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Research Article

IN-VITRO HEPATOPROTECTIVE ACTIVITY OF *ALBIZIA LEBBECK, CASSIA OCCIDENTALIS AND SWERTIA CHIRATA* ON HEPG2 CELLS

ANIL KUMAR[1], MANJU O PAI[2], NISHANT RAI[1]*

[1]Department of Biotechnology, Graphic Era University, Dehradun, Uttarakhand, India. [2]Department of Medical Microbiology & Medical Lab Technology, SBS PG Institute of Biomedical Sciences and Research, Dehradun, Uttarakhand, India. Email: nishantrai1@gmail.com

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ABSTRACT

Objective: The aim of this study was to investigate the *in-vitro* hepatoprotective activity of solvent extracts of *Albizia lebbeck, Cassia occidentalis,* and *Swertia chirata* on HepG2 cell line.

Methods: The methanolic, ethanolic, and acetone seed extracts of *A. lebbeck, C. occidentalis,* and leaves extract of *S. chirata* were used in this study. The different extracts of *A. lebbeck, C. occidentalis,* and *S. chirata* were assessed for their hepatoprotective activity on human liver hepatocellular carcinoma (HepG2) cell line against paracetamol (PCM) as a liver damage inducing agent. The cell line viability was assessed by 3-(4,5-dimethyl thiazol-2-yl)-5-diphenyltetrazolium bromide assay.

Results: The percentage cell viability was determined with respect to the normal control cells. Control cells showed 100% cell viability in all tested plant extracts. The PCM treated HepG2 cells showed a maximum cell viability (46.6±2.49%) in presence of all seed extracts of *A. lebbeck*. The silymarin and PCM treated HepG2 cells showed maximum cell viability (156.6±2.49%) presence of leaves extract of *S. chirata*. The maximum cells viability of 131.6±9.39% was observed in methanolic seed extract of *A. lebbeck* (50 µg/mL), and the minimum cell viability of 107.3±3.68% was observed in acetone seed extract of *C. occidentalis* (50 µg/mL) comparatively.

Conclusions: The methanolic, ethanolic, and acetone extracts of seeds/leaves from *A. lebbeck, C. occidentalis,* and *S. chirata* were showed the hepatoprotective activity. Further *in vivo* and clinical studies are required to confirm their therapeutic efficacy.

Keywords: *Albizia lebbeck, Cassia occidentalis*, *Swertia chirata*, Paracetamol, HepG2, [3-(4,5-dimethyl thiazol-2-yl)-5-diphenyltetrazolium bromide] assay.

