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Antioxidant and hepatoprotective effects of mixed micellar lipid formulation of phyllanthin and piperine in carbon tetrachloride-induced liver injury in rodents

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Running Head: MMLF of phyllanthin and piperine for hepatoprotection.

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Abstract

Phyllanthin, a sparingly water-soluble hepatoprotective lignin obtained from *Phyllanthus amarus* Schum. et Thonn. (Euphorbiaceae) possesses restricted bioavailability issue. Phyllanthin along with piperine (a nutraceuticals bioenhancer) was formulated as mixed micellar lipid formulation (MMLF) in present studies and investigated to resolve the restricted bioavailability and enhance hepatoprotective effects on oral administration. Hepatoprotective, antioxidant and bioavailability study of MMLF, a complexed phosphatidylcholine formulation of phyllanthin (CP-PC), phyllanthin + piperine (CP-P-PC) and its corresponding non-formulated phyllanthin has been carried out. Phyllanthin (30 mg/kg p.o.), CP-PC (30 mg/kg p.o.), CP-P-PC (30 mg/kg p.o.) and reference drug silymarin (100 mg/kg, p.o.) were administered daily to rats for 10 days, followed by liver damage by administering a 1:1 (v/v) mixture of CCl_4 and olive oil (1 ml/kg, i.p.) for 7 days from day 4 to day 10. The degree of protection was evaluated by determining the level of marker enzymes (SGOT and SGPT), bilirubin (TB) and total proteins (TP). Further, the effects of MMLF on lipid peroxidation (LPO), glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GR) were estimated in liver homogenates to evaluate antioxidant activity. Finally the concentration of phyllanthin was evaluated in plasma. EC_{50} value for *in vitro* antioxidant assay by DPPH was found to be 19.99, 15.94 and 13.5 for phyllanthin, CP-PC and CP-P-PC, respectively. CP-P-PC (30 mg/kg p.o.) showed significant ($p < 0.05$) hepatoprotective effect by reducing the levels of serum marker enzymes (SGOT, SGPT, and TB), whereas, elevated the levels of depleted total protein (TP), lipid peroxidation and antioxidant markers enzymes activities such as, GSH, SOD, CAT, GPX, and GR. Complex MMLF normalized adverse conditions of rat liver more efficiently than the non-formulated phyllanthin. The present findings indicate that the MMLF is helpful in solving the problem of low bioavailability of phyllanthin.

Keywords: Bioavailability, Hepatoprotection, Lignin, Pharmacokinetics.

Introduction

Bioavailability is one of the major hurdle and limiting factor in the translation of the preclinical potential to clinical application of many botanical extracts, especially for those, whose active ingredients show poor water solubility and strong tendency to self-aggregate.[1,2] The genus *Phyllanthus* (Euphorbiaceae) contains 550-750 species in 10-11 subgenera that are distributed in all tropical regions of the world.[3] *Phyllanthus amarus* Schum. et Thonn. (Euphorbiaceae) is the most widespread species of *Phyllanthus* genus and is widely utilized for medicinal and nutritional purposes. The plant traditionally used for the treatment of diabetes, liver, kidney and bladder problems.[4] The major lignins of the genus, namely, phyllanthin and hypophyllanthin, have been reported to possesses hepatoprotective activity and protect liver cell damage through its antioxidant activity.[5,6,7,8] Phyllanthin has poor aqueous solubility but high lipid permeability and classified as class II drug under the biopharmaceutics classification system (BCS).[9] Malabsorption of phyllanthin through intestine as reported in pharmacokinetic studies warrants its high doses to reach therapeutic levels, which is many times inconvenient.[10] Piperine (1-piperoyl piperidine) is a major alkaloid of *Piper nigrum* Linn. (Piperaceae) and *Piper longum* Linn. (Piperaceae) and has been reported to possesses bioavailability enhancing activity by increasing absorption. This might be achieved due to alteration in membrane lipid dynamics and change in the conformation of enzymes in the intestine.[11,12] Piperine was also reported to possess hepatoprotective activity.[13,14] Mixed micellar lipid formulations (MMLF) are phospholipids based drug delivery systems that have been formed by incorporation of target drug along with bioenhancer moiety to improve poor aqueous solubility and absorption.[15,16,17] MMLF are advanced novel drug delivery systems that are better absorbed, utilized, and produce better result in comparison to conventional system.[18,19] MMLF are produced via a process whereby the individual components of the herbal extracts or active chemical rich fractions are bound to phospholipid phosphatidylcholine (PC).[20] Hence phyllanthin and piperine were incorporated with phosphatidylcholine in the present studies for advancement in delivery via design of MMLF. Characterization of the complex was conducted by adopting various reported spectroscopic methods.[20] The present study showed the comparative effect of MMLF and its corresponding non-formulated

phyllanthin for protective property against carbon tetra chloride- induced hepatotoxicity in rats, antioxidant activity, and plasma level concentration.

