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Hepatoprotective Study of Curcumin-Soya Lecithin Complex

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Abstract

The purpose of present study was to prepare and characterize the complex between curcumin and soya lecithin, and to evaluate its hepatoprotective activity. The curcumin-soya lecithin complex was prepared by dissolving curcumin and soya lecithin in equimolar ratio in dichloromethane and heating at 60°C for 2 h. The curcumin-soya lecithin complex was characterized by DSC, FTIR and NMR spectroscopy. The prepared complex provided a 3-fold increase in solubility of curcumin. On evaluation of *in vitro* intestinal permeability of curcumin across the everted sheep gut sac, the complex was found to provide the higher intestinal permeation of curcumin. On *in vivo* evaluation of curcumin-soya lecithin complex in paracetamol-induced hepatotoxicity in mice, it was observed that the complexed curcumin afforded a significantly higher protection against paracetamol-induced rise in serum aspartate aminotransferase and alanine aminotransferase levels as compared to pure curcumin.

Keywords

Curcumin • Soya lecithin Complex • Hepatoprotective

Introduction

Curcumin, a naturally occurring polyphenolic phytoconstituent, is isolated from the rhizomes of *Curcuma longa* Linn.(Zingiberaceae). Curcumin is chemically (1*E*,6*E*)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione. It has a pKa_1, pKa_2 and pKa_3 value of 7.8, 8.5 and 9.0, respectively for three acidic protons [1]. It is insoluble in water under acidic or neutral conditions but dissolves in alkaline conditions. Curcumin is highly

unstable undergoing rapid hydrolytic degradation in neutral or alkaline conditions to feruloyl methane and ferulic acid [2]. It is reported to be stable below pH 6.0. Thus, the use of curcumin is limited by its poor aqueous solubility in acidic or neutral conditions and instability in alkaline conditions.

It is commonly used as the coloring pigment and spice in food. A plethora of therapeutic uses of curcumin has been documented in traditional system of medicine and has been scientifically validated by modern system of medicine. On pharmacological evaluation, curcumin was found to possess anti-cancer [3–6], anti-oxidant [7–9], anti-inflammatory [10–12], hyperlipidemic [13, 14], anti-bacterial [15], wound healing [16] and hepatoprotective [17–19] activities. Apart from its pharmacological actions, it has also been investigated as photostabilizing agent to protect photo-labile drugs in solution, topical preparations and soft gelatin capsules [20]. Despite the presence of large number of pharmacological actions, the therapeutic efficacy of curcumin is limited due to its poor oral bioavailability [21–23]. The poor oral bioavailability of curcumin has been attributed to its poor aqueous solubility and extensive first pass metabolism [24].

Enhancing the absorption of poorly water-soluble drugs is a real challenge for pharmaceutical research. Soya lecithin is a phospholipid that is a major constituent of cell membranes. Soya lecithin is freely compatible with other nutrients, and when co-administered may enhance their absorption. It is used for preparation of vesicle suspensions in pharmaceutical industry. Several studies have demonstrated that complexation of phospholipids with phytoconstituents increases their bioavailability [25, 26] and as a result there is an increase in therapeutic effect also.

In the present investigation, curcumin-soya lecithin complex was prepared [27] and characterized by infra-red (IR), nuclear magnetic resonance (NMR) spectroscopy and differential scanning calorimetry (DSC). The *in vitro* release of curcumin from the complex was evaluated using everted gut sac method. The *in vivo* hepatoprotective activity of curcumin-soya lecithin complex was evaluated and compared with pure curcumin in paracetamol-induced hepatotoxicity in mice model.

Results and Discussion

Tab. 1. Solubility characteristics of curcumin, physical mixture and complex of curcumin-soya lecithin.

Sample	**Aqueous Solubility (µg/ml)***
Curcumin	8.7 ± 0.0233
Curcumin soya lecithin physical mixture	20.2 ± 0.021
Curcumin-soya lecithin complex	29.4 ± 0.045

* Values are mean± SEM (*n*=3)

The method of preparation of curcumin-soya lecithin complex was found to be reproducible yielding 82.0 ± 3.7% of product. On assaying the complex, it was found to contain 42.55 ± 0.88% of curcumin (n=3). The results of solubility study (Tab.1) reveal that

curcumin-soya lecithin complex provided a 3-fold increase in solubility of curcumin as compared to pure curcumin. Further, the physical mixture of curcumin and soya lecithin also provided a higher solubility of curcumin. This can be attributed to the surface activity of soya lecithin.