INTRODUCTION

Herbal medicines have the ability to affect body systems. The effects are dependent on the chemical constituents present in the plant used. Scientists first started extracting and isolating chemicals from plants in the 18th century and since that time, we have grown a custom of looking at herbs and their effects in terms of the active constituents they contain. *Albizia lebbeck* (Shirish, *Leguminosae*) is a deciduous tree with compound leaves, flat an oblong fruit, round cream colored seeds, and grows wild. The plant is found throughout India, Bangladesh, tropical and subtropical Asia, and Africa [1,2]. Therapeutically bark is used in bronchitis; bark and seeds in piles; root in hemicranias; flowers in a cough, bronchitis, tropical pulmonary eosinophilia, and asthma [3]. The seeds contain e-chinocystic acid and β-sitotosteroland quercetin. It has been reported that *A. lebbeck* has antibacterial, analgesic, anti-inflammatory, antioxidant, anti-asthmatic and anti-anaphylactic, nootropic, anxiolytic and anticonvulsant activities [4]. *Swertia chirata,* Indian name: *Chirayata* (*Gentianaceae*) is a robust annual herb which grows up to about 1.5 meters in height. It has leaves in opposite pair about 10 cm long, without stalks, pointed at the tip. The plant has numerous flowers, pale green in color, tinged with purple, with long white or pink hairs and minute sharp pointed fruits. The whole plant, collected in its flowering stage and dried, constitutes the drug. It is found in the Himalayan ranges of India from Kashmir to Bhutan at an altitude of 1200-3000 m. It is also found in the Khasi Hills of Meghalaya at an altitude of 1200-1500 m [5]. It has long been used by the Ayurvedic physicians as a bitter tonic. The plant contains a bitter glycoside chiratin, which yields on hydrolysis, two bitter principles, ophelic acid and chiratin. The ophelic acid is a brown hydroscopic substance which is soluble in water and alcohol. Chirata is an effective drug for reducing fever [6]. It has been widely used as herbal medicine in Asian countries particularly in India, Nepal, Myanmar, Arab and some parts of the European countries. Reported studies showed extracts of this plant has attributable properties as hypoglycemic, antipyretic, anti-inflammatory, antibacterial, antiviral, antimalarial, antihepatotoxic, and wound healing activity [7]. *Cassia occidentalis* belongs to the genus *Cassia* and the Family *Caesalpiniaceae*. It is called Stinking Weed. In Hausa, it is known as "Rai dore." In Igbo, it is called "Osiisi" while "Gaya" in Nupe [8]. It is an erect herb, commonly found by road sides, ditches and waste dumping sites. *C. occidentalis* has many applications in traditional medicine. All the parts of the plant have medicinal uses. Traditionally, its roots, leaves, flowers and seeds are used as laxatives and purgative. Phytochemically, the aqueous extract of *C. occidentalis* contains tannins, anthraquinone, sterol, cardiac glycosides, saponin, and alkaloids. The World Health Organization estimated that 80% of the population of developing countries relies on traditional medicines, mostly plant drugs, for their primary health care needs. Medicinal plants play an important role in the discovery of novel drugs used in modern medicine [9]. Traditional medicines have been used for many centuries by a substantial proportion of the population of India. India has a rich heritage of traditional knowledge and is home to several important time-honored systems of health care like Ayurveda, Siddha, and Unani. It has been estimated that the proportion of medicinal plants in India (7,500 of the 17,000 higher plant species are medicinal plants) is higher than any country of the world with respect to the existing flora of that respective country [10]. There are a number of studies which highlight the uses of herbal drugs using the plant extracts of *S. chirata*, *Albizia,* and *Andrographis paniculata* to name a few in hepatoprotection [11,12]. These observations have drawn attention for the present study which is an attempt to explore the *in-vitro* hepatoprotective activity of seeds of *A. lebbeck* and *C. occidentalis* and leaves of *S. chirata* on human liver hepatocellular carcinoma cells.

METHODS

Collection and preparation of the plants extracts

A. lebbeck and *C. occidentalis* were collected from the area of Nakronda forest range, (30°14'19.60"N, 78°07'01.70"E), Dehradun, and *S. chirata* was collected from Chobattakhal (29°57'16.40"N, 78°52'41.20"E), Pauri Garhwal, Uttarakhand region of North India. The above-mentioned plants were examined, identified, and authenticated. The seeds of *A. lebbeck*, *C. occidentalis,* and leaves of *S. chirata* were air dried and pulverized into powder. About 30 g of the powdered sample of each medicinal plant were weighed and extracted in 100 mL each of methanol (ME), ethanol (ET) and acetone (AC) extract in a soxhlet apparatus separately and the process is carried out for 7 days at 40-45°C. The filtrate was evaporated to dryness at 40°C in a rotary evaporator. The above process was repeated for several times until the sufficient amount of extract is produced. The concentrated extract of each plant was stored at 4°C until when required for use.

Chemicals

3-(4,5-dimethyl thiazol-2-yl)-5-diphenyltetrazolium bromide (MTT), fetal bovine serum (FBS), phosphate buffered saline (PBS), Dulbecco's Modified Eagles Medium (DMEM), trypsin, ethylenediaminetetraacetic acid (EDTA), glucose, dimethyl sulfoxide, and hydrogen peroxide were obtained from Hi-Media Laboratories Ltd., Mumbai.

HepG2 cell line

Human liver hepatocellular carcinoma (HepG2) cell lines were obtained from the National Centre for Cell Science (NCCS) Pune, India. The cells were regularly maintained in DMEM containing 10% FBS in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with 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 microtiter plates (Tarsons India Pvt. Ltd., Kolkata, India). The cells were seeded at a density of 1×10^5 cells/mL. The culture medium was changed twice a week.