Materials and methods

Plant material and preparation of extract

The aerial parts of *Phyllanthus amarus* plant was collected from the locality of Vadodara (Gujarat, India) in July, 2011. The plant was identified by Dr. P.S. Nagar (Department of Botany, The M S University of Baroda, Vadodara, India). A voucher specimen (PS/AN/PA - 1101) was deposited in the herbarium of the Department of Pharmacy, The M S University of Baroda, Vadodara, India. The plant material was shade dried and pulverized into a coarse powder for extraction[21]: 1 kg was extracted with petroleum ether (2000 ml) to yield 10.24% w/w of petroleum ether extract.

Drugs and chemicals

The soy phosphatidylcholine (Lipoid S 100) was obtained as a gift sample from Lipoid, Ludwigshafen, Germany. Pure standard of phyllanthin was purchased from Natural Remedies Pvt Ltd, Bangalore, India. Silymarin was purchased from Micro Labs, Hosur, Tamilnadu, India. All other chemicals and reagents were procured from S.D. Fine Chem, Mumbai, India.

Isolation of phyllanthin

Preparation of unsaponified fraction of petroleum ether extract of plants

The dried petroleum ether extract was subjected to saponification by refluxing with 10% methanol KOH for 2 h. Unsaponified matter was extracted by partitioning with diethyl ether (three times) and then washed with water. Diethyl ether fraction obtained from above method was concentrated using rotary vacuum evaporator and collected to get 5 g of an orange solid mass residue.

Column chromatography

The residue (5 g) obtained was mixed with silica gel (10 g) and chromatographed over silica gel column (500 mm length × 20 mm diameter, 100-200 mesh size). Gradient elution was carried out in the following sequence, hexane (200 mL) → hexane:ethyl acetate (300 mL; 90:10, v/v) → hexane:ethyl acetate (300 mL; 80:20, v/v) → hexane:ethyl acetate (300 mL; 70:30, v/v) → hexane:ethyl acetate (300 mL; 60:40, v/v) to obtained 100 fraction of 15 ml each. Thin layer

chromatography (TLC) was performed using petroleum ether:chloroform:ethyl acetate:methanol (7:1:1.5:0.5 v/v) as the mobile phase. The fractions (40-76) collected were combined, re-chromatographed and the solvent evaporated to give 2.0 g of phyllanthin (96.24%; Fig. 1). Phyllanthin was characterized by comparing melting point, UV–Visible spectrophotometry, FT-IR, ^{1}H NMR, ^{13}C NMR and mass spectral analysis with that of pure standard of phyllanthin.[22]

Preparation of complexes

Two sets of complexes were prepared. In one set phyllanthin (419 mg) and phosphatidylcholine (3 × 760 mg) is optimized in 1:3 ratios, whereas in another set phyllanthin (419 mg), piperine (285 mg) and phosphatidylcholine (3 × 760 mg) was optimized in 1:1:3 ratios for attaining maximum complexation. Both the complexes were taken in two separate round bottom flasks and suspended in 20 ml dichloromethane each. Both mixtures were refluxed for 2 h at 40°C with continuous stirring on magnetic stirrer. The resultant clear solutions of phyllanthin-phosphatidyl choline (CP-PC) and phyllanthin-piperine-phosphatidyl choline (CP-P-PC) were washed with n-hexane (10 ml) to remove traces of PC and then dried under vacuum.[23,24,25,26] The CP-PC and CP-P-PC complexes were kept in amber colored glass bottle and stored at room temperature.

Characterization of complexes

The CP-PC and CP-P-PC complexes were characterized, on the basis of solubility profile, melting point, FT-IR and TLC studies.[27]

Solubility study

Solubility studies were performed by adding an excess amount of the phyllanthin, CP-PC and CP-P-PC complexes to 10 ml of different solvents on the basis of their polarity and shaking the contents using wrist shaker for 18-24 h in volumetric flasks.[28]

Melting Point

A capillary melting point apparatus was used to determine the melting point of phyllanthin, CP-PC and CP-P-PC complexes.[29]

FTIR

Fourier transform infra-red spectroscopy was used to determine the functional group confirmation in phyllanthin, and CP-PC complexes. FTIR spectra were obtained on the 8400 Shimadzu FTIR spectrometer with the wave number 500~3500 cm^{-1} using KBr pellets.[28]

