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DEVELOPMENT AND CHARACTERIZATION OF HEPATOPROTECTIVE PHYTOSOMES OF *ABUTILON INDICUM* AND *PIPER LONGUM*

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ABSTRACT

Background: *Evidences from ethnopharmacological practices have shown that combination of Abutilon indicum and Piper longum are traditionally used by tribals of orissa to treat symptoms of liver disorder. The hypothesis is Phytosomes of 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 biherbal ethanolic extract of both drugs to meet the need for better effectiveness and safety. Method: Phytosomes were formulated by using Indena's patented process. Characterization involved following parameters: particle size determination, percentage yield, entrapment efficiency, and drug content evaluation, DSC, SEM and FTIR. Phytosomes and biherbal extract were compared for their antioxidant activity.* ***Result:*** *phytosomes were prepared using soy phosphatidylcholine and methylene chloride and n-hexane as solvent. The phytosomal formulation with maximum 79% drug entrapment was developed and phytosomes shows potent antioxidant activity.* ***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, Piper longum, characterization, phytosome, antioxidant activity.

INTRODUCTION

According to ethnobotanical literature of Odisha, paste of *A. indicum* leaves and *P. 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], antidiarrheal 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-setosterol, 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 CCl_4 induced liver injury is lipid peroxidation (LPO) by free radicals derivatives of CCl_4. [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) have been demonstrated for its bioavailability enhancing property may be due to inhibition of drug 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 soylecithin. It is also a cellular component which is biodegradable and has reported hepatoprotectve activity. Phytosomes have improved pharmacokinetic and pharmacological parameter. [21-23].

MATERIAL AND METHOD

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

Soy phosphatidylcholine and all solvants and chemicals used were of analytical grade and were obtained from Hi-Media Laboratories Pvt. Ltd, Mumbai.

Experimental Animals

Adult Charles Foster albino rats (150±10 g) of either sex, were procured from 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 & Mice Feed Pvt. Ltd., Sangli, India) and water *ad libitum*. Animals were acclimatized for at least one week before commencement of experiments and exposed only once to every experiments. All experimental protocols were performed after approval from 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 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 gm in ratio of 1:1 respectively were mixed with ethanol (1 gm in 25 ml of ethanol) followed by preleaching for 5 minutes, then irradiated in microwave in an intermittent way, i.e. irradiation: cooling: irradiation for 6 minutes. 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 seven days. The filterate was dried in vaccum rotary evaporator to yield solid residue of 7.4 gm (yield, 7.4%) [24].

Acute Toxicity Studies

Oral acute toxicity study of biherbal extract was performed according to OECD (economic cooperation and development) guidelines number 26/241, Nulliparous and non-pregnant healthy rats were used for this study. To the overnight fasted rats, combined ethanolic extract of both drugs administered orally as per the guidelines and the rats were observed individually up to 48 h for any behavioural and neurological changes such as tremors, convulsions, salivation, diarrhoea, sleep, lacrimation and feeding behaviour 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.

HPTLC Quantification and standardization of Quercetin in *Abutilon indicum*

Instrument CAMAG Linomat 5 “Linomat5_170634”.

Application parameters

Inert gas used as Spray gas, methanol as sample solvant, Dosage speed was 150 nl/sec, Syringe size was 100 µl, Predosage volume was 0.2 µl, Number of tracks were 14, Application position Y was 8.00 mm, Band length was 8.00 mm.

Development

Chamber type was Twin trough chamber 20×10cm, Mobile phase used toluene: ethylacetate: formicacid (5:3:0.5), Solvant front position was 70.00 mm, Volume as 10.00 ml, Drying device used was CAMAG TLC plate heater III, Temperature upto 60°C, Time was 5 minutes.

Formulation of Phytosomes

Phytosomes were prepared by reaction of natural phospholipid (soy phosphatidylcholine) 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 by vaccum evaporator and 10 ml of n-hexane was added to it with continuous stirring, the precipitate was filtered and dried under vacuum evaporater to remove traces of solvents, resulting to 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 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 non-volatile recipients.

$$Percent\ yield = \frac{weight\ of\ phytosomes\ formed}{weight\ of\ drugs\ and\ non\ volatile\ excipients}\ X\ 100$$

Drug Entrapment Efficiency

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

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

Drug Content evaluation

Drug content was determined by taking 5 mL of phytosomal suspension was agitated with 1 mL of 0.1 % triton X-100 (non ionic surfactant) for 1 hr and kept at room temperature for 24 h without disturbing. Then, diluted with phosphate buffer (PBS) pH 7.4 and resulted solution was analyzed spectrophotometrically at 278 nm.