MTT assay

The cells were seeded at density of 1.0×10^5 cells/mL in 96-well flat bottomed plates, and incubated at 37°C in a humidified incubator with 5% CO_2. After 24 hrs, when partial monolayer was formed, the supernatant was flicked off and the monolayer was washed once. The final volume of all the treated and the control wells was kept same, i.e., 500 µl. Paracetamol (PCM) (1%), silymarin, (100 µg/ml), different concentrations (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL) of methanolic, ethanolic and acetone extract of *A. lebbeck, C. occidentalis and Swertia chirata* were added to specified wells. The silymarin was used as a control. Microscopic examination was carried out after 60 minutes of PCM intoxification. Cytotoxicity was assessed by estimating the percentage viability of HepG2 cells by MTT reduction assay. The absorbance was measured by enzyme-linked immune sorbent assay reader at 540 and 630 nm [13]. The wells containing medium only served as a blank while the wells containing untreated wells were used as a control in the assay. The test was performed in duplicates. The percentage cell viability was calculated using the formula.

$$\text{Percent cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of normal control cells}} \times 100$$

Statistical analysis

The results of hepatoprotective activity were expressed as the mean±SEM. A statistical analysis was performed with one-way analysis of variance followed by Tukey's multiple comparison tests using Graph Pad Instat Software. p<0.05 was considered to be statistically significant (p<0.05).

RESULTS

The percentage cell viability with respect to the normal control (NC) cell lines (HepG2) at different concentrations of methanolic, ethanolic and acetone seeds extracts of *A. lebbeck*, *C. occidentalis* and leaves of *S. chirata* were determined. The NC cells showed 100±0.02% cell viability in all tested plant samples (Fig. 1). The PCM treated HepG2 cells showed 46.6±2.49% cell viability in all extracts of *A. lebbeck*; 44.3±1.69% cell viability in all extracts of *C. occidentalis* and 46.3±1.24% cell viability in all extracts of *S. chirata* (Fig. 2). The silymarin and PCM treated HepG2 cells showed 156.3±1.69% cell viability in all extracts of *A. lebbeck*; 154±1.63% cell viability in all extracts of *C. occidentalis* and 156.6±2.49% cell viability in all extracts of *S. chirata* (Fig. 3).

The methanolic seed extract of *A. lebbeck* at concentration 100 µg/mL, 50 µg/mL, 25 µg/mL and 12.5 µg/mL showed 140.6±1.69%, 154.3±5.90%, 105.3±2.49%, 87.6±4.02% cell viability, respectively. The ethanolic seed extract of *A. lebbeck* at concentration 100 µg/mL, 50 µg/mL, 25 µg/mL and 12.5 µg/mL showed 134±3.74%, 147.6±3.85%, 100±4.32%, 79.3±1.88% cell viability, respectively. The acetone seed extract of *A. lebbeck* at concentration 100 µg/mL, 50 µg/mL, 25 µg/mL and 12.5 µg/mL showed 123.3±7.36%, 136.6±7.71%, 94±5.09%, 79.6±5.43% cell viability, respectively. Out of all, 50 µg/mL concentration was selected for checking its protective efficacy in presence of PCM. 50 µg/mL methanolic extract (ME) and PCM showed 131.6±9.39% cell protection, 50 µg/mL ethanolic extract (ET) and PCM showed 120.3±7.03% cell protection and 50 µg/mL AC and PCM showed 115.3±8.37% cell protection (Figs. 4a and 5).

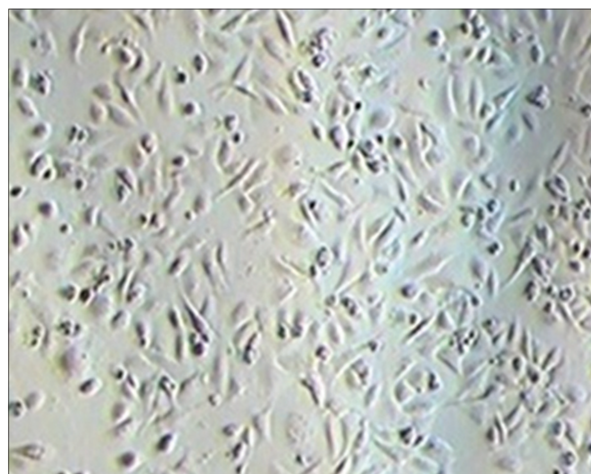

Fig. 1: Normal control cells. The HepG2 cells pretreated with Dulbecco's Modified Eagles Medium showing normal cells surface architecture (×40)