TLC

TLC (Pre-coated silica gel 60F254; Merck, Germany) of phyllanthin, CP-PC and CP-P-PC complexes was performed using petroleum ether: chloroform:ethyl acetate:methanol (7:1:1.5:0.5 v/v) as solvent system. After development, the plate was sprayed with 10% methanol sulphuric acid and heated at 110°C for 10 min and R_F values were calculated for all the spots.[30]

Preparation of micellar lipid formulations

Two sets of micellar lipid formulations were prepared. In first set CP-PC complex and cholesterol in 7:3 ratio were dissolved in chloroform: methanol (4:1) for preparation of micellar lipid formulation, while in second set CP-P-PC complex and cholesterol in 7:3 ratio were dissolved in chloroform: methanol (4:1) for preparation of mixed micellar lipid formulations. Each complex was then introduced into separate 250 ml round bottom flask with round glass neck attached to separate rotary evaporator and rotated at 70 rpm. After complete removal of the organic solvent, the casted film was dispersed in phosphate buffer saline (pH 7.4). Upon hydration the lipid swell and peeled off from the wall of round bottom flask and vesiculate forming micellar lipid formulations.

Microscopic view of micellar lipid formulations

Optical microscope (Leica Microsystems, Switzerland) was used for microscopic characterization of the phyllanthin based micellar lipid formulations. The MMLF were suspended in distilled water, and then a drop of the suspension was placed on a slide, covered with a coverslip and viewed at a magnification of 400×.[27]

Vesicle Size

The size of micellar lipid formulations was determined by particle size analyzer (Malvern instrument Ltd., UK). For measurement of vesicle size, vesicular suspension was diluted with PBS (pH 7.4) and the size was measured.[29]

***In vitro* antioxidant assays**

A comparative antioxidant study of phyllanthin, CP-PC and CP-P-PC complexes were performed using 1, 1-diphenyl-2-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and phosphomolybdenum complex assays.[31,32,33]

Hepatoprotective activity

In vivo hepatoprotective activity of the phyllanthin, CP-PC and CP-P-PC complexes were evaluated against CCl_4 induced hepatotoxicity.[34,35]

Animals

A total of 36 male albino Wistar rats (150-200 g) used in the present study were procured from Zydus Cadila Laboratory, Ahmedabad, India. All the animals were kept under standard laboratory conditions (temperature 25 ± 2°C , relative humidity 55% ± 10% and 12 h light/ dark cycle) and acclimatized for 1 week before commencement of the experiment.[36] They were allowed to have free access to standard dry pellet diet (Hindustan Lever Ltd., Bangalore, India) and water *ad libitum*. The experimental protocols were approved (Reg. No. MSU/PHARM/IAEC/2011/09; dated, 27 January 2012) by the Institutional Animal Ethics Committee, The M.S. University of Baroda, Vadodara, Gujarat, India, accordance with the guidelines for the care and use of laboratory animals set by committee for the purpose of control and supervision of experiments on animals (CPCSEA).

Grouping and experimental procedure

Animals, after acclimatization (7 days) in the animal quarters, were fasted overnight and randomly segregated into six groups of six animals each. Group I - served as normal control and received olive oil (1 ml/kg, i.p.) daily for 7 days. Group II - served as negative control and received 1:1 (v/v) mixture of CCl_4 and olive oil (1 ml/kg, i.p.) daily for 7 days. Group III - served as standard drug treatment and received silymarin (100 mg/kg, p.o.) for 10 days and a 1:1 (v/v) mixture of CCl_4 and olive oil (1 ml/kg, i.p.) for 7 days from day 4 to day 10. Group IV - served as test drug treatment and received phyllanthin (30 mg/kg p.o.) daily for 10 days and a 1:1 (v/v) mixture of CCl_4 and olive oil (1 ml/kg, i.p.) for 7 days from day 4 to day 10. Group V - served as test drug treatment and received CP-PC (30 mg/kg 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 VI - served as test drug treatment and received CP-P-PC (30 mg/kg p.o.) for 10 days and a 1:1 (v/v) mixture of CCl_4 and olive oil (1 ml/kg, i.p.) for 7 days from day 4 to day 10.