Differential Scanning Calorimetry (DSC)

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

Scanning Electron Microscopy (SEM)

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

FTIR Spectroscopy

FTIR Spectra of pure drug *Abutilon indicum*, phosphatidylcholine and formulated Phytosomes were obtained by the conventional KBr pellet/disc method using FT-IR (SHIMADZU, Model 8400, Japan). The scanning range was 400 to 4000cm^{-1}.

***In vitro* antioxidant activity**

Estimation of DPPH free radical scavenging activity

DPPH is usually used as a reagent to evaluate scavenging activity of antioxidants. The DPPH molecule is stable molecule that accepts electron from analyte. The ability to scavenge stable DPPH molecule is widely used method to evaluate radical scavenging capacity.The antioxidant activity of the samples, on the basis of scavenging activity of the stable DPPH free radical, was determined by the method described by Braca et al., 2001. One ml of various concentration of the sample was added to 3 ml of a 0.1 mmol/l methanolic solution of DPPH. Absorbance at 517 nm was determined after 30 min. Ascorbic acid was used as positive control [27].

Estimation of Nitric Oxide Scavenging Activity

Nitric oxide radical generated from the sodium nitropruside and measured by the Greiss reduction. Sodium nitropruside at physiological pH spontaneously generates nitric oxide, which thereby interacts with oxygen to produce nitrate ions that can be estimated by use of Greiss reagents. Thus the scavengers of nitricoxide compete with the oxygen, leading to reduced production of nitric oxide. Sodium nitroprusside (5 mM) in phosphate–buffered saline (PBS) was mixed with 3.0ml of different concentrations (10-100µg/ml) of the drugs dissolved in the suitable solvent systems and incubated at 25^{0}C for 150 min. The samples from the above were

reacted with Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% napthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine was measured at 546nm. Ascorbic acid was used as positive control [28].

Statistical Analysis

All values were expressed as mean ± Standard Error of Mean (SEM). Statistical significance differences between control and treatment groups were determined by one way analysis of variance (ANOVA) followed by Student-Newman-Keuls test unless otherwise stated. Statistical significance were determined at level of confidence $p<0.05$. Graph pad Prism (version 5.0) software was used for all statistical analysis.

RESULTS AND DISCUSSION

Acute Toxicity Study

Biherbal 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 wt.

HPTLC Quantification and standardisation of Quercetin in *Abutilon indicum*

After performing the HPTLC Quantification of Quercetin in ethanolic extract of *Abutilon 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 [Figure 1b] respectively. Statistical analysis proved that the HPTLC method of quantification will 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 0.451and zeta potential -28.6 mV shown in [Table 1]. This nanoscale range is useful to circumvent the issues of poor bioavailability and solubility of the herbal conventional drug delivery.

Avg. dia (nm)	Polydispersity Index	D (10%) (nm)	D(50%) (nm)	D(90%) (nm)	Zeta potential (mV)
1357.6	0.451	302.5	511.2	15254.4	-28.6

Table 1: Particle Size and Polydispersity Index

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 %, and minimum was 48.30 % 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 drug.

Drug content evaluation

Drug content analyses of different batches are listed in [Table 3], the maximum drug content was 91.02 % w/w, and minimum was 32.46 % w/w.

Sr. No.	Batch no.	Entrapment Efficiency (% w/w) ± SD
I	B1	48.30±0.78
II	B2	69.40±0.86
III	B3	79.02±0.48
IV	B4	52.46±0.69
V	B5	65.35±0.72

Table 2: Entrapment efficiency of different batches of phytosomes

Sr. No.	Batch no.	Drug content (%w/w)± SD
I	B1	63.09±0.02
II	B2	80.07±0.08
III	B3	91.02±0.01
IV	B4	32.46±0.06
V	B5	53.21±0.01

Table 3: drug content evaluation different batches of phytosomes

Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry or 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 physical mixture of phosphatidylcholine 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 (SEM)