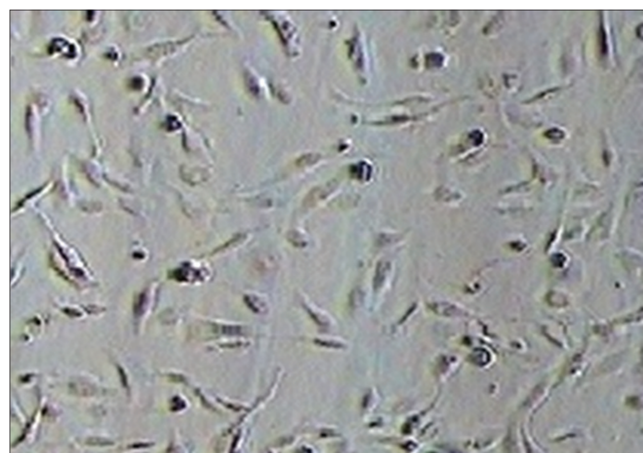

Fig. 2: Paracetamol (PCM) treated. PCM induced toxicity to hepatic cells (HepG2) showed detachment of cells from the surface of plate, rounding up of cells and alteration in cellular meshwork indicating cytotoxicity and necrosis (×40)

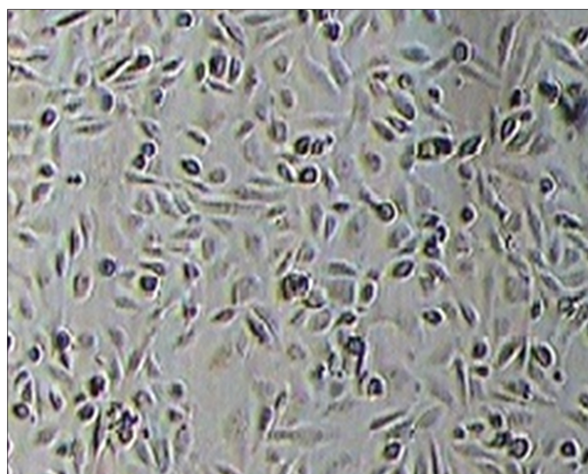

Fig. 3: Silymarin treated. HepG2 cells pretreated with silymarin (hepatoprotective drug), at concentration of 100 µg/mL showed intact cells and a confluent meshwork of cells (×40)

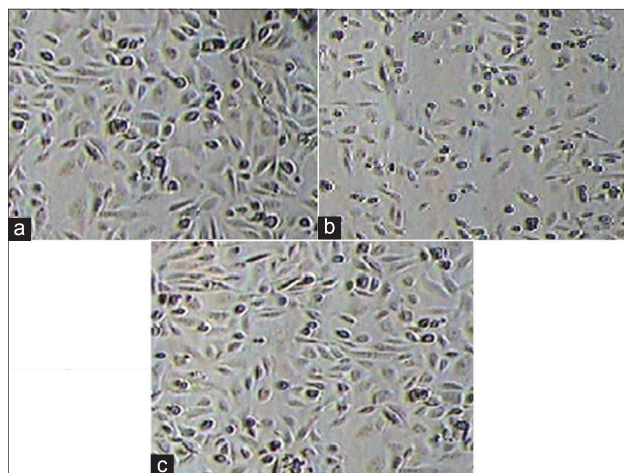

Fig. 4: Pretreatment with plant extracts (50 µg/mL). (a) Protection of HepG2 pre-treated with methanolic extract (ME) of *Albizia lebbeck* seed, (b) protection of HepG2 pre-treated with ME of *Cassia occidentalis* seed, (c) protection of HepG2 pre-treated with ME of *Swertia chirata* leaves (×40)