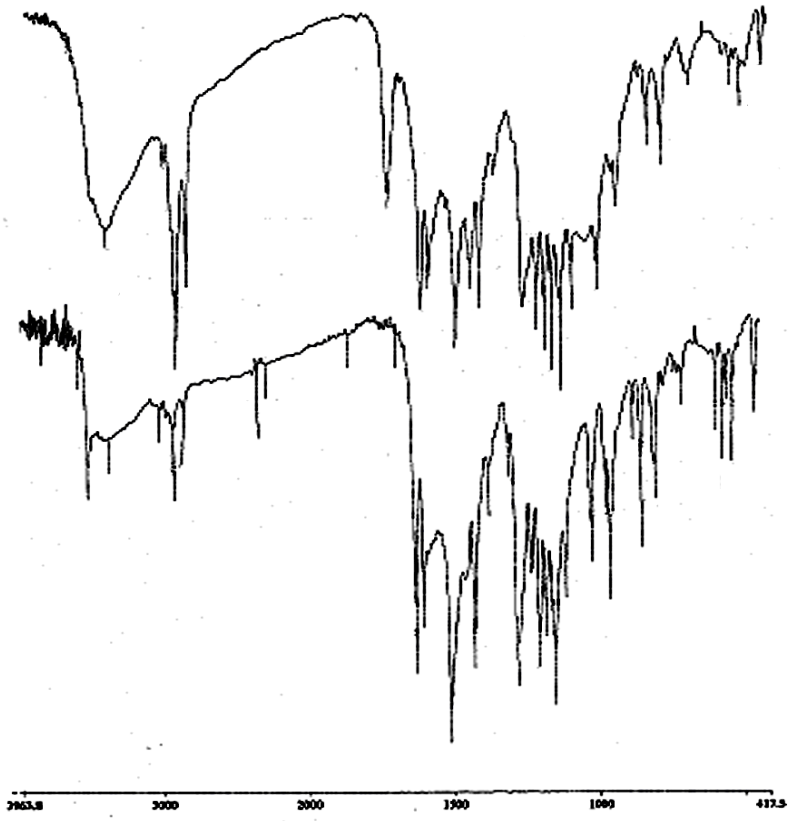


Fig. 1. FTIR spectra of curcumin (A) and curcumin-soya lecithin complex (B)

Fig. 1 compares the IR spectra of curcumin and curcumin-soya lecithin complex. The characteristic IR (KBr) peaks of curcumin appear at 3573.1**,** 1699.5, 3015.0, 1281.5, and 1628.0 cm^{-1}. The curcumin-soya lecithin complex shows its IR (KBr) peaks at 3401.1, 1739.9, 3011.0, 1282.8 and 1628.2 cm^{-1}. The IR spectra of curcumin show a sharp peak of hydroxyl (-OH) group at 3573.1 cm^{-1}, which indicates the presence of free hydroxyl (-OH) group. While in the IR spectra of complexed curcumin a broad peak for hydroxyl group appears at 3401.1 cm^{-1}, which shows that some interaction has occurred at hydroxyl (-OH) group [28].

The proton-NMR spectra of cucrcumin, soya lecithin and curcumin-soya lecithin revealed the presence of different peaks as follows–

NMR data for Curcumin

^{1}H NMR (δ ppm): 6.85–7.07 (m, 6H, Ar-H), 7.54–7.59 (d, 2H, adjacent to benzene ring) 8.63 (s, 2H, OH), 3.90–3.92 (s, 6H, OCH_3) 5.81–5.83 (s, 2H of CO-CH_2-CO).

