

Development, Characterization, and Evaluation of Hepatoprotective Effect of *Abutilon indicum* and *Piper longum* Phytosomes

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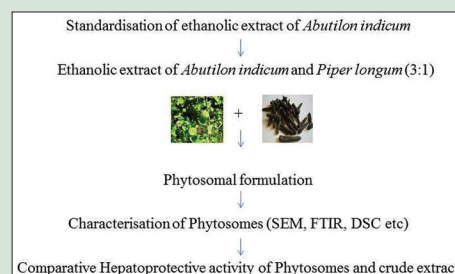
ABSTRACT

Background: Evidences from ethnopharmacological practices have shown that combination of *Abutilon indicum* and *Piper longum* are traditionally used to treat symptoms of the liver disorder. The hypothesis is phytosomes of a combination of both crude drug extract will be more effective and safe as hepatoprotective agent. **Aim:** Present work is aimed at development and characterization of phytosomes containing ethanolic extract of both drugs to meet the need for better effectiveness and safety. **Materials and Methods:** Phytosomes were formulated by using Indena's patented process. Characterization involved following parameters: Particle size determination, percentage yield, entrapment efficiency, differential scanning calorimetry, scanning electron microscope, fourier transform infrared spectroscopy, and high performance thin liquid chromatography. Liver damage was induced in adult Charles foster rats (150 ± 10 g) with CCl₄ in olive oil (1:1 v/v, i.p) 1 ml/kg once daily for 7 days. LIV 52 (1 ml/kg per oral [p.o]), ethanolic extract of *A. indicum* and *P. longum* combination (100, 200, and 400 mg/kg p.o) and phytosomes (100 mg/kg p.o.) was given 3 days prior to CCl₄ administration. Estimation of liver marker enzymes and histopathological studies were done. Result was analyzed by using (analysis of variance) followed by Student-Newman-Keuls test. **Result:** Combined extract has shown hepatoprotective activity but phytosomal formulation has more potent hepatoprotective effect on CCl₄ induced liver toxicity at very low dose comparative to a higher dose of combined extract. **Conclusion:** Novel approach for herbal drug delivery is more prominent than conventional which improves bioavailability of polar extract and also patient compliance. **Key words:** *Abutilon indicum*, Characterization, Hepatoprotective activity, Phytosome, *Piper longum*

SUMMARY

- Standardised ethanolic extract of leaves of *abutilon indicum* and *piper longum* fruits by microwave assisted extraction was used for phytosomal complex

formation and phytosomal complex was characterised by various parameters and finally the hepatoprotective activity of phytosomes and crude extract was evaluated by different biochemical markers and histopathological study.



Abbreviations Used: DSC: Differential scanning calorimetry, SEM: Scanning electron microscope, FTIR: Fourier transform infrared spectroscopy, HPTLC: High performance thin liquid chromatography, p.o: Per oral, *A. indicum*: *Abutilon indicum*, *P. longum*: *Piper longum*.

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INTRODUCTION

According to the ethnobotanical literature of Odisha, paste of *Abutilon indicum* leaves and *Piper longum* fruits were used to treat symptoms of jaundice by tribes of Kalahandi district, Odisha, India.^[1] The crude drug used were leaves of *A. indicum* (Malvaceae) and fruits of *P. longum* (Piperaceae). *A. indicum* (Malvaceae) commonly known as Indian Mallow, Atibala in Sanskrit and Kanghi in Hindi is an important medicinal plant of Indian traditional system of medicine and found to possess hypoglycemic activity,^[2] anti-inflammatory activity,^[3] antibacterial activity,^[4] anticonvulsant activity,^[5] lipid lowering activity,^[6] antiulcer activity,^[7] anti-diarrheal activity,^[8] immunomodulatory activity,^[9] analgesic activity,^[10] wound healing activity,^[11] acetylcholinesterase inhibitory activity,^[12] and hepatoprotective activity.^[13] Chemically, the active constituents present in *A. indicum* are fatty acids, flavonoids, quercetin, glycosides, alkaloids, steroids, terpenoids, saponins, sesquiterpenes, lactones, gallic acid, β-sitosterol, geraniol, caryophyllene, and phenolic compound.^[14] Quercetin is the bioactive marker of *A. indicum*

and ethanolic extract of leaves contains 72% more quercetin than flowers.^[15] The mechanism mainly involved in CCl₄ induced liver injury is lipid peroxidation by free radicals derivatives of CCl₄.^[16] *P. longum* (Piperaceae) also known as Indian long pepper, it has many medicinal and dietary uses and its fruits are taken in combination with other herbs in Ayurvedic medicine. The main active constituent piperine (1-piperoyl piperidine) has been demonstrated for its bioavailability enhancing property may be due to inhibition of drug