After 24 h of the last injection, the rats of all six groups were anesthetized and the blood samples were collected by puncturing retro-orbital plexus. The blood samples were allowed to clot for 30 min. The serum was separated by centrifugation at 2000 × *g* for 10 min at 4°C and used for the assay of biochemical parameters such as serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), total bilirubin (TB) and total proteins (TP).[27] After collection of blood samples, the rats were sacrificed and their liver excised, rinsed in ice-cold normal saline followed by 0.15 M Tris–HCl (pH 7.4), blotted dry and weighed. The slices of liver tissue were fixed in Bouin's solution and used for histopathological studies.[37]

Determination of *in vivo* antioxidant activity

Liver tissue homogenate (10 %) was prepared in 1.15 % KCl and centrifuged at 7000 × *g* for 30 min at 4°C.[34] The obtained supernatant was collected and used for the following experiments.

Determination of thiobarbituric acid reactive substances (TBARS) for lipid peroxidation

The amount of malondialdehyde (MDA) in liver homogenate was determined by reaction with thiobarbituric acid (TBA) and used as an index of lipid peroxidation. The amount of TBARS so, formed in each of the samples was assessed by measuring optical density of the supernatant at 535 nm using spectrophotometer against a reagent blank. The results were expressed as nM TBARS/min/mg tissue at 37°C using molar extinction coefficient of 1.56×10^{-5} M cm^{-1}.[38]

Reduced glutathione estimation

Reduced glutathione was estimated in the liver homogenate using 1, 2-dithio-bis nitro benzoic acid (DTNB) by the method of Ellman.[39] The absorbance of yellow color developed was read immediately at 412 nm and the results were expressed as μM of GSH/g of wet tissue.

Antioxidant enzyme assays in liver homogenate

Estimation of SOD was done by autoxidation of hydroxylamine at pH 10.2, which cause production of nitrite by reduction of NBT. The activity of SOD was inversely proportional to the concentration of its oxidation product, which was measured spectrophotometrically at 320 nm. CAT was estimated by determining the decomposition of H_2O_2 at 240 nm in assay mixture containing phosphate buffer in unit time for routine studies of CAT kinetics. GPX in the tissue homogenate oxidizes glutathione and simultaneously H_2O_2 is reduced to water. This reaction is arrested at 10 min using trichloroacetic acid

and the remaining glutathione is reacted with DTNB solution to result in a colored compound, which is measured spectrophotometrically at 420 nm. In the presence of GR, oxidized glutathione undergoes reduction and simultaneously, NADPH is oxidized to $NADP^+$. GR enzyme activity is quantified by measuring the disappearance of NADPH/min at 340 nm spectrophotometrically.[34]

Pharmacokinetic studies *in vivo*

For pharmacokinetic studies standard reported methods were adopted.[40] Male albino Wistar rats (150-200 g) were divided into three groups (n = 6). Group 1, Group 2, and Group 3 were orally administered phyllanthin, CP-PC and CP-P-PC complexes respectively. The dosages for all treatment were fixed at 30 mg/kg body weight. The blood plasma samples (0.5 ml) were collected by retro orbital plexus at 8.0, 10, and 12 h after oral administration. Samples were preserved at - 80°C.

Sample preparation for plasma concentration study

Blood plasma was allowed to clot at room temperature for about 1 h and serum was separated by centrifugation at 3000 rpm for 15 min. To the serum deproteinizing agent [chloroform:methanol (2:1), 20 times volume of serum] was added and again centrifuged at 7000 rpm for 15 min. Proteins were settled at the bottom and the remaining supernatant was transferred to glass micro vials. The solvent was evaporated (< 50°C). The serum samples were reconstituted with 100 µl of methanol and centrifuged at 10,000 rpm for 10 min.[41] Afterwards, 20 µl of the supernatants was analyzed using HPTLC method.[42]

Quantification of phyllanthin by HPTLC

Precoated and preactivated TLC plates of silica gel 60 F_{254} with the support of aluminum sheets 0.1 mm thick and 10 × 10 cm were used. Pure standard of phyllanthin (1 mg) was dissolved in methanol (1 ml). The quantity of standard applied was 1000-6000 ng phyllanthin per spot and quantity of plasma sample applied was 20 µl. All the samples were applied in the form of a band using CAMAG LINOMAT V, an automatic sample application device, maintaining a band width 6 mm, space 10 mm, 250 nl/s.[43] The solvent system used was petroleum ether:chloroform:ethyl acetate:methanol (7: 1: 1.5: 0.5 v/v). The plates were developed by placing in presaturated tanks (8 cm height) with the respective solvent system. After drying the plates, sprayed with 10% methanolic sulphuric acid, heated at 110°C for 10 min. and scanned in visible mode at 580 nm.

Statistical analysis

Data were expressed as mean ± standard error of mean (SEM). For the hepatoprotective and antioxidant activity studies, statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Dunnett's and bonferroni multiple comparison test using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). P values < 0.05 were considered significant. For the plasma concentration study, animal data were analyzed by Student's *t* test. Again, P values < 0.05 were considered significant.