NMR data for Soya Lecithin

^{1}H NMR (δ ppm): 3.7–4.3 (m, 5H, O-CH_2-CH-CH_2-O-), 1.25–1.29 (m, 20 H, CO-$(CH_2)_5$), 8.35 (s, 1 H, OH), 1.5-2.77 (s, 9H, H of N $(CH_3)_3$), 3.28 (t, 2H, CH_2)

NMR data for Curcumin Soya-Lecithin Complex

^{1}H NMR (δ ppm): 3.9 (m, 5H, O-CH_2-CH-CH_2-O-), 1.1–1.20 (m, 20 H, CO-$(CH_2)_5$), 8.39 (s, 2H, OH), 6.45–6.49 (d, 2H, adjacent to benzene ring)

In the case of the curcumin soya lecithin complex there is the formation of H-bonds between the phenolic hydroxyl of curcumin moiety and the phosphate ion on phosphatidyl choline. This can be evidenced by the appearance of the prominent broad peak at 8.4 ppm of ^{1}H NMR spectra of complex. This can be deduced from the comparison of the NMR of the complex and with the pure precursors that the signals of the fatty acid chains are almost unchanged. Such evidences inferred that the two long aliphatic chains of phosphatidyl choline are wrapped around the active principle.

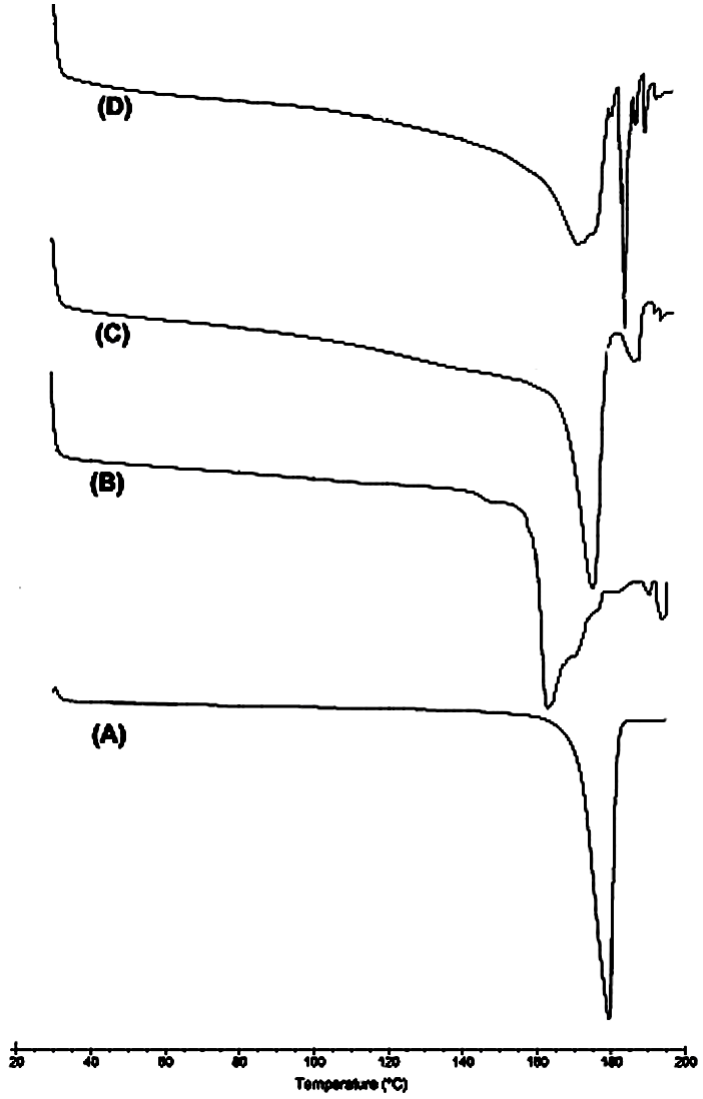


Fig. 2. DSC thermogram of pure curcumin (A), L-*α*-phosphatidylcholine (B), Physical mixture (C) and complex (D) of curcumin and L-*α*-phosphatidylcholine.

Fig. 2 exhibits the DSC plots of curcumin and curcumin-soya lecithin complex. Thermogram of curcumin shows a sharp endothermic peak characteristic of crystalline substances with onset at 171.31°C and maximum occurrence at 179.32°C.