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

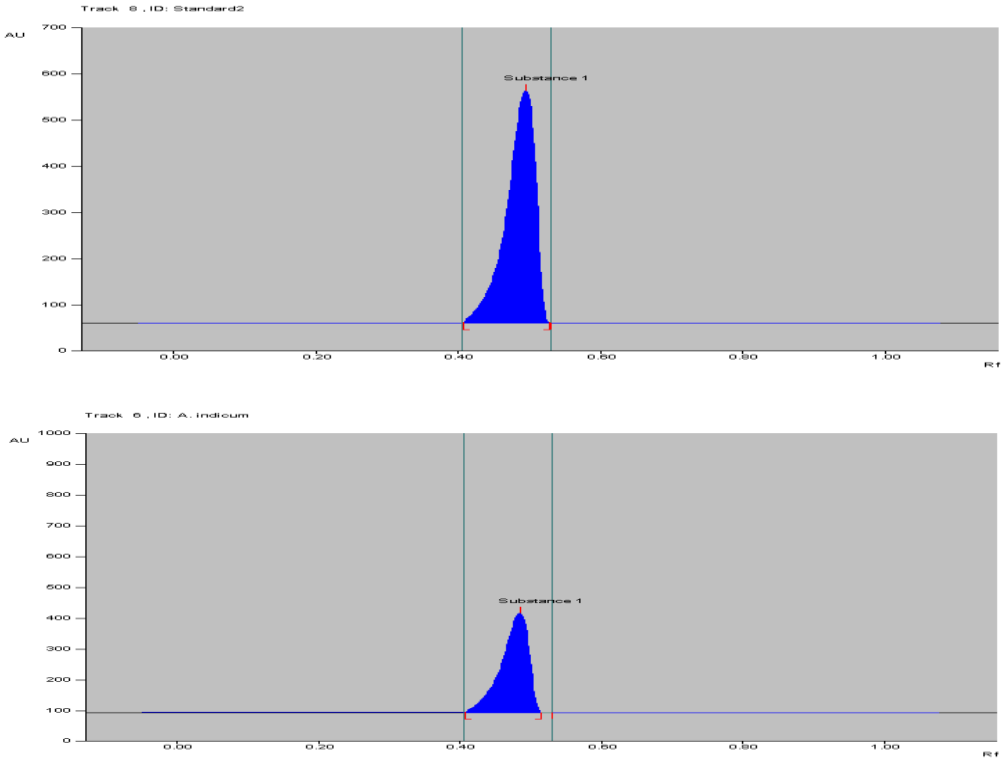


Figure 1: HPTLC Chromatogram of Standard Quercetin and ethanolic extract of *Abutilon indicum*.