Results

Extraction, isolation, and spectral confirmation of phyllanthin

The saponified petroleum ether extract in petroleum ether:chloroform:ethyl acetate:methanol (7:1:1.5:0.5 v/v) showed ten prominent spots on TLC plates with spot of phyllanthin at retention factors of 0.33. After extensive column chromatography, complete separation of white crystal of phyllanthin was achieved using gradient elution with hexane:ethyl acetate solvent mixture. The isolated compound showed a single spot in TLC with petroleum ether:chloroform:ethyl acetate:methanol (7:1:1.5:0.5 v/v), R_F 0.33 (Fig. 1). The isolated phyllanthin was recrystallized from acetone; giving a melting point range of 281-283°C (282°C for reference standard of phyllanthin). To identify the structure the UV, IR, ^{1}H, ^{13}C NMR and MS were recorded and confirmed by comparing physicochemical and spectral data for isolated and reference standard of phyllanthin.[22] The UV λ_{max}/nm (methanol) was found to be 232 and 280 (Fig. 1). IR (KBr) shown major peak at 3100 (C-H aromatic stretch); 2999, 2917, 2869 (C-H aliphatic stretch) and 1182, 1140 (C-O-C stretch). The ^{1}H NMR spectral values were compared for both and match with major signals at δ 6.75, 6.65, 6.60, 3.83, 3.78, 3.30, 3.25, 2.65 and 2.05. ^{13}C NMR spectrum revealed the presence of only 12 distinct carbon resonances. The presence of 3 oxygens could also be suggested by the methoxy signals at δH 3.32 (δC 58.8), 3.83 (55.7) and 3.88 (55.9). Mass spectrum in both showed a molecular ion peak at *m*/*z* 418 and a base peak at *m*/*z* 151 corresponding to dimethoxy benzylic fragment. The probable fragmentation pattern revealed ion peaks at *m*/*z* 386 and 354 due to successive elimination of methanol with the corresponding molecular formula were $C_{24}H_{34}O_6$ (Fig. 1).

Mixed micellar lipid formulation (MMLF) complexes formation and characterization

In this study, MMLF of CP-PC and CP-P-PC complexes was formed, in which 85.60 ± 1.22% of phyllanthin was entrapped with PC. The drug loading efficiency of CP-PC was 3.34 ± 0.12% (n = 3), which was lower than that of CP-P-PC (7.54 ± 0.14%). The solubility study reveals the characteristics of the complex, which is of outer lipid layer and produce micelle with water, while dissolved in other organic solvents. The R_F values of phyllanthin, in CP-PC and CP-P-PC complexes were found to be 0.33 respectively. The physical mixture of CP-PC and CP-P-PC showed one and two more additional spots, respectively. This difference in number of spots with newer R_F value indicates the presence of other chemical entity utilized in the formation of complex.

FT-IR of MMLF specify CP-PC and phyllanthin are presented in Fig. 2. The characteristic FT-IR absorption bands at 2917.93 cm^{-1}, 2959.97 cm^{-1}, 2998.9 cm^{-1} [C-H stretching (aromatic)], 2850.37 cm^{-1} [C-H stretching (aliphatic)], 1041. 73 cm^{-1}, 1108 cm^{-1}, 1140.61 cm^{-1}, 1159.18 cm^{-1}, 1179.69 cm^{-1} (C-O-C stretching) were observed in the FT-IR spectra of phyllanthin. While MMLF shows characteristic absorption band at 2850.33 cm^{-1}, 2917.85 cm^{-1}, 2983.94 cm^{-1} due to C-H stretching and 1081.41 cm^{-1}, 1140.41 cm^{-1}, 1159.67 cm^{-1} due to COC stretching. In the case of MMLF the characteristic C-H and C-O-C stretching band shifted to lower side with reduced intensity. All the peaks seen in the FT-IR spectra of the phyllanthin are also seen in FT-IR of the MMLF, without disappearance and any change in the position indicating that the interaction is between PC and phyllanthin.

Characterization of vesicles

Vesicles size

The average size of the vesicles (in nm) for CP-PC (1:3, v/v) and CP-P-PC (1:1:3, v/v) was found to be 286.76 ± 4.61 and 304.52 ± 5.97, respectively.

Vesicle shape

The microscopic view of the MMLF showed a vesicular structure. The vesicles consist of phospholipid, while phyllanthin and piperine is present in the lipid bilayer in intercalated form.

