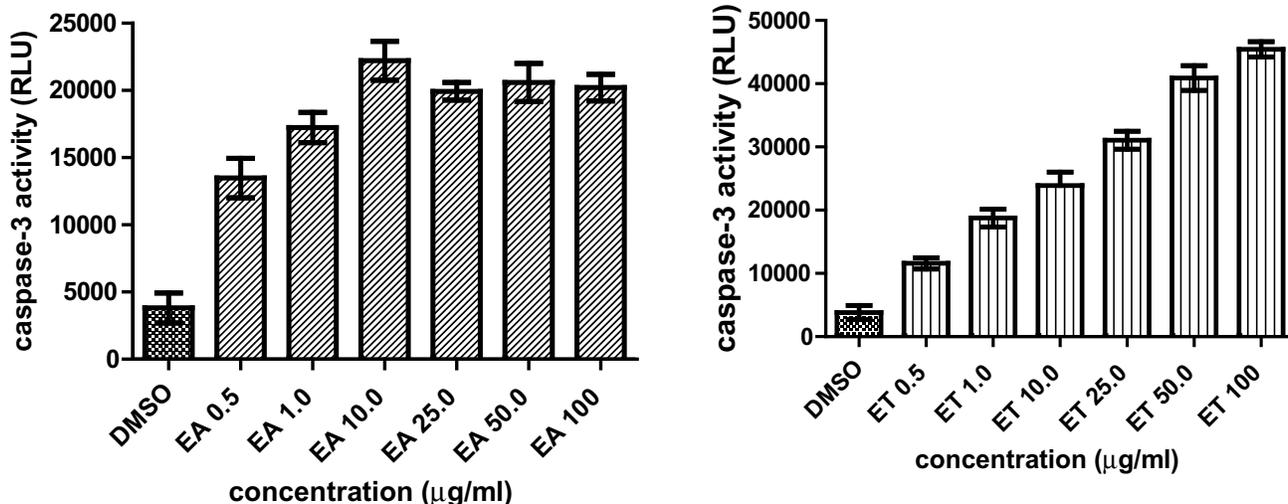


Figure 2 : Characterizing the anti-cancer property of (A) EA and (B) ME extracts of MP seeds. Huh-7 cells were treated with at concentrations of 0.5, 1, 10, 25, 50 and 100 µg/ml for 24 h and subjected to caspase-3 activity determination (n=3). Activity was expressed as relative luminescence units (RLU) and compared against a DMSO-containing vehicle control. Statistical differences were demonstrated between control and Test groups for both extracts (one-way ANOVA, P<0.001) at all concentrations.

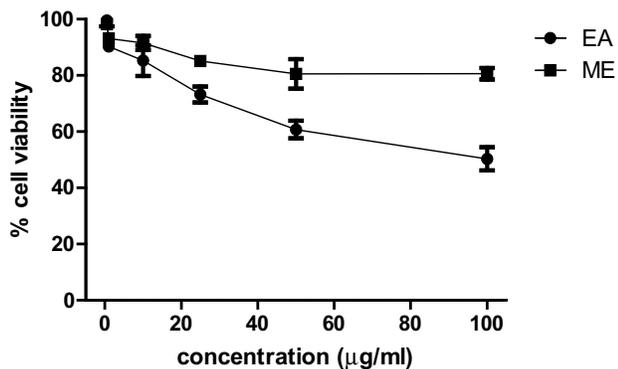


Figure 3 :Viability of THLE-2 cells after treatment with EA and ME extracts. THLE-2 cells were treated with respective compounds at 0.5, 1, 10, 25, 50 and 100 µg/ml for 72 h before subjecting to MTT assay. All samples were prepared in triplicate (n=3). Viability is expressed as a percentage of vehicle-treated control and data was expressed as mean ± S.D. Statistically significant differences were observed between EA and ME extracts (paired t-test, P<0.05) at 25, 50 and 100 µg/ml.