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metabolizing enzymes and renal clearance, enhancing blood supply for absorption or modulating active transporters.^[17,18] Phytosomes are defined as “phyto” means plants and “some” means cell-like. The phytosome (technology was developed by Indena s.p.a of Italy), are used to enhance the bioavailability of phytomedicines by incorporating phospholipids into standardized plant extract.^[19] It is novel drug delivery system in which hydrophilic choline moiety (head) binds to phytoconstituents (polar) and lipophilic phosphatidyl moiety surrounds choline bound phytoconstituents or form outer layer, hence water soluble phytoconstituents become lipid soluble.^[20] Phytosomes contains naturally occurring phospholipid, phosphatidylcholine (PC) like soylcithin. It is also a cellular component which is biodegradable and has reported hepatoprotective activity. Phytosomes have improved pharmacokinetic and pharmacological parameter.^[21-23]

MATERIALS AND METHODS

Plant material

The fresh green leaves of *A. indicum* were collected in the month of November from the botanical garden of Department of Dravyaguna, Banaras Hindu University, Varanasi. Dried fruits of *P. longum* were brought from Gola market of Varanasi, and identification of these drugs were done by Prof. N. K. Dubey, Department of Botany, Faculty of Science, Banaras Hindu University, Varanasi.

Drugs and chemicals

LIV 52 (Himalaya Drug Company) was purchased from the local medicinal shop of Varanasi. Carbon tetrachloride used to induce hepatotoxicity was obtained from the Merck Ltd. Mumbai. The biochemical estimation was performed by standard procedures in biochemical kit obtained from RAGINI DIAGNOSTIC, Varanasi. All other chemicals used were of analytical grade and were obtained from the Hi-Media Laboratories Pvt. Ltd, Mumbai.

Experimental animals

Adult Charles Foster albino rats (150 ± 10 g) of either sex, were procured from the Central Animal House (CPCSEA), Institute of Medical Science, Banaras Hindu University, Varanasi, India. The certified pathogen free animals were housed in polypropylene cages under standard condition (ambient temperature of 25°C ± 1°C and 45–55% RH, with a 12:12 h light/dark cycle). They were fed with commercially available rat feed (Amrut Rat and Mice Feed Pvt. Ltd., Sangli, India) and water *ad libitum*. Animals were acclimatized for at least 1 week before commencement of experiments and exposed only once to every experiments. All experimental protocols were performed after approval from the Central Animal Ethical Committee of Banaras Hindu University (No. Dean 10-11/60 dated 07/01/2011) and were conducted in accordance with accepted standard guidelines of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

Extraction procedure

Dried leaves of *A. indicum* and fruits of *P. longum* were dried and coarsely powdered, accurately weighed homogenous powder 100 g in ratio of 3:1, respectively, were mixed with ethanol (1 g in 25 ml of ethanol) followed by preleaching for 5 min, then irradiated in microwave in an intermittent way, that is, irradiation: Cooling: Irradiation for 6 min. The microwave irradiation time was 1 min and cooling time of 1 min. After irradiation, the samples were centrifuged at 4000 rpm and the supernatant evaporated under reduced pressure. Dried residue was defatted with petroleum ether and then extracted in ethanol by maceration process for 7 days. The filtrate was dried

in vacuum rotary evaporator to yield solid residue of 7.4 g (yield, 7.4%).^[24]

Acute toxicity studies

Oral acute toxicity study of plant extract was performed according to the Organisation for Economic Cooperation and Development guidelines number 26/241, Nulliparous and nonpregnant healthy rats were used for this study. To the overnight fasted rats, combined extract of both drugs administered orally as per the guidelines and the rats were observed individually up to 48 h for any behavioral and neurological changes such as tremors, convulsions, salivation, diarrhoea, sleep, lacrimation, and feeding behavior in extract treated rats as a sign of acute toxicity. The observation was further extended up to 14 days to see any sign of mortality.

High performance thin liquid chromatography quantification of quercetin in extract

Instrument CAMAG Linomat 5 “Linomat5_170634”.

Application parameters

Inert gas used as spray gas, methanol as sample solvent, dosage speed was 150 nl/s, syringe size was 100 µl, predosage volume was 0.2 µl, number of tracks were 14, application position Y was 8.00 mm, and band length was 8.00 mm.

Development

Chamber type was Twin trough chamber 20 cm × 10 cm, Mobile phase used toluene:ethyl acetate:formic acid (5:3:0.5), solvent front position was 70.00 mm, volume as 10.00 ml, drying device used was CAMAG TLC plate heater III, temperature up to 60°C, Time was 5 min.