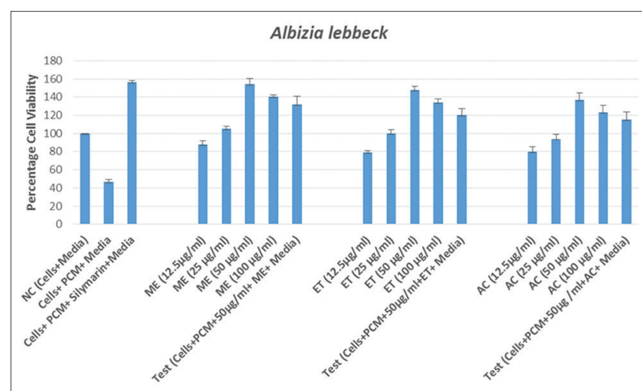

Fig. 5: Cell cytotoxicity assay (3-(4,5-dimethyl thiazol-2-yl)-5-diphenyltetrazolium bromide assay) of *Albizia lebbeck* seed extract. Percentage cell viability of NC, paracetamol (PCM) treated, silymarin treated, ME, ME, ET and AC (at different concentrations), test extract (50 µg/mL) with PCM in respect to normal cell control (HepG2 cell). NC: Normal control, ME: Methanolic extract, ET: Ethanolic extract, AC: Acetone extract

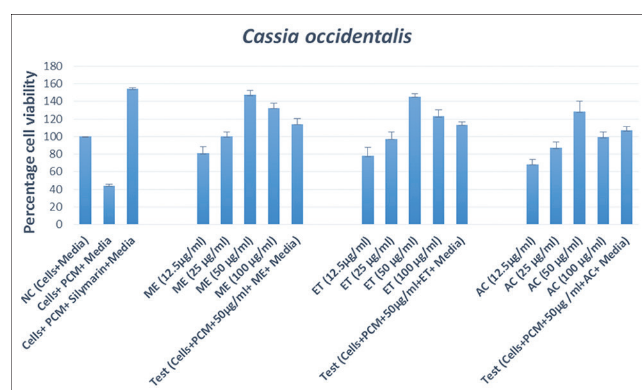

Fig. 6: Cell cytotoxicity assay (3-(4,5-dimethyl thiazol-2-yl)-5-diphenyltetrazolium bromide assay) of *Cassia occidentalis* seed extract. Percentage cell viability of NC, paracetamol (PCM) treated, silymarin treated, ME, ET and AC (at different concentrations), test extract (50 µg/mL) with PCM in respect to normal cell control (HepG2 cell). NC: Normal control, ME: Methanolic extract, ET: Ethanolic extract, AC: Acetone extract

The methanolic seed extract of *C. occidentalis* at concentration 100 µg/mL, 50 µg/mL, 25 µg/mL and 12.5 µg/mL showed 136.6±5.31%, 147.3±4.98%, 100.3±4.92%, 81.3±7.40% cell viability respectively. The ethanolic seed extract of *C. occidentalis* at concentrations 100 µg/mL, 50 µg/mL, 25 µg/mL and 12.5 µg/mL showed 123.3±6.79%, 145.6±3.09%, 97.6±7.84%, 78.3±9.28% cell viability respectively. The acetone seed extract of *C. occidentalis* at concentrations 100 µg/mL, 50 µg/mL, 25 µg/mL and 12.5 µg/mL showed 99.6±5.90%, 128.6±11.4%, 87.3±6.54%, 68.6±5.24% cell viability respectively. Out of all, 50 µg/mL concentration was selected for checking its protective efficacy in presence of PCM. 50 µg/mL ME and PCM showed 114.3±6.34% cell protection, 50 µg/mL ET and PCM showed 113.6±3.29% cell protection and 50 µg/mL AC and PCM showed 107.3±3.68% cell protection (Figs. 4b and 6).