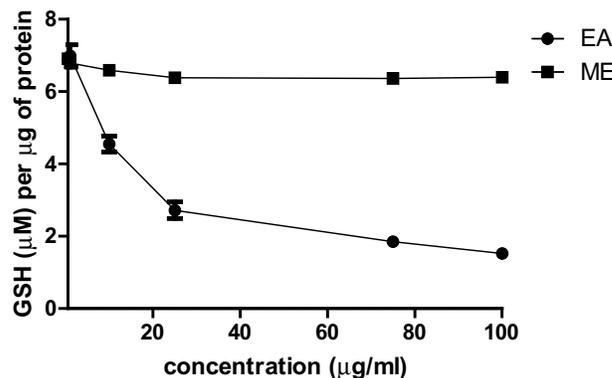


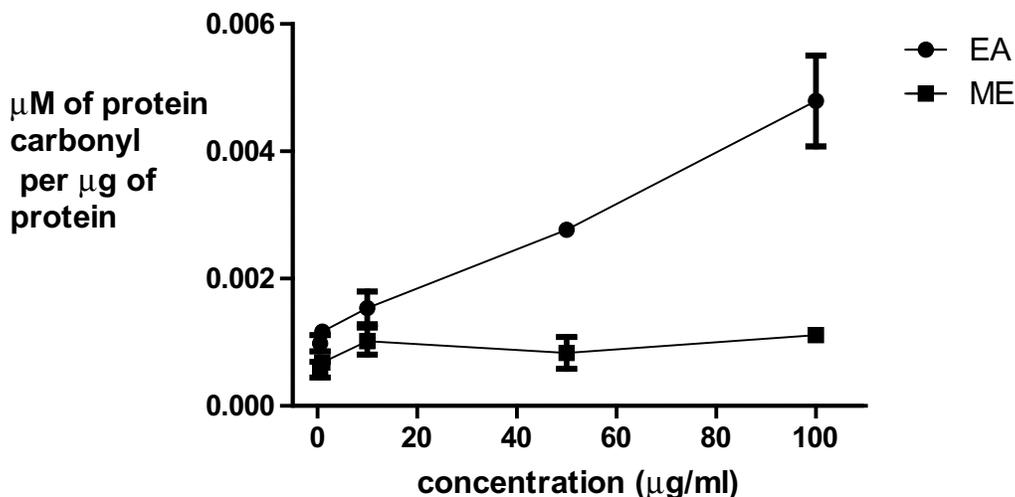
Figure 4 : GSH depletion in THLE-2 cells after treatment with EA and ME extracts. THLE-2 cells were treated with various concentrations of respective extracts for 72 h before subjecting to GSH content determination. All samples were prepared in triplicate (n=3). The amounts detected were normalized to the total cellular protein content of each sample. Statistical differences were demonstrated between EA and ME extracts (paired t-test, P<0.05) at and above 10 µg/ml.

and ME extracts (paired t-test, P<0.05) at 25, 50 and 100 µg/ml.

### GSH depletion assay

GSH depletion was used as a complementary indicator of cytotoxicity and oxidative stress in normal hepatic cells. Determination of GSH-DTNB adducts was per-

formed in THLE-2 cells after 72 h incubation to measure cytotoxic effects. Here, we observed that the EA extract depleted more GSH than the ME extract after 1.0 µg/ml concentration (Figure 4). This assay signified that the EA extract was more toxic than the ME extract. Statistical differences were demonstrated between EA and ME extracts (paired t-test, P<0.05) at and above 10 µg/ml.



**Figure 5 :**Protein carbonyl assay in THLE-2 cells after treatment with EA and ME extracts. THLE-2 cells were treated with various concentrations of extracts for 48 h before subjecting to protein carbonyl determination using 2,4-dinitrophenolhydrazine as trapping agent. All samples were prepared in triplicate (n=3). Statistical difference was demonstrated between EA and ME extracts (paired t-test,  $P < 0.05$ ) at 50 and 100 µg/ml.

### Protein Carbonyl (PC) assay

PC content was measured in THLE-2 cells after 48 h of incubation between EA and ME extracts. EA extract produced more PC as compared to ME extract at and above 10 µg/ml concentration (Figure 5). Statistical difference was demonstrated between EA and ME extracts (paired t-test,  $P < 0.05$ ) at 50 and 100 µg/ml.