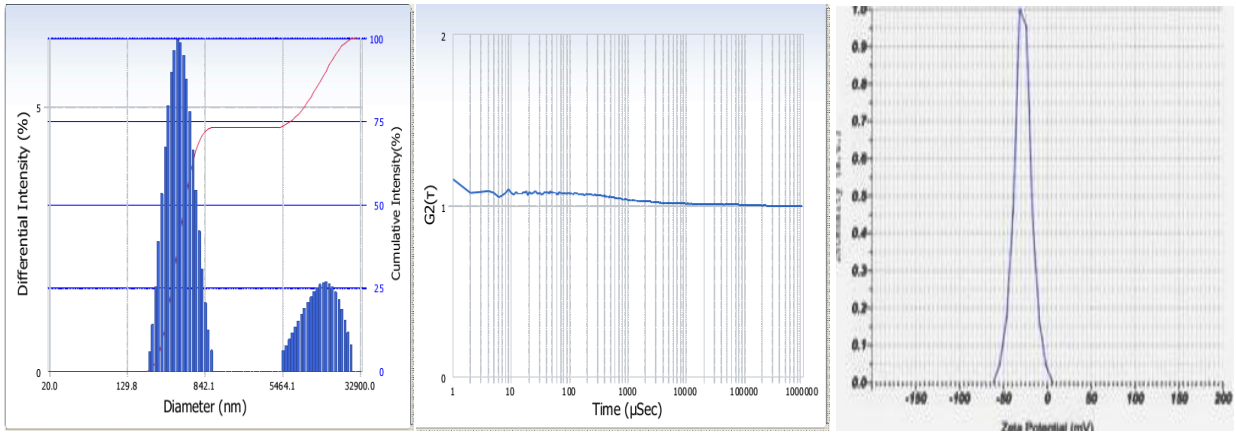


Figure 2: Particle size and Zeta potential of Phytosomes

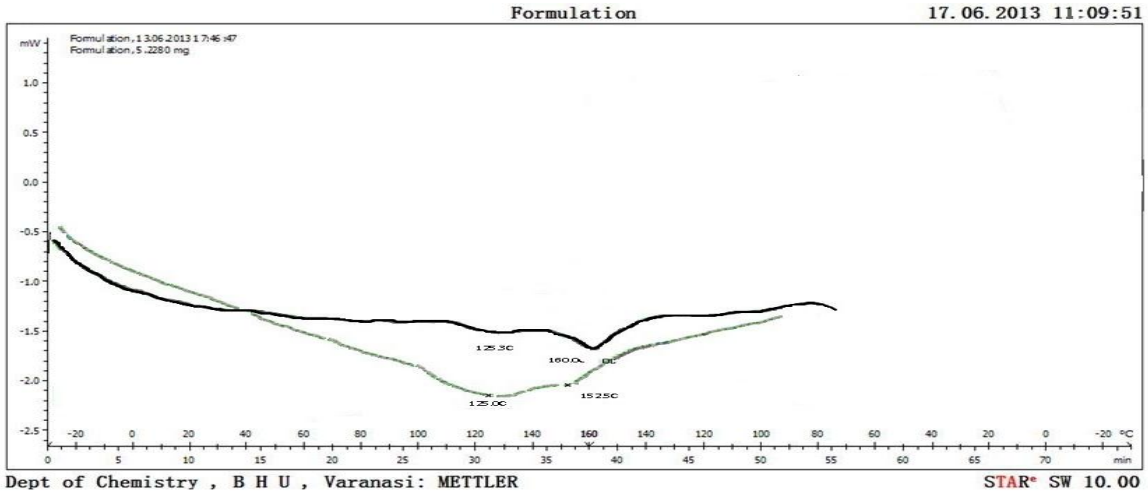


Figure 3: DSC curve of phytosomal formulation shown by black solid line and DSC curve of physical mixture of phosphatidylcholine and extract is shown by green solid line.

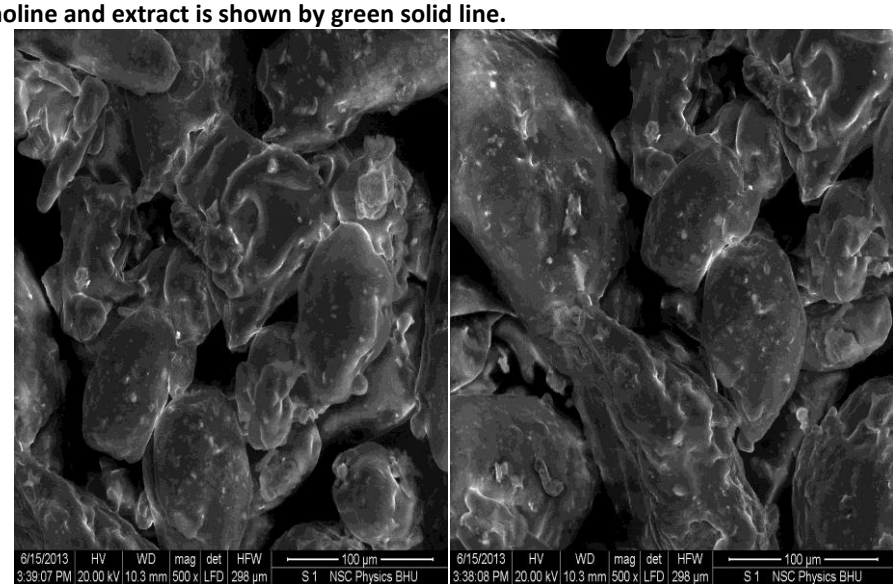


Figure 4: SEM images of Phytosomes developed.

FTIR 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-5c] shows the additive effect of individual components i.e. spectrum of biherbal extract and spectrum of phosphatidylcholine. The presence of biherbal extract of 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 phosphatidylcholine 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).