Thermogram of soya lecithin exhibits three broad endothermic peaks characteristics of amorphous substances at 163.0°C, 190.5°C and 194.8°C. Thermal curve of complexed curcumin shows two peaks at 175.2°C and 187.6°C. Thermogram of physical mixture shows a broad endotherm at 171.5°C and a sharp peak with onset at 182.0°C and maximum occurrence at 183.8°C. It can be concluded from the comparison of the peak temperatures and endothermic transition contours of curcumin, soya lecithin, the physical mixture of curcumin and soya lecithin and complex of curcumin and soya lecithin that an interaction has occurred between curcumin and soya lecithin.

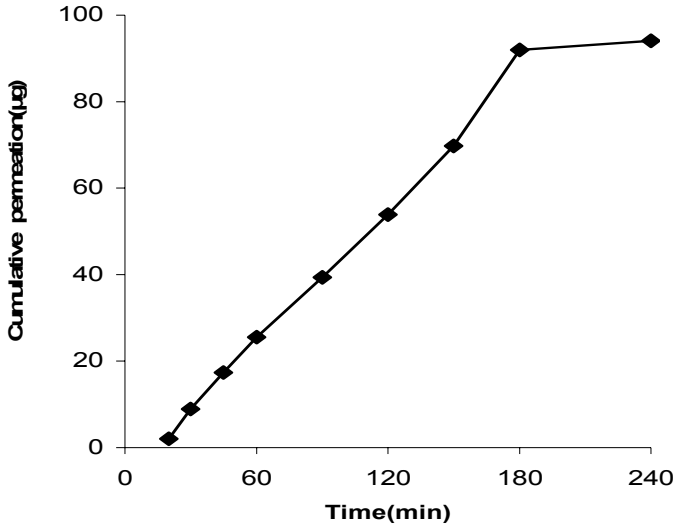


Fig. 3. *In vitro* intestinal permeation profile of curcumin from curcumin-soya lecithin complex

Fig. 3 represents the *in vitro* permeation profile of curcumin across the isolated everted sheep gut sac. Curcumin is characterized by poor aqueous solubility and absorption. At the end of 4 h only about 100 µg of curcumin could permeate across the everted gut sac from the complexed curcumin formulation. On the other hand the permeation of curcumin from the uncomplexed curcumin was too small to be detected spectrophotometrically. The higher intestinal permeability of curcumin from the complex can be attributed to its higher solubility.

The paracetamol-induced hepatotoxicity in mice model was selected for evaluating the hepatoprotective activity of curcumin. An overdose of paracetamol is known to cause acute hepatic necrosis in both experimental animals [29, 30] and humans [31, 32]. Paracetamol-induced hepatotoxicity has been shown to be due to its conversion to a highly reactive intermediate, N-acetyl-p-benzoquinone-imine (NAPQI), by cytochrome P-450 mixed function oxidases [30, 33, 34]. This toxic metabolite is normally rapidly conjugated with reduced glutathione (GSH) and eventually excreted in the urine as cystine

and mercapturic acid conjugates of paracetamol [35]. However, on administration of over dose of paracetamol, hepatic GSH is depleted, and free NAPQI binds to macromolecules in the hepatocytes causing cell necrosis [35, 36]. Curcumin is known to protect liver against the toxic effects of agents like CCl_4 [17, 19] acetaminophen [37] and *Aspergillus* aflatoxin [38]. Curcumin is a bifunctional antioxidant, which contains Michael-acceptor functionalities (olefins or acetylenes conjugated to electron withdrawing groups) and phenolic hydroxyl groups, which directly scavenge the free-radical moieties and induce the cytoprotective enzymes like glutathione-S-transferase, which facilitate the removal of toxins from the body [39].

Serum AST and ALT levels were selected as parameters to measure the hepatoprotective effect of curcumin. Tab. 2 compares the effect of pure curcumin and curcumin-soya lecithin complex on the paracetamol-induced rise in serum AST and ALT levels of mice.

Tab. 2. Effect of Curcumin and curcumin-soya lecithin complex on paracetamol-induced serum AST and ALT levels.