## DISCUSSION

Treatment of cancer in epithelial region of human body remains disappointing. The usage of synthetic antiproliferative chemotherapeutic agents have declined due to their potential toxicity to the human body. Compounds obtained from natural sources are perceived as being safer and less toxic. Hence, it is necessary to investigate potential therapeutic agents for clinical benefits in cancer patients. Present chemotherapy treatment of liver cancer is not satisfactory. Specific anticancer agents against different types of liver cancer need to be developed. Recent investigations suggest that isoquinoline alkaloids from natural origin have good antiproliferative effect via inhibition of topoisomerase II enzymes.<sup>11</sup> The seed of MP contains higher amount of isoquinoline alkaloids.<sup>9</sup> The question arose whether MP seed extract had any antiproliferative effect due to the presence of isoquinoline alkaloids. In this present study, we investigated the antiproliferative action of MP seeds using various extracts (PE, EA and ME) and examined the effects of these extract using liver cancer cells (Huh-7 cells).

Antiproliferative action was determined on Huh-7 cells using MITT as the detecting reagent. Huh-7 cells are hepatic carcinoma cells which are very useful for primary drug screening *in vitro*.<sup>15</sup> We observed that both the EA and ME extracts of MP seed possessed antiproliferative action at lower concentration (1 mg/ml) whereas the PE extract had no such effect. The PE extract was not examined in the other assays due to its poor antiproliferative activity. Lee and Ho (2012) investigated similar effects using gambogic acid isolated from *Garcinia hanburyi* in Huh-7 cancer cells with an  $IC_{50}$  of 2.0 µM.<sup>16</sup> Apoptotic properties of the EA and ME extracts were evaluated through caspase-3 activity determination and expressed as relative luminescence units (RLU). The results were compared with a DMSO-containing vehicle control. This assay is an indication for the measurement of apoptotic activity in treated cells as a phenotype of anticancer potential. The ME extract produced caspase-3 enzyme activity twice that of the EA extract. Similar trends were also observed when in a study investigating several synthetic compounds using the Huh-7 cells.<sup>17</sup>

As these two extracts produced antiproliferative action on hepatoma cells, it was important to determine whether they had any cytotoxic action on normal human hepato-

cytes. We performed MTT based cell viability assay using normal primary hepatic cells (THLE-2 cells).<sup>18</sup> We confirmed that the ME extract had no effect on THLE-2 cells toxicity whereas the EA extract had slightly higher toxicity than ME extract. To further examine the toxicity, we performed GSH depletion assay and demonstrated that the ME extract depleted less GSH than the EA extract. Follow-on PC assays that compared the formation of reactive oxygen species-induced protein oxidation showed a similar trend. Investigated the cytotoxic effect of troglitazone and its analogue on THLE-2 cells and they performed MTT viability, GSH depletion and PC assays to check the cytotoxic effects of that compounds.<sup>19</sup> We followed the similar experiments in our investigation to support our hypothesis. All these experiments are reliable and supportive to prove our hypothesis.

## CONCLUSION

All biochemical assays suggested that both the EA and ME extract of MP seed had antiproliferative effect in Huh-7 cells. The ME extract showed higher effect than EA extract in antiproliferation, as well as it demonstrated doubled caspase-3 enzyme action than the EA extract. Later, cytotoxicity assay on THLE-2 cells revealed that the ME extract expressed less toxicity on normal human hepatocytes using MTT reagent. Other assays like GSH depletion and PC assays expressed the same trends. Collectively, we concluded that ME extract of MP seeds might have anticancer effect on hepatoma cells and protective effect on normal hepatocytes.

There is no literature available for anticancer activity of MP seeds. We reported for the first time that MP seed has antiproliferative effect on Huh-7 cells. Isolated four isoquinoline alkaloids from MP seeds.<sup>9</sup> It may be possible that these isolated isoquinoline alkaloids might be responsible for this antiproliferative effect.

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## CONFLICT OF INTEREST

Authors declare that they have no conflict of interest.

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