The methanolic leave extract of *S. chirata* at concentration 100 µg/mL, 50 µg/mL, 25 µg/mL and 12.5 µg/mL showed 134.6±6.23%, 149.6±7.03%, 106.6±9.56%, 85.3±6.18% cell viability, respectively. The ethanolic leaves extract of *S. chirata* at concentrations 100 µg/mL, 50 µg/mL, 25 µg/mL and 12.5 µg/mL showed 120.6±8.05%, 144.3±3.09%, 108.6±10.6%, 71±5.71% cell viability, respectively. The acetone leaves extract of *S. chirata* at concentrations 100 µg/mL, 50 µg/mL, 25 µg/mL and 12.5 µg/mL showed 119±7.87%, 134.6±8.9%, 90.3±10.3%, 71.3±7.71% cell viability, respectively. Out of all, 50 µg/mL concentration was selected for checking its protective efficacy in presence of PCM. 50 µg/mL ME and PCM showed 120±2.44% cell protection, 50 µg/mL ET and PCM showed 117±1.63% cell protection and 50 µg/mL AC and PCM showed 110±1.63% cell protection (Figs. 4c, 7 and Table 1).

DISCUSSION

Medicinal plants are crucial for about 80% of the world population in developing as well as the developed nations for their primary and basic health care needs owing to better tolerability, superior compatibility with human body and having lesser side effects. The herbal drugs are rapidly becoming popular due to the study of the extensive activity of different plant components and their therapeutic principles [14].

Among the plant species, *A. lebbeck, C. occidentalis* and *S. chirata* also seem to possess numerous pharmacological properties. Bioactive compounds such as saponins, alkaloids, flavonoids, and phenolic compounds are reported to be highly active against cytotoxicity in tumor cancer cells. They are widely used as anti-asthma, anti-septic,

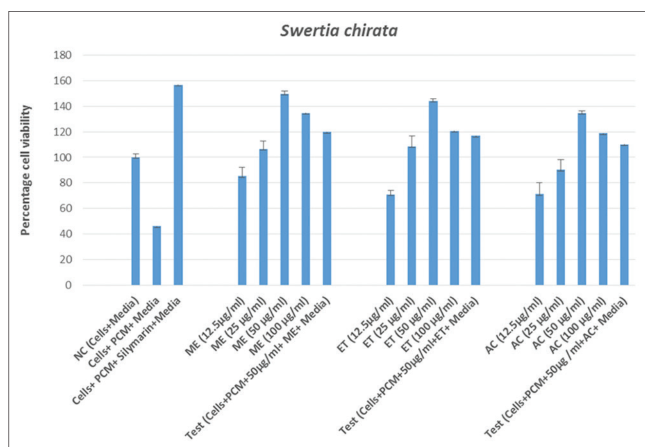

Fig. 7: Cell cytotoxicity assay (3-(4,5-dimethyl thiazol-2-yl)-5-diphenyltetrazolium bromide assay) of *Swertia chirata* leaves extract. Percentage cell viability of NC, paracetamol (PCM) treated, silymarin treated, ME, ET and AC (at different concentrations), test extract (50 μg/mL) with PCM in respect to normal cell control (HepG2 cell). NC: Normal control, ME: Methanolic extract, ET: Ethanolic extract, AC: Acetone extract

Table 1: Percentage cell viability of HepG2 cell lines with different plant extracts treated groups on HepG2 cell lines

Samples	Percentage cell viability		
	(*A. lebbeck*)	**(*C. occidentalis*)**	**(*S. chirata*)**
NC	100±0.02	100±0.02	100±0.02
PCM treated	46.6±2.49	44.3±1.69	46.3±1.24
Silymarin+ PCM treated	156.3±1.69	154±1.63	156.6±2.49
50 μg/mL ME extract+ PCM treated	131.6±9.39	114.3±6.34	120±2.44
50 μg/mL ET extract+ PCM treated	120.3±7.03	113.6±3.29	117±1.63
50 μg/mL AC extract+ PCM treated	115.3±8.37	107.3±3.68	110±1.63

The values represent mean of triplicate tests with their respective standard deviations. ME: Methanolic extract, ET: Ethanolic extract, AC: Acetone extract, *A. lebbeck: Albizia lebbeck*, *C. occidentalis: Cassia occidentalis*, *S. chirata: Swertia chirata*, NC: Normal control, PCM: Paracetamol

anti-dysenteric, anti-tubercular, antioxidant and anti-microbial agents and also possess hepatoprotective activity [10,15]. *In-vitro* bioassays of biological extracts possessing >200 μg/mL are not considered meaningful as it is unlikely that such concentrations will be reached *in-vivo* and due to the probability that high concentrations of plant extracts will significantly change the physiochemical environment in terms of ionic strength, pH and osmolarity which will impact the cell viability independent of the extract itself leading to artificial toxicity [16,17]. Therefore keeping in mind this fact, this study used a maximum concentration of 100 μg/mL of the plant extracts in all the experiments.