Assessment of *in vitro* antioxidant studies

It was reported that reactive oxygen species and superoxide radicals have key roles in hepatoprotection and scavenging of these free radicals by antioxidants can lessen hepatotoxins.[22,44]

Therefore, the antioxidant properties of phyllanthin, CP-PC and CP-P-PC complexes were investigated herewith by:

DPPH method

The radical scavenging effects of phyllanthin, CP-PC and CP-P-PC complexes are represented in Fig. 3A. The minimum and maximum EC_{50} value was 13.50 and 19.99 µg/mL for CP-P-PC and non-formulated phyllanthin, respectively. The order of the activity was CP-P-PC > CP-PC > phyllanthin, suggests that complexation improves activity by enhancing solubilities. The activity of complexes and phyllanthin were found to be lower than that of standard ascorbic acid (EC_{50} 9.78 µg/mL).

Ferric reducing antioxidant power method

CP-PC and CP-P-PC complexes were found to be very effective in reducing ferric ion in FRAP assay and activity is higher than that of phyllanthin (Fig. 3B).

Phosphomolybdenum antioxidant method

The total antioxidant capacity of CP-PC and CP-P-PC complexes were found to be higher than that of phyllanthin and shown in Fig. 3C.

Assessment of hepatoprotective activity

The hepatoprotective activities of silymarin, phyllanthin, CP-PC and CP-P-PC complexes are summarized in Fig. 4 and 5.

Effect of MMLF complexes on hepatic markers

The effects of silymarin, phyllanthin, CP-PC and CP-P-PC on serum SGOT, SGPT, TB and TP are summarized in Fig. 4. Hepatic damage induced by CCl_4 caused increase in the levels of SGOT, SGPT, and TB compared with normal animals. Oral administration of phyllanthin significantly ($p < 0.05$) reduced the elevated levels of these marker enzymes, but the effect is lower. In contrast, CP-PC and CP-P-PC reduced significantly ($p < 0.05$) the elevated enzyme levels but also produced a higher effect in comparison to non-formulated phyllanthin. The TP level was significantly ($p < 0.001$) reduced in CCl_4-treated animals compared with normal animals. Treatment with phyllanthin increased the depleted protein level in lesser extent in comparison to CP-PC and CP-P-PC.

Histopathological studies

The effects of phyllanthin, CP-PC and CP-P-PC and silymarin on liver histopathology of CCl_4 treated rat are presented in Fig. 5. Histopathological observations of liver sections from the normal control group showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and a central vein (Fig. 5A). In contrast, the CCl_4 control group showed massive fatty changes, necrosis, ballooning degeneration, broad infiltration of lymphocytes, and the loss of cellular boundaries (Fig. 5B). Changes were also improved in silymarin pretreated rats, which exhibited areas of normal liver architecture and patches of necrotic hepatocytes (Fig. 5C). The liver sections of the rats treated with phyllanthin, CP-PC and CP-P-PC (30 mg/kg body weight) showed a relatively normal (Fig. 5 D; E; F) lobular pattern with a mild degree of fatty change, necrosis, and lymphocyte infiltration compared to Group II (CCl_4 control). The higher degree of normalization of architecture of liver cells suggests improvement of protection by CP-P-PC respective of phyllanthin. The complex produced hepatoprotective activity for a longer time and normalized adverse conditions of rat liver more efficiently than the free drug or its extract. The results even showed that the complex equivalent to 30 mg/kg phyllanthin exerted a similar effect to 60 mg/kg phyllanthin in the long term.

Assessment of *in vivo* antioxidant studies

The effects of MMLF of on antioxidant biochemical parameters are summarized in Table 1. The free radical formation by lipid peroxidation in terms of the MDA content in the liver homogenate was increased in the CCl_4 treated group in comparison with the normal group and was significantly reversed by MMLF treatment. Both silymarin and CP-P-PC brought down MDA to normal levels. Levels of GSH were decreased significantly with CCl_4 treatment. Pretreatment with MMLF as well as silymarin prevented this decrease and restored it to also normal. SOD activity in the CCl_4 treated group was found to be lower than that in the normal group. Both MMLF and silymarin elevated the SOD levels. The CAT activity of liver homogenate in the CCl_4 treated group was found to be lower than that of normal group. GBP and silymarin increase the CAT levels than those in the CCl_4 toxicant group. There is decline in liver GPX activity in the CCl_4 group as compared with normal animals was reversed by MMLF treatments. Both types of MMLF induced a significant increase in GR activity in CCl_4-treated rats as compared with CCl_4 treatment alone.