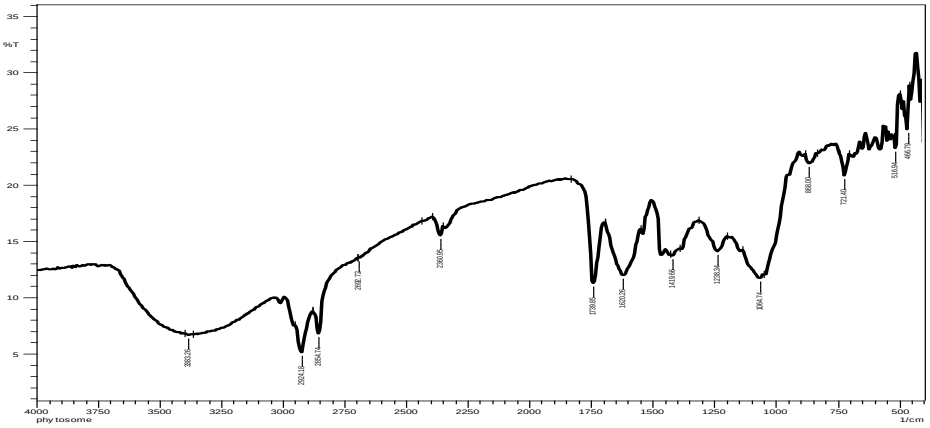


Figure 5a: FTIR spectrum of Phytosomal formulation showing combined effect of ethanolic biherbal extract and phosphatidylcholine

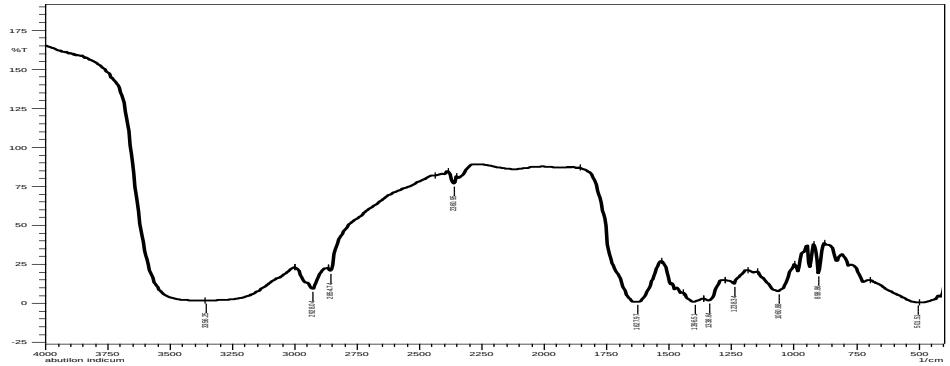


Figure 5b: FTIR spectrum of ethanolic extract *Abutilon indicum*

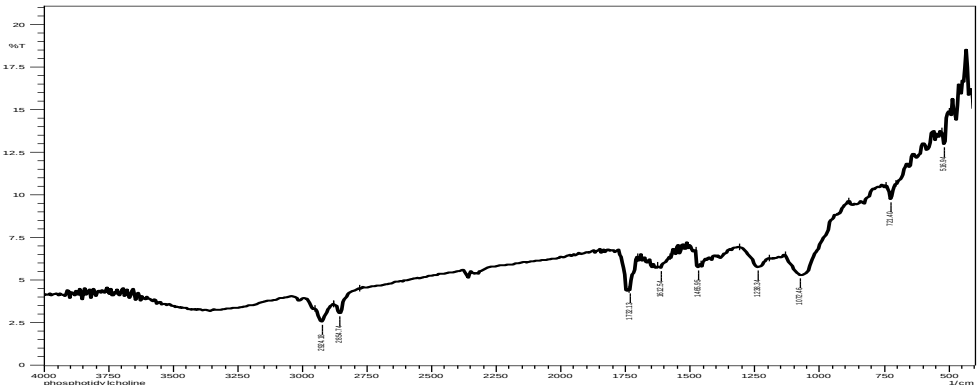


Figure 5c: FTIR spectrum of Soy Phosphatidylcholine

***In vitro* antioxidant activity**

Estimation of DPPH free radical scavenging activity

The evaluation of scavenging effect of combined ethanolic extract and phytosomes on DPPH radical was investigated using Ascorbic acid as standard. The results of IC_{50} are given in [Table 3] and Percentage inhibition is shown in [figure 6]. IC_{50} value of biherbal extract, phytosomes and ascorbic acid were 44.2 µg/ml, 35.1 µg/ml and 12.2 µg/ml respectively. This showed that extract have low antioxidant activity as compared to phytosomal complex.