Group	**AST Levels (IU/L)***	**ALT Levels (IU/L)***
Group-I (Tween 80)	44.8 ± 0.44	38.9 ± 1.52
Group-II (Tween 80 + PCM)	82.2 ± 1.60‡	86.8 ± 2.83‡
Group-III (Tween 80+ PCM+CN,50mg/kg)	60.2 ± 1.20[a]	49.9 ± 2.36 [a]
Group-IV (Tween 80+ PCM+CN,100mg/kg)	51.6 ± 2.30 [a]	36.6 ± 1.69 [a]
Group-V (Tween 80+ PCM+CS,50mg/kg)	52.3 ± 1.20 [a,b]	39.4 ± 1.53 [a,b]
Group-VI (Tween 80+ PCM+CS,100mg/kg)	46.0 ± 1.60 [a,c]	39.6 ± 1.79 [a,c]

* Values are mean ± SEM (n=6), ‡ significant difference *($p<0.001$*) as compared to group-I,[a]significant difference *($p<0.001$*) as compared to group-II,[b]significant difference *($p<0.001$*) as compared to group-III, [c]significant difference *($p<0.001$*) as compared to group-IV, PCM-paracetamol, CN-curcumin, CS-curcumin soya lecithin complex

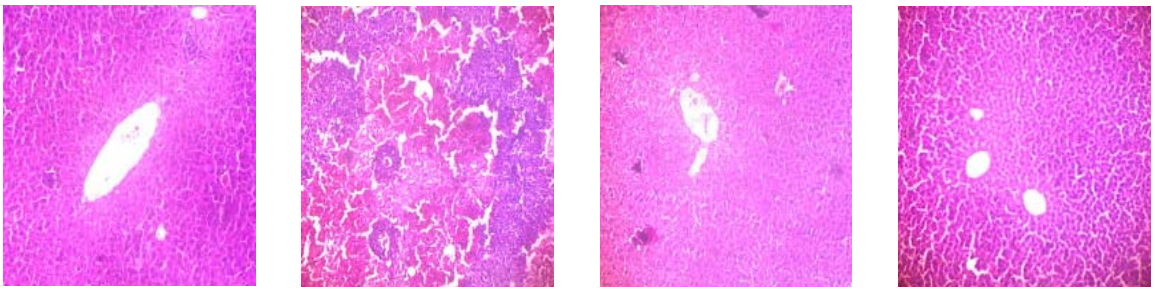
It can be observed from comparison of serum AST and ALT levels of mice of group-I and group-II that treatment with paracetamol (350 mg/kg) in group-II led to hepatotoxicity indicated by highly significant ($p<0.001$) increase in level of serum AST and ALT.

The treatment of animals with curcumin at a dose of 50 mg/kg (group-III) and 100 mg/kg (group-IV), afforded a highly significant ($p<0.001$) protection against the paracetamol-induced enhanced serum AST and ALT levels. It can be observed from the results that in mice treated with higher dose of curcumin (100mg/kg) a significant ($p<0.01$) reduction in paracetamol-induced enhanced serum level of AST, and a highly significant ($p<0.001$) reduction of paracetamol–induced enhanced serum ALT levels, occurred in comparison to mice treated with lower dose of curcumin (50mg/kg). Further, treatment with higher dose of

curcumin prevented the paracetamol-induced rise in serum ALT levels, and no significant difference was observed between the serum ALT levels of group-I and group-IV.

Treatment of animals with curcumin-soya lecithin complex at 50 mg/kg (group-V) and 100 mg/kg (group-VI) provided a highly significant protection ($p< 0.001$) against paracetamol-induced rise in serum AST levels as compared to pure curcumin (group-III and group-IV) at the respective doses. On comparing the serum ALT levels it was observed that curcumin-soya lecithin complex at the dose of 50 mg/kg (group-V) afforded a significant protection against paracetamol-induced serum ALT level as compared to pure curcumin at the comparable dose (group-III). The treatment with curcumin-soya lecithin complex at 100 mg/kg (group-VI) prevented the paracetamol-induced rise in serum ALT levels and a no significant difference was observed between the serum ALT levels of group-VI and group-IV or group-VI and group-I. Thus, a dose dependent hepatoprotective activity of curcumin-soya lecithin complex was observed. Further, analyses of results revealed that curcumin-soya lecithin complex at the dose of 50 mg/kg produced the hepatoprotective effect comparable to that produced by pure curcumin at the dose of 100 mg/kg.

Histological studies of liver give a visual assessment of hepatic architecture. The comparison of normal architecture with hepatotoxicity caused by hepatotoxin (paracetamol) can be clearly distinguished by degeneration of hepatocytes, necrotic areas and non-visible portal tract. Fig. 4 (A–D) shows the representative photomicrographs of liver section of mice.