In this study, we evaluated the hepatoprotective activity of methanol, ethanol and ACs of seeds from *A. lebbeck, C. occidentalis* and leaves extract from *S. chirata* against PCM induced cytotoxicity. Incubating HepG2 cells with PCM for 2 hrs caused a significant loss in the cell viability. Pretreatment with methanolic, ethanolic and ACs of the above-mentioned plants for the same time interval resulted in a dose-dependent increase in cell viability at concentrations ranging from 12.5 to 50 μg/mL and then after, an appreciable decrease at a concentration of 100 μg/mL. The highest percentage cell viability (154.3±5.90%) was observed at 50 μg/mL concentration of methanolic seed extract of *A. lebbeck,* therefore in the test treated group, this concentration was selected along with PCM. The treated group showed 131.6±9.39% cell viability confirming its heaptoprotective potential, while acetone seed extract of *C. occidental* is showed lowest cell viability (128.6±11.4%) at the same concentration (i.e., 50 μg/mL) and in test treated group it was observed 110±1.63% confirming its heaptoprotective potential. Further, on comparing with the reference standard where silymarin showed 156.3±1.69% cell viability in seed extracts of *A. lebbeck*, 154±1.63% cell viability in seed extracts of *C. occidentalis* and 156.6±2.49% cell viability in leaves extract of *S. chirata* against PCM induced hepatotoxicity on HepG2 cell line. In a similar study by Drever *et al.*, [18] it was shown that ET of *C.* species exhibit remarkable neuroprotection in mouse hippocampal cultures. Studies have promisingly shown hepatoprotection and immunomodulation by methanolic and ETs of *A. lebbeck* and *S. chirata* [6,19,20]. Promising results have also been shown in the model of Wister rats proving histologically the beneficial effect of the ET of *A. lebbeck* in hepatoprotection [21]. Our study also revealed the hepatoprotective activity of *C. occidentalis.*

Hence, overall results indicates that methanolic, ethanolic and acetone seed extracts of *A. lebbeck, C. occidentalis* and leaves extract of *S. chirata* possess a potent hepatoprotective activity against PCM induced hepatic damage, and the main mechanism involved in the protection could be associated with its strong capability to reduce the intracellular level of reactive oxygen species. The effective components which might act against oxidative damage are mainly phenolic compounds, flavonoids, and tannins [22]. Many phytochemical reports revealed that the ME of the plants was found to contain higher concentration of terpenoids in addition to flavonoids [23]. Elucidating the exact cellular mechanism of action of these plant extracts on hepatic cells is outside the scope of the present study, and it can only be speculated whether any or all of these extracts act at a common point involved in inhibiting distinct pathways involved in cell death. Indeed, the very nature of herbal extracts means it is impossible to attribute actions seen to specific compound, given the number of pharmacologically active substances in such preparations their potentially complex interaction. In addition, these extracts can also be tried as a combination therapy *in-vivo* and *in-vitro* to know and understand the synergistic action of these compounds if any, cellular protection. Our findings are relevant for future therapeutic considerations related to methanolic, ethanolic and ACs of these plants alone or in combination for hepatic disorders.

CONCLUSION

ME of *A. lebbeck, C. occidentalis,* and *S. chirata* showed the most efficient hepatoprotective activity against PCM on HepG2 cell lines with silymarin as control compared to ethanolic and AC of *A. lebbeck, C. occidentalis* and *S. chirata,* respectively. The above study indicates positive hepatoprotective activity of the above mentioned plants. Characterization and identification of active principles in ME of *A. lebbeck* are further required to evaluate its potential as a potent hepatoprotective agent for liver drug formulation. Further *in-vivo* and clinical studies are also required to confirm its therapeutic efficacy.

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