Formulation of phytosomes

Phytosomes were prepared by reaction of natural phospholipid (soy PC) with the extract at a molar ratio of 1:1. The reaction is carried out by refluxing in 100 ml round bottom flask with 20 ml aprotic solvent, methylene chloride, the mixture was refluxed at a temperature not exceeding 40°C for 2 h. The resultant clear solution was evaporated and 10 ml of *n*-hexane was added to it with continuous stirring, the precipitate was filtered and dried under vacuum to remove traces of solvents, resulting in a thin film formation. This thin film was separated and kept in an amber colored glass bottle, flushed with nitrogen, and stored at room temperature.^[25,26]

Evaluation of phytosomes

Particle size determination

The particle size of the phytosomes was determined by Dynamic Light Scattering (NANO ZS Malvern instrument) and Zeta potential was estimated on the basis of electrophoretic mobility under an electric field. Particle size measurement was performed following 1/100 (v/v) dilution of phytosomal suspension in redistilled water at 25°C. Zeta potential was measured using the same instrument at 25°C following the same dilution in 1 mM NaCl solution.

Percentage yield

The prepared phytosomes were dried properly and weighed accurately. This weight was divided by total weight of drugs nonvolatile recipients.

$$\text{Percent yield} = \frac{\text{weight of phytosomes formed}}{\text{weight of drugs and nonvolatile excipients}} \times 100$$

Drug entrapment efficiency

100 mg of prepared phytosomes were dried properly and dispersed in 50 ml distilled water. The content was stirred for 2 h and filtered through Whatman filter paper of pore size 45 μm . It was then suitably diluted and analyzed spectrophotometrically at λ_{max} 266. The amount of drug entrapped was calculated from the standard calibration curve.

$$\text{Entrapment efficiency} = \frac{\text{actual amount of drug in phytosomal formulation}}{\text{theoretical amount of drug in phytosomal formulation}} \times 100$$

Differential scanning calorimetry

Thermal curves of phytosomes and physical mixture of PC extract were obtained using Differential Scanning Calorimeter (Mettler-Toledo TGA/differential scanning calorimetry [DSC] STAR SW9.20, USA). Each sample weighing was scanned at a rate of 10°C/min over the range of -25–200°C. The flow rate of nitrogen was maintained at 5 ml/min.

Scanning electron microscopy

Morphological characteristics were observed by environmental scanning electron microscope (SEM)-quanta 200. The phytosomes were spread on a circular aluminium stub precoated with silver glue (for enhancing conductivity of electrons) and placed observation area of the instrument. It was then observed under the SEM in varying magnifications and micrographs were recorded. Samples were analyzed in SEM at low vacuum. The pressure was maintained in the range of 5.99–6.02e⁻¹ torr. The detector used was secondary electron detector.

Fourier transform infrared spectroscopy

FTIR spectra of pure drug *Abutilon indicum*, PC and formulated phytosomes were obtained by the conventional KBr pellet/disc method using FT-IR (SHIMADZU, Model 8400, Japan). The scanning range was 400–4000 cm⁻¹.

Drug treatment

Animals after acclimatization (6–7 days) in the animal house, animals were fasted overnight and randomly divided into seven groups of six animals each and treatment is given

- Group I - Served as a normal control and received olive oil (1 ml/kg, i.p) daily for 7 days
- Group II - Served as toxicant control and received 1:1 (v/v) mixture of CCl₄ and olive oil (1 ml/kg, i.p.) daily for 7 days
- Group III - Treated with standard drug Liv-52 at a dose of 1 ml/kg, per oral (p.o.) for 10 days and a 1:1 (v/v) mixture of CCl₄ and olive oil (1 ml/kg, i.p.) for 7 days from day 4 to day 10
- Group IV - Treated with extract 100 mg/kg, p.o. daily for 10 days and a 1:1 (v/v) mixture of CCl₄ and olive oil (1 ml/kg, i.p.) for 7 days from day 4 to day 10
- Group V - Treated with extract 200 mg/kg, p.o. daily for 10 days and a 1:1 (v/v) mixture of CCl₄ and olive oil (1 ml/kg, i.p.) for 7 days from day 4 to day 10
- Group VI - Treated with extract 400 mg/kg, p.o. daily for 10 days and a 1:1 (v/v) mixture of CCl₄ and olive oil (1 ml/kg, i.p.) for 7 days from day 4 to day 10
- Group VII - Treated with phytosomes 100 mg/kg, p.o. daily for 10 days and a 1:1 (v/v) mixture of CCl₄ and olive oil (1 ml/kg, i.p.) for 7 days from day 4 to day 10.

Animals were killed 24 h after the last dosing. Blood was collected by a cardiac puncture and allowed to clot for 30 min at room temperature. The serum was separated by centrifugation and used for the assay of marker enzymes and histopathological studies.^[27,28]

Estimation of liver marker enzymes

Lysosomal enzymes serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT),^[29] total bilirubin (TB) and direct bilirubin (DB),^[30] and alkaline phosphate (ALP)^[31] were analyzed according to standard method.