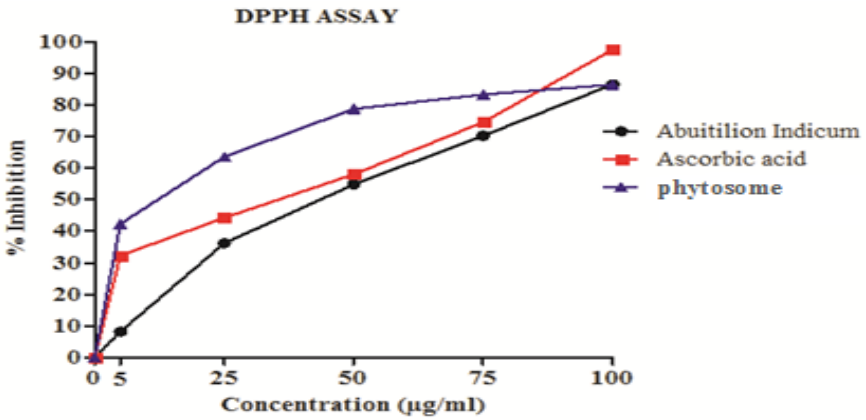


Figure 6: Curve for DPPH Radical scavenging Activity

Estimation of Nitric Oxide Scavenging Activity

Nitric Oxide scavenging activity of combined ethanolic extract and phytosomes was investigated using Ascorbic acid as standard. The results of IC_{50} are given in [Table 4] and Percentage inhibition is shown in [Figure 7] IC_{50} value of extract, phytosomes and ascorbic acid were 40.12 µg/ml, 33.4 µg/ml and 31.2 µg/ml respectively. This showed that extract have low antioxidant activity and phytosomal complex have high scavenging potential for free radicals.

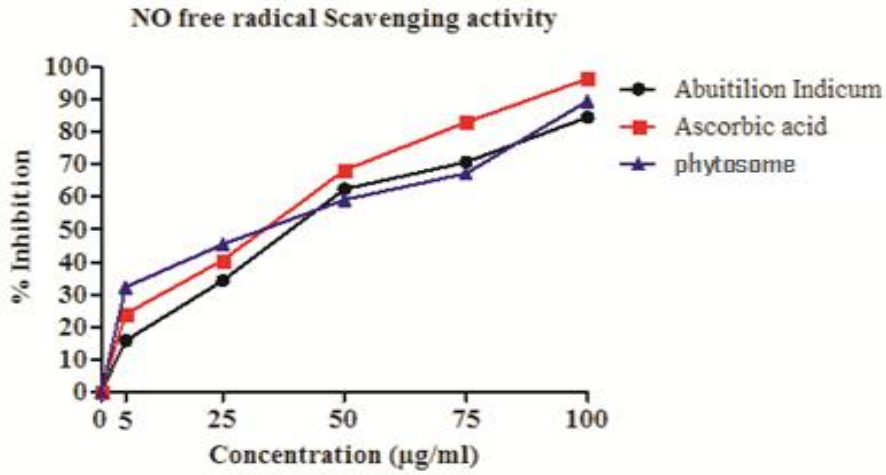
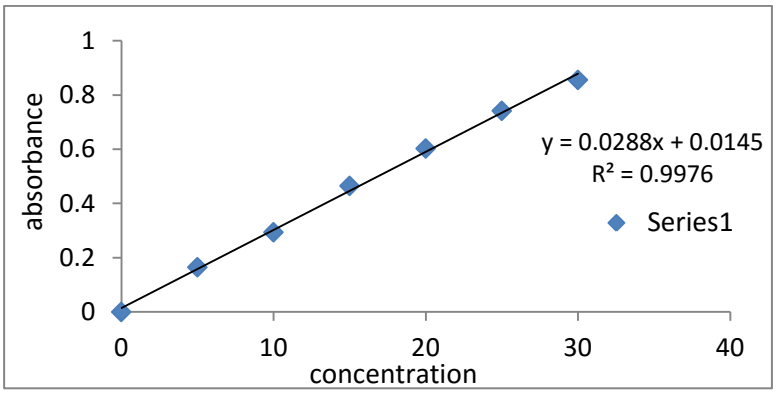


Figure 7: Curve for Nitric Oxide scavenging activity



Graph 1- Standard Curve of Entrapment Efficiency

CONCLUSION

In the present study phytosomes containing biherbal extract of *A. indicum* and *P. longum* was developed by indena's patented process and characterised for various physicochemical parameters, found to exhibit significant antioxidant activity and. The characterization showed that extract formed complex with phospholipids with better lipid solubility drug entrapment with 79.02 % w/w of drug. FTIR shows biherbal extract and soy lecithin form non-covalent bond. phytosomal formulation shows promising physico chemical properties and shows better drug delivery system. Phytosomes shows better antioxidant potential than crude biherbal extract. This biherbal 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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