Assessment of plasma concentration of phyllanthin and content estimation in serum by HPTLC

Bioavailability experiments in rats showed the presence of phyllanthin in serum. A validated HPTLC method was adopted for the quantification of phyllanthin in serum. The calibration plots were linear in the range of 1000-6000 ng phyllanthin per spot and the correlation coefficient (r) of 0.96105 was indicative of good linear dependence of peak area on concentration. The calibration curve was represented by the linear equation y = 1618.95 + 0.707x (where y is the response as peak area and x is the concentration). Peak serum concentration was attained rapidly at 1st h of CP-PC and CP-P-PC treatment respectively. The MMLF preparation displayed higher serum concentration of phyllanthin (Fig. 6). The results of pharmacokinetic study of phyllanthin, CP-PC and CP-P-PC complexes in combination with piperine have shown that the bioavailability has been improved after oral administration. The higher concentration was also maintained for a longer period of time. Thus in complexed form, the phosphatidylcholine and piperine enhanced the plasma concentration of phyllanthin in a significant manner and the effect persisted for a longer period of time.

Discussion

It was reported in literature that for achieving good bioavailability, natural products must have a good balance between hydrophilicity (for dissolving into the gastrointestinal fluids) and lipophilicity (to cross lipidic biomembranes).[20] Phyllanthin has poor aqueous solubility but high lipid permeability and has its widespread use in treatment of jaundice, hepatitis etc. Poor absorption of phyllanthin through intestine as reported in pharmacokinetic studies warrants high doses to reach therapeutic levels of phyllanthin, which is sometimes inconvenient. Duc-Hanh et al. (2015) attempted to improve oral bioavailability of phyllanthin by developing self-microemulsifying drug delivery system (SMEDDS) and reported that oral absorption of phyllanthin in rats was significantly enhanced by SMEDDS as compared with plain phyllanthin.[9] However, till date there is no research has been conducted to improve hepatoprotective and antioxidant activity of phyllanthin by formulating MMLF along with piperine. Piperine has been reported to be a biological catalyst that enhances the bioavailability of the drug in the body by promoting rapid absorption from the gastrointestinal tract or by protecting the drug from being metabolised in the first passage through the liver after being absorbed or by a combination of both.[11,12] Johri et al. (1992) studied the absorptive function of piperine in the intestine

and reported that piperine (25-100 μM) significantly stimulated γ-glutamyl transpeptidase activity, enhanced the uptake of amino acids and increased lipid peroxidation in freshly isolated epithelial cells of rat jejunum.[45] They suggested that piperine may interact with the lipid environment to produce effects which lead to increased permeability of the intestinal cells. It is reported to be least toxic to humans and does not undergo any metabolic change during absorption.[46,47] There are also reports on significant protection of liver against various hepatotoxins by piperine.[48,49,50] An increasing number of studies in experimental animals suggest that phospholipids other than enhancing absorption might be of benefit in the treatment of liver disease by improving bile fluidity and protection of the bile pole of the hepatocyte.[51] This raises the possibility that synthetic or naturally occurring phospholipid isolates could be used in combination with hepatoprotective nutraceuticals.[52] Improved absorption by complexation with phospholipids has been reported in number of cases.[53,54] Mixed micelles are emerging novel drug delivery vehicles and have been adopted in recent years to improve the bioavailability and solubility of poorly soluble hydrophobic drugs.[17] So, phyllanthin was isolated from *P. amarus* and was incorporated with piperine as mixed micellar lipid formulation (MMLF) in the present study for improved hepatoprotective activity.

Naturally derived antioxidants counteract the oxidative stress induced by many hepatotoxins.[44] In the present study, the antioxidant activity of phyllanthin, CP-PC and CP-P-PC and the possible mechanisms had been investigated by assessing their roles on DPPH, FRAP radicals scavenging activity and phosphomolybdenum assay. It was found that the complex shows better *in vitro* radical scavenging and antioxidant activity than non-formulated phyllanthin. CCl_4 is one of the most commonly used hepatotoxins and converted to its active metabolite, the trichloromethyl (CCl_3^-) radical by Cytochrome P-450.[55] These radical readily reacts with oxygen to form free radicals like trichloromethylperoxyl radical ($CCl_3O_2^-$), which trigger damage to hepatic tissue by formation of lipid peroxides, which in turn yield products like MDA. Assessment of liver function can be performed by estimating the activity of enzymes SGOT and SGPT originally present in high concentrations in the cytoplasm of liver cells.[34] When liver cells are damaged or destroyed, the enzymes present in the liver cells leak out into the blood. This may cause elevated concentration of liver enzymes (SGOT and SGPT) in the blood. Pretreatment with the MMLF as well as the silymarin significantly reduced the