Histopathological studies

Rats were killed on the day of withdrawal of blood and livers were dissected out immediately, washed with ice-cold saline, and fixed in 10% formalin solution. After dehydration, the liver pieces were embedded in paraffin wax, sections were cut into 4–6 mm, and stained using hematoxylin and eosin and observed under a microscope for histopathological changes in liver architecture.

Statistical analysis

All values were expressed as a mean \pm SEM. Statistical significance differences between control and treatment groups were determined by one-way analysis of variance followed by Student-Newman-Keuls test unless otherwise stated. Statistical significance were determined at the level of confidence $P < 0.05$. GraphPad Prism (version 5.0) software, Inc. La Jolla, California, (USA) was used for all statistical analysis.

RESULTS AND DISCUSSION

Acute toxicity study

The combined ethanolic extract was found to be practically nontoxic when administered orally to rats and its LD50 value was found to be higher than 5000 mg/kg body weight.

High performance thin liquid chromatography quantification of quercetin in ethanolic extract of *Abutilon indicum*

After performing the high performance thin liquid chromatography

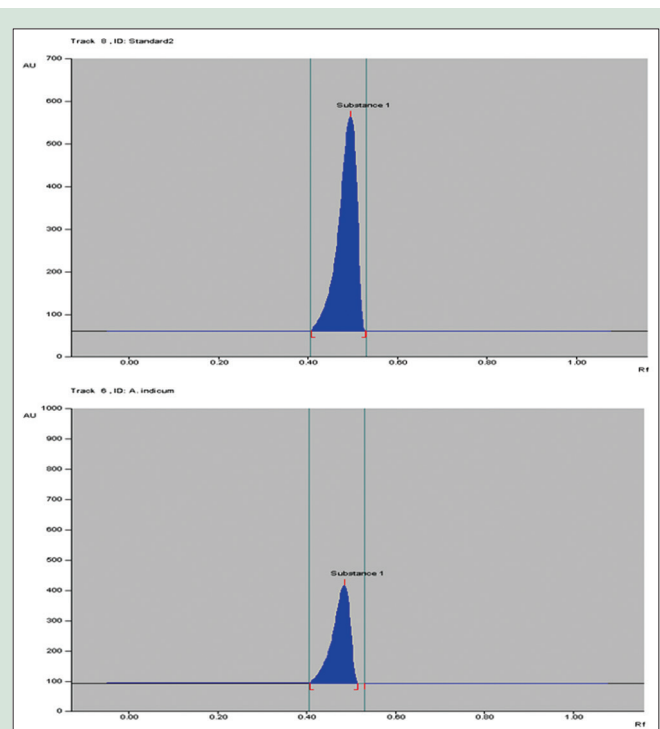


Figure 1: High performance thin liquid chromatography chromatogram of standard quercetin and ethanolic extract of *Abutilon indicum*

(HPTLC) quantification of quercetin in ethanolic extract of *A. indicum*, the quantity of quercetin measured was found to be 3.65% w/w. Chromatogram of standard quercetin and extract shown in Figure 1a and b, respectively. Statistical analysis proved that the HPTLC method of quantification will be useful for quality control and standardization of extract and also useful in differentiating the desired species from the adulterant and act as a biochemical marker for this important medicinal plant.

Preparation of phytosomes

Phytosomes were prepared by patented simple and reproducible method. Phytosomes were found to be as free flowing particles. Formulation was further characterized.

Evaluation of phytosomes

Particle size determination

The sample was diluted with distilled water (1:10) before analysis. After performing the particle size determination, the average diameter of phytosomal formulation was found to be 1357.6 nm [Figure 2] and polydispersity index was 0.451 shown in Table 1. This nanoscale range is useful to circumvent the issues of poor bioavailability and solubility of the herbal conventional drug delivery.

Percentage yield

Percentage yield varies with batches, so the average percentage yield was found to be 66.2%

Drug entrapment efficiency

Entrapment efficiency of different batches of phytosomal formulations are listed [Table 2], the maximum entrapment efficiency was 79.02 ± 0.48 ,

Table 1: Particle size and polydispersity index

Average diameter (nm)	Polydispersity index	D (10%) (nm)	D (50%) (nm)	D (90%) (nm)
1357.6	0.451	302.5	511.2	15,254.4

Table 2: Entrapment efficiency of different batches of phytosomes

Batch number	Entrapment efficiency (%)±SD
B1	48.30±0.78
B2	69.40±0.86
B3	79.02±0.48
B4	52.46±0.69
B5	65.35±0.72

SD: Standard deviation

and the minimum was 48.30 ± 0.78 shown in Graph 1. Phytosomes showed a good percent loading of the extract which might be an essential aspect for feasibility of the clinical or therapeutic delivery of the drug.