A B C D

Fig. 4. Photomicrographs of liver section taken from the mice of group-I (A), group-II (B), group-III (C) and group-V (D).

The mice treated with a vehicle control (group-I) (Fig. 4A) show a normal hepatic architecture and visible portal tract. Treatment of animals with paracetamol (350 mg/kg) (group-II) results in acute hepatotoxicity as can be observed from necrotic patches and degenerative hepatocytes with mild inflammation and unremarkable portal tract (Fig. 4B). Pre-treatment of animals with curcumin (50 mg/kg, group-III) resulted in hepatoprotection, as can be observed by absence of necrotic areas, degenerated hepatocytes and visible portal tract which indicate normal hepatic architecture (Fig. 4C). The similar results were observed on pre-treatment of animals with curcumin-soya lecithin complex at 50 mg/kg (group-V) (Fig. 4D).

In the present study, curcumin-soya lecithin complex was successfully prepared and characterized by IR and DSC studies. The curcumin-soya lecithin complex provided a

higher aqueous solubility and *in vitro* intestinal permeability of curcumin. *In vivo* assessment of hepatoprotective activity of complex revealed a dose dependent hepatoprotective activity of curcumin. The curcumin-soya lecithin complex at 50 mg/kg provided the hepatoprotective effect comparable to pure curcumin at 100 mg/kg. The enhanced hepatoprotective activity of curcumin appears to be due to the better solubility and absorption of curcumin. The increased aqueous solubility of curcumin from the complex partially offsets the limited therapeutic use of curcumin. However, further stability studies to evaluate the protection offered by curcumin-soya lecithin complex to curcumin against hydrolytic and photochemical degradation should be conducted to comment on the potential usefulness of curcumin-soya lecithin complex.

Experimental

Materials

Curcumin and L-α-phosphatidyl choline were procured from Hi-Media Laboratories Pvt. Ltd. (Mumbai, India). Paracetamol was obtained as the gift sample from GMH Laboratories (Pvt.) Ltd., (Baddi, India). Assay kits for serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were purchased from Trans Asia Biomedical Ltd., (Mumbai, India). Animals used in the study were obtained from small animal house of CCS Haryana Agriculture University, (Hisar, India) and were kept under pathogen-free conditions. All other reagents used were of analytical grade and were used as received.

Preparation of curcumin-soya lecithin complex [27]

An accurately weighed 338 mg of curcumin and 776 mg of soya lecithin (1:1 molar ratio) were dissolved in 50 ml of dichloromethane. The mixture was refluxed at a temperature not exceeding 60 °C for 2 h. The resultant clear solution was evaporated under vacuum up to 10 ml, followed by addition of 10 ml of n-hexane with continuous stirring. The curcumin-soya lecithin complex was precipitated and the precipitate so obtained was dried under vacuum. The dried powder was weighed and tightly sealed in storage vial and stored in a dessicator.

Assay

The contents of curcumin in complex were determined spectrophotometrically. A bolus of 5 mg of the complex was dissolved in 10 ml of methanol and stirred for 2 h on a magnetic stirrer. The concentration of curcumin in complex was determined by measuring the absorbance of the solution so obtained at 422 nm.

FT-IR spectroscopy

FT-IR spectroscopy of curcumin and curcumin-soya lecithin complex was performed on fourier-transformed infrared spectrophotometer (1700, Shimadzu). The pellets of sample and potassium bromide were prepared by compressing the powders at 20 psi for 10 min on KBr press and the spectra were scanned on the wave number range of 3600-400 cm^{-1}.

Differential scanning calorimetry

Thermograms of curcumin, soya lecithin, curcumin–soya lecithin complex and physical mixture (1:1 molar) of curcumin and soya lecithin, were recorded using Q10 differential

scanning calorimeter (TA Systems, USA). The samples (3-5 mg) were sealed in the aluminum crimp pan, and heated at the speed of 10°C/min from 30 to 200°C in nitrogen atmosphere.

NMR study

To confirm the complexation between curcumin and soya lecithin NMR spectra of curcumin, soya lecithin and curcumin soya lecithin complex were taken on Bruker Avance II 400 NMR Spectrometer.