Differential scanning calorimetry

DSC is a thermoanalytical technique in which the difference in the amount of heat required to increase the temperature of a sample and reference is measured as a function of temperature. The result of a DSC experiment is a curve of heat flux versus temperature. In this experiment, DSC analysis of phytosomal complex and the physical mixture of PC and extract is reported. DSC profile phytosomes shown by black solid line in Figure 3 shows an endothermic peak with maximum at about 160°C corresponding to a 3% weight loss, and a less intense and broader endothermic signal with a maximum at about 148°C DSC profile of physical mixture green solid line Figure 3 show two broad endothermic peaks, the first one with a maximum at about 125°C and corresponding to a weight loss of 2%, and the second one at about 152°C with a loss of weight of 3% followed by the massive weight loss expected for degradation.

Scanning electron microscopy

The scanning electron microscopic view indicated the presence of spheroid or irregular shape with rough surface morphology of the phytosomes [Figure 4].

Fourier transform infrared spectroscopy

The formation of the complex can be confirmed by FTIR spectroscopy comparing the spectrum of the complex with the individual components and their complex. The spectrum of phytosomal formulation Figure 5a-c shows the additive effect of individual components, that is, the spectrum of ethanolic extract of *A. indicum* and spectrum of PC. The presence of ethanolic extract of *A. indicum* in the phytosomal complex can be revealed by absorption peaks at 2924.18 cm^{-1} and 2360.95 cm^{-1} describes the presence of N-H structure groups. Absorption peaks at 1060.74 cm^{-1} and 1060.88 cm^{-1} describes the presence of C-O (Primary alcohol). The PC can be revealed by absorption peaks at 2854.74 cm^{-1} and 2924.18 cm^{-1} describes the presence of N-H group (Ammonium ion), absorption peaks at 1238.34 cm^{-1} for C-O (Aromatic ether), absorption peak at 721.40 for C-X (chloromethane) and 516.94 cm^{-1} for C-X (bromomethane).

Estimation of liver marker enzymes

Administration of carbon tetrachloride to rats caused significant liver damage, and this damage results in altered liver marker enzymes that may result from the interference with cytochrome p450, which results

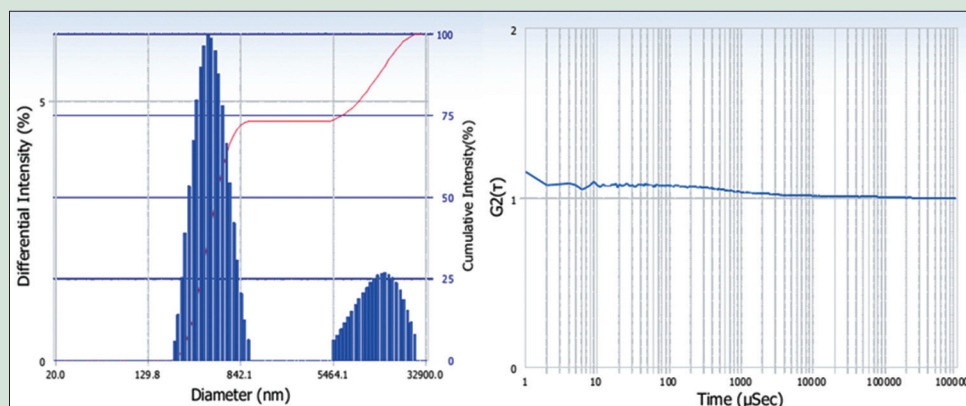


Figure 2: Particle size and Zeta potential of phytosomes

in the hindrance of the formation of hepatotoxic free radicals which in turn alters the structure and function of liver cells. Treatment of rats with combined ethanolic extract exhibited remarkable protection against carbon tetrachloride induced hepatotoxicity in dose dependent manner, which is shown in Table 3. 400 mg/kg of combined ethanolic extract of *A. indicum* and *P. longum* showed significant hepatoprotective activity against injurious effect of carbon tetrachloride comparable with the standard LIV 52 and Phytosome at 100 mg/kg has shown better effect than 400 mg/kg of combined ethanolic extract. The higher dose of herbal extract is required due to poor absorption of their polar

phytoconstituents. Phospholipid-based drug delivery system have been found a promising effect for better and effective delivery of drug and can enhance the rate and extent of drug absorption across the lipoidal biomembrane. SGOT, SGPT, ALP, TB, and DB levels of different groups are shown in Figure 6a-e.

Histopathological studies

Histopathological images of the liver are shown in Figure 7a-e.

CONCLUSION

In this study, the combined ethanolic extract of *A. indicum* and *P. longum* in ratio of 3:1 found to exhibit significant hepatoprotective activity in carbon tetrachloride induced hepatotoxicity in rats by reducing carbon tetrachloride induced rise in liver injury markers such as SGPT, SGOT, ALP, and bilirubin. Extract-phospholipid complex or phytosomes were prepared and characterized for various physicochemical parameters. The

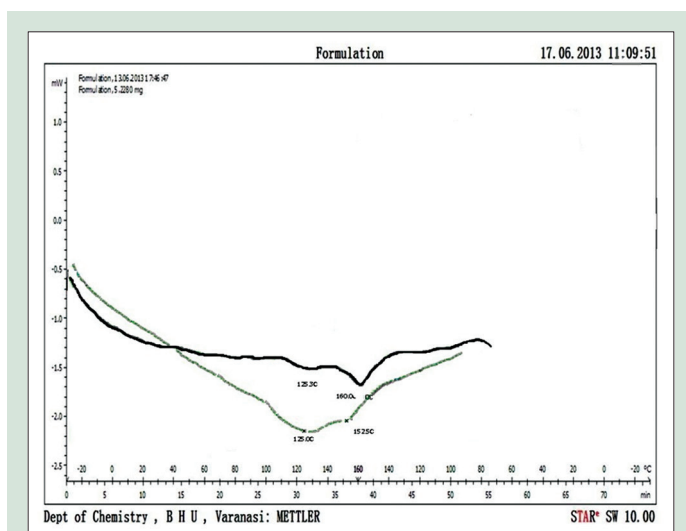


Figure 3: Differential scanning calorimetry curve of phytosomal formulation shown by black solid line and differential scanning calorimetry curve of physical mixture of phosphatidylcholine and extract is shown by green solid line