Solubility study

Solubility of curcumin, curcumin-soya lecithin complex and the physical mixture of curcumin and soya lecithin were obtained by adding excess of the samples to 10 ml of water in a glass container at room temperature. The samples were shaken for 24 h and centrifuged at 4000 rpm for 15 min. To 1 ml of clear supernatant, 9 ml of methanol was added and resulting sample was analyzed for the contents of curcumin spectrophotometrically (Varian-Cary 5000 UV-Vis-NIR) at 422 nm.

In vitro drug release

The *in vitro* release of curcumin from curcumin-soya lecithin complex was determined using everted sheep gut sac method [40]. The fresh intestine of sheep was brought from the local butcher's shop in cold (4 °C) Kreb's bicarbonate solution within half an hour of slaughtering of the animal. After discarding approx. 10–15 cm section from the pyloric end, the entire intestine was everted, using a blunt headed glass rod. The everted gut was stretched under a weight of 10 gm. A sac of everted intestine was made by ligating the distal end and attaching the proximal end with the cannula. A weight of 10 gm was attached to the ligated end to keep the sac in a vertical position. The sac was suspended in tissue bath containing 120 ml of dispersion of curcumin in Kreb's bicarbonate solution (mucosal) by attaching the distal end to the aeration tubing. An aliquot of 5 ml of the Kreb's bicarbonate solution (serosal) was introduced into the sac. The samples of serosal solution were withdrawn at various time intervals and analysed for the contents of curcumin spectrophotometrically at 422 nm.

In vivo hepatoprotective activity

Evaluation of *in vivo* hepatoprotective activity of curcumin formulations was done using paracetamol-induced hepatotoxicity in mice model. The animal handling and *in vivo* experiments were carried out in accordance with the guidelines of the "Committee for the purpose of control and supervision on experiments on animals (CPCSEA), Ministry of Environment and Forests, Govt. of India". An experimental protocol was designed and approval of institutional animal ethics committee (IAEC) (Regn. No. 0436) was obtained. Swiss albino mice of either sex (25–40 g) were obtained from the disease free small animal house of CCS Haryana Agriculture University Hisar and maintained on standard chow diet and water ad libitum. The animals were housed in a special animal room with the temperature maintained at 25°C and were kept on a light/dark cycle of 12 h.

Hepatic injury in mice was induced by administering paracetamol orally in a dose of 350 mg/kg. Paracetamol suspension in saline was administered 1 h after the last dose of the

respective treatments. Thirty six mice were divided into 6 groups of 6 animals each as follows:

Group-I animals received a single daily dose of 2% (v/v) Tween-80 for 4 days, group-II animals received a single daily dose of 2% (v/v) Tween-80 for 4 days and single dose of paracetamol suspension in saline (350 mg/kg) after 1h of last dose of Tween 80 on the 4^{th} day. Animals in the group-III and group-IV received a single daily dose of 50 mg/kg and 100 mg/kg of curcumin suspension in Tween 80 (2%, v/v) respectively for 4 days followed by single dose of paracetamol suspension in saline (350 mg/kg) after 1h of the last dose of curcumin. Animals in the group-V and group-VI were administered a single daily dose of test formulation of curcumin-soya lecithin complex in Tween 80 (2%, v/v) at a dose of 50 mg/kg and 100 mg/kg of curcumin, respectively for 4 days followed by single dose of paracetamol suspension in saline (350 mg/kg) after 1h of the last dose of complex.

Biochemical analysis

Animals were anesthetized under mild ether anesthesia, 24 h after paracetamol administration. Blood samples were collected by cutting carotid artery and serum was prepared from the collected blood and subjected to biochemical estimations of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) using commercial assay kit [41].

Histopathology

A portion of liver tissue in each group was fixed in 10% formalin, and 7µ thick paraffin sections of formalin fixed liver were prepared and stained with Ehrlich's hematoxylin and eosin [42].

Statistical analysis

The statistical significance between groups was analyzed using one way ANOVA followed by Tukey-Kramer multiple comparison test. A p value (<0.05) was considered significant.

Authors' Statements

Competing Interests

The authors declare no conflict of interest.

Animal Rights

The institutional and (inter)national guide for the care and use of laboratory animals was followed. See the experimental part for details.

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