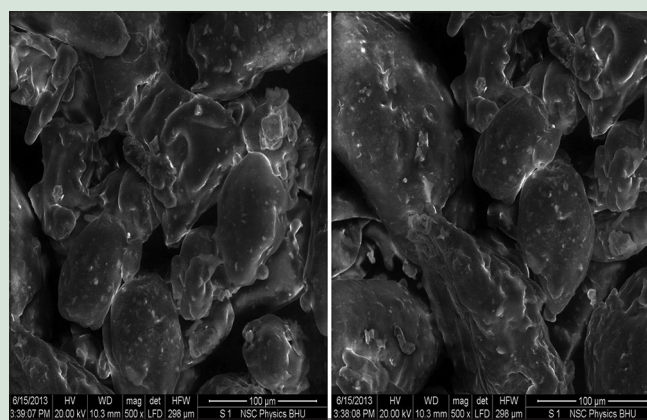


Figure 4: SEM images of phytosomes developed

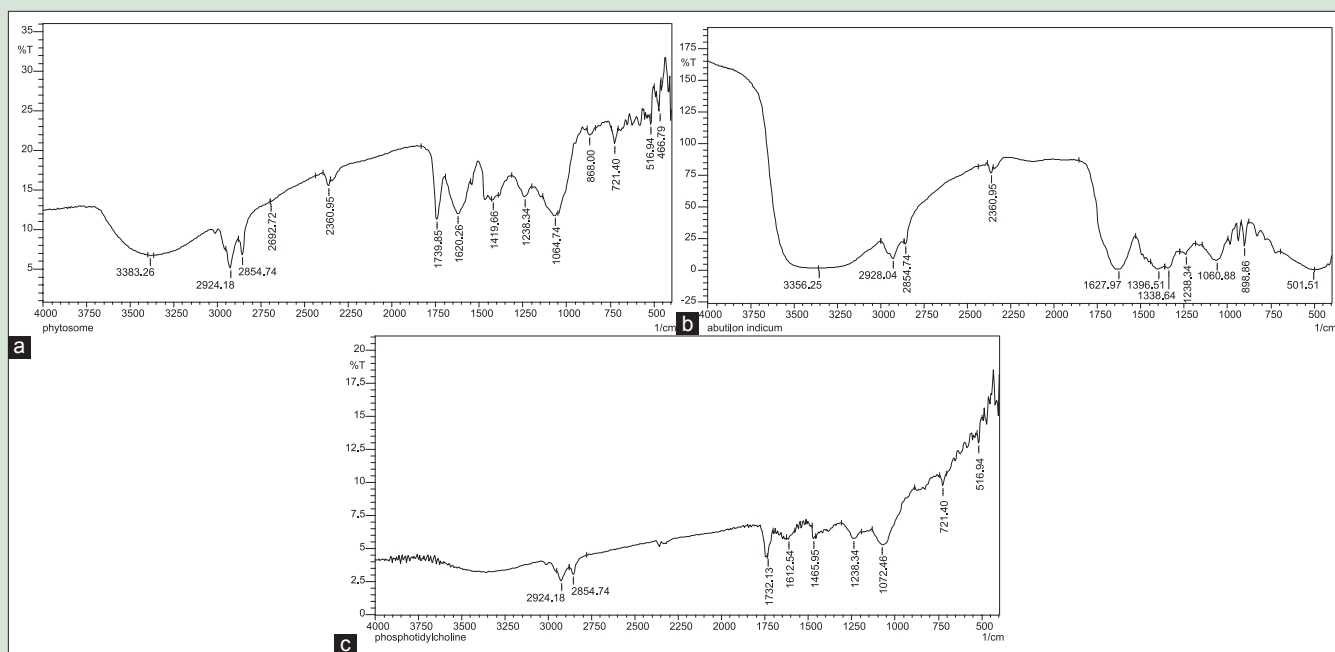
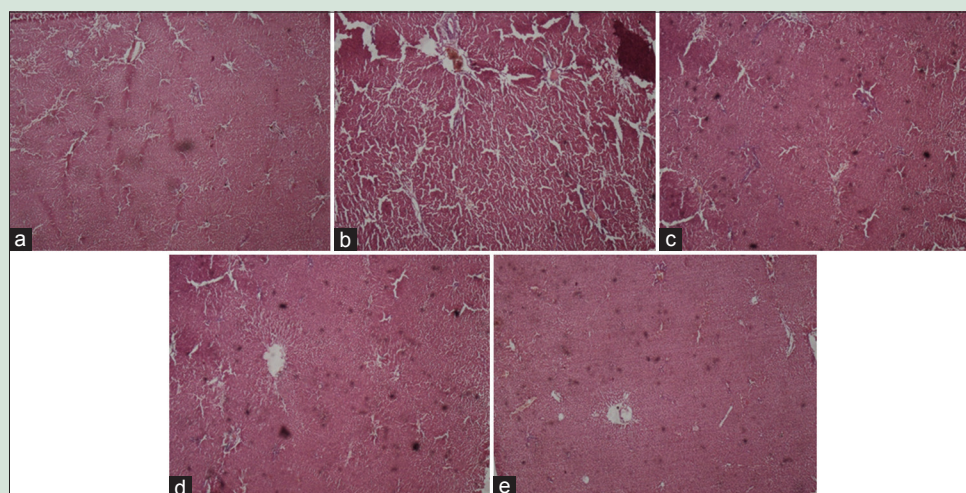
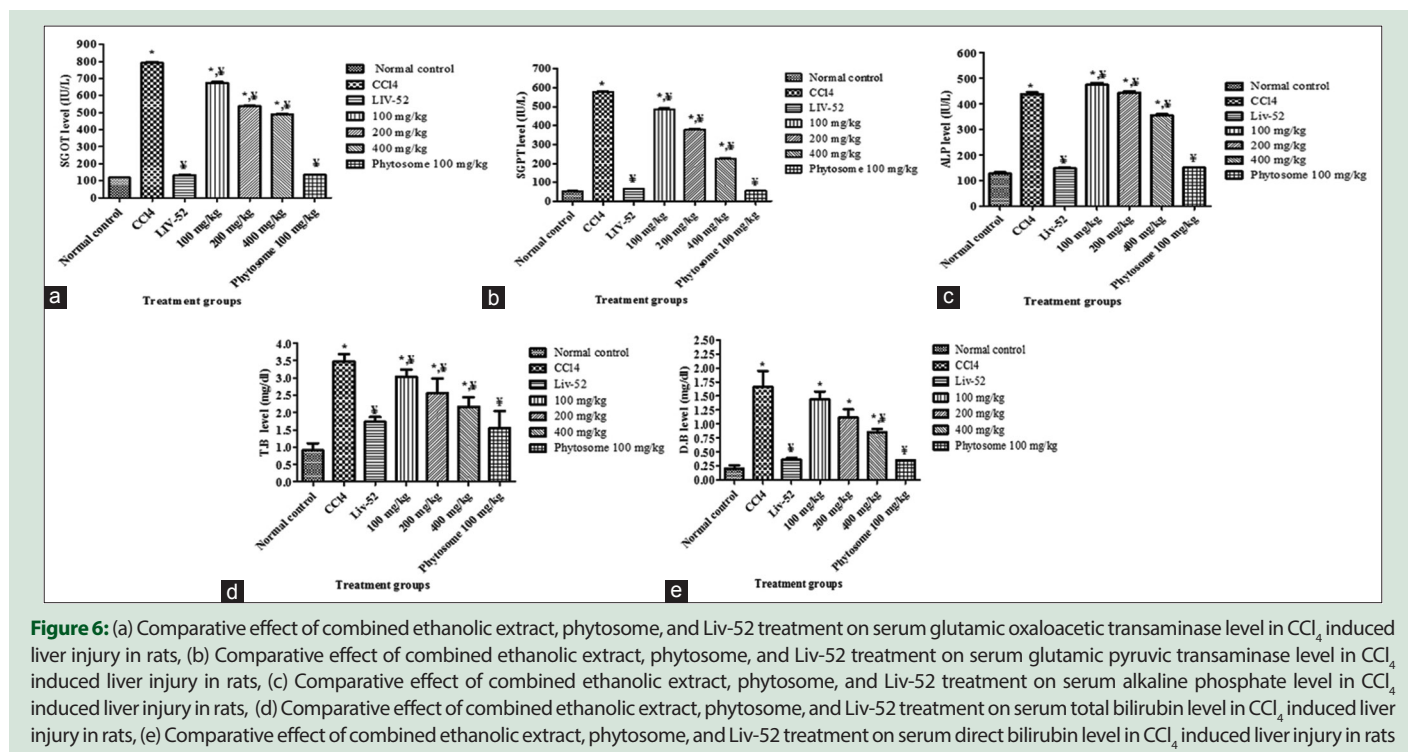


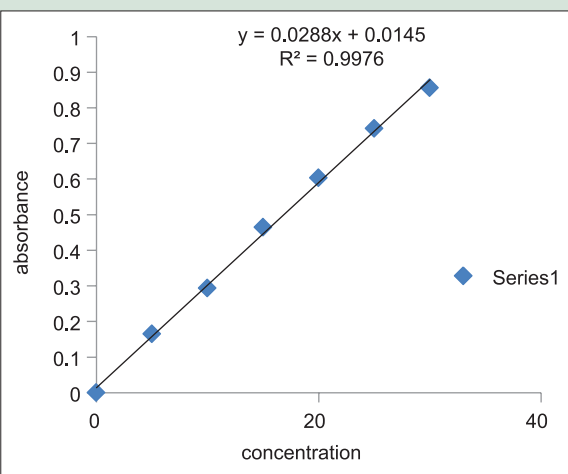
Figure 5: (a) Fourier transform infrared spectroscopy spectrum of phytosomal formulation showing combined effect of ethanolic biherbal extract and phosphatidylcholine, (b) Fourier transform infrared spectroscopy spectrum of ethanolic extract *Abutilon indicum* (c) Fourier transform infrared spectroscopy spectrum of soy phosphatidylcholine

Table 3: Values are mean±SEM, (n=6)

Group	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)	TB (mg/dl)	DB (mg/dl)
Normal control	117.65±2.49	53.32±3.48	126.40±5.96	0.91±0.20	0.20±0.06
CCl ₄ treated control	791.91±6.04*	575.41±7.38*	438.20±6.64*	3.48±0.21*	1.66±0.29*
CCl ₄ + Liv-52	132.66±4.46 ^y	63.03±2.21 ^y	147.37±4.93 ^y	1.73±0.14 ^y	0.36±0.03 ^y
CCl ₄ + 100 mg/kg	674.06±6.61 ^{*y}	485.38±7.67 ^{*y}	474.25±8.19 ^{*y}	3.03±0.21*	1.44±0.14*
CCl ₄ + 200 mg/kg	535.71±4.77 ^{*y}	379.39±4.58 ^{*y}	442.11±6.14 ^{*y}	2.57±0.42*	1.11±0.15*
CCl ₄ + 400 mg/kg	487.95±4.47 ^{*y}	225.32±2.90 ^{*y}	354.34±5.53 ^{*y}	2.17±0.27 ^{*y}	0.85±0.06 ^{*y}
CCl ₄ + phytosome 100 mg/kg	134.5±0.7 ^y	55.65±2.4 ^y	150.2±1.2 ^y	1.55±0.5 ^y	0.35±0.001 ^y

*P<0.05 normal control, ^yP<0.05 versus CCl₄ treated control. SGPT: Serum glutamic pyruvic transaminase; ALP: Alkaline phosphatase; SGOT: Serum glutamic oxaloacetic transaminase; TB: Total bilirubin; DB: Direct bilirubin; CCl₄: Carbon tetrachloride; SEM: Standard error of mean





Graph 1: Standard curve of entrapment efficiency

characterization showed that extract formed complex with phospholipids with better lipid solubility. Hepatoprotective activity of developed phytosomes was found to be more potent than crude extract of both drugs. Phytosomes 1 ml/kg, p.o was more potent than 400 mg/kg p.o dose of combined ethanolic extract in reducing liver injury markers up to control level. Hence, phytosomal formulation of this herbal drug combination can be used for clinical application to enhance the therapeutic effect and patient compliance. Hence, further study is required to investigate *in vitro* drug release profile and pharmacokinetic activity.

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Conflicts of interest

Authors declare that there are no conflicts of interest.

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