416 elevation in liver enzymes. Further, MMLF increases the levels of total proteins and bilirubin in the
417 serum, which indicates hepatoprotective activity. The complex produced hepatoprotective activity for
418 a longer time and normalized adverse conditions of rat liver more efficiently than the free drug or its
419 extract. Although there is need to incorporate two more groups viz., one group treated with complex
420 of piperine alone and another with pure phospholipid for better understanding of the result in the
421 present hepatoprotective studies. But from literature it was revealed that the complex of piperine
422 showed better hepatoprotective effects than uncomplexed piperine, whereas phospholipid itself find
423 use in hepatoprotection guided our present experimental design.[14,52] The results even showed that the
424 complex (at 30 mg/kg) phyllanthin exerted a similar effect to 60 mg/kg phyllanthin in the long term.
425 The effect produced by MMLF of phyllanthin may be due to sustained release action and improved in
426 bioavailability. The result of improvement in bioavailability and pharmacokinetic property of
427 phytochemicals having poor aqueous solubility and high lipid permeability was reported to be
428 resolved in many previous studies by designing similar formulation.[23]
429 An elevation in the MDA levels in the liver suggests enhanced peroxidation leading to tissue damage
430 and failure of the antioxidant defence mechanisms.[56] Pretreatment with MMLF significantly reversed
431 these changes by restoring the SOD, CAT, GPX and GR levels. Hence, it is likely that the mechanism
432 of hepatoprotection of MMLF is due to its antioxidant effect. The results of present studies are also
433 supplemented by a histopathological examination of the rat livers. This clearly indicated that
434 pretreatment of MMLF enhanced hepatocyte regeneration in a manner comparable to those of
435 silymarin treated group.
436 The results of pharmacokinetic study of phyllanthin, CP-PC and CP-P-PC complexes have shown
437 improvement in bioavailability after oral administration of CP-P-PC. Likewise, several other studies
438 have indicated the beneficial role of phospholipids in enhancing the therapeutic efficacy of some
439 molecules having poor oral absorption.[26] The improved bioavailability of complex may be due to
440 increased aqueous and lipid solubility of phyllanthin in complex form. Complexation plays a major
441 role in sustaining phyllanthin release from the MMLF, which is evident from the experimental results.
442 The results of present study clearly indicates the superiority of CP-P-PC complex in combination with
443 piperine over non-formulated phyllanthin, in terms of better absorption, enhanced bioavailability and

improved pharmacokinetics. Based on the above observations, it can be concluded that complexation of a phospholipid with phyllanthin and piperine may solve the problem of rapid clearance and lower elimination half-life associated with phyllanthin in all the terms of hepatoprotective study.

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Conflict of Interest

Author does not have any conflict of interest.

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554 **TABLE LEGENDS**

555 **Table 1** Effect of mixed micellar lipid formulation of phyllanthin and piperine on liver TBARS, GSH,
556 SOD, CAT, GPX and GR in CCl_4 intoxicated rats.

557

558 **FIGURE LEGENDS**

559 **Fig. 1** (A) Chemical structure of phyllanthin; (B) UV overlain spectra of standard and isolated
560 phyllanthin; (C) TLC of standard and isolated phyllanthin (R_F 0.33); (D) HPTLC chromatogram of
561 isolated phyllanthin.

562 **Fig. 2** FTIR spectra of (A) CP-PC; (B) phyllanthin

563 **Fig. 3** (A) Antioxidant by DPPH; (B) Ferric reducing antioxidant power assay; (C)
564 Phosphomolybdenum antioxidant assay

565 **Fig. 4** Effect of phyllanthin, CP-PC and CP-P-PC on biochemical parameters percentage of CCl_4
566 damaged livers in rats

567 **Fig. 5** Liver micrograph of phyllanthin, CP-PC, CP-P-PC and silymarin (100 mg/kg) treated rats: (A)
568 vehicle control group; (B) CCl_4 control showing degeneration, massive fatty changes with
569 inflammatory changes; (C) Silymarin and CCl_4 treated group (D) Phyllanthin and CCl_4 treated group;
570 (E) CP-PC and CCl_4 treated group; (F) CP-P-PC and CCl_4 treated group.

571 **Fig. 6** Plasma concentration of phyllanthin, CP-PC and CP-P-PC in rats after oral administration.
572 Values are mean ± SEM (n = 6 per group and time point).

573

Table 1 Effect of mixed micellar lipid formulation of phyllanthin and piperine on liver TBARS, GSH, SOD, CAT, GPX and GR in CCl_4 intoxicated rats.

Groups⟶ **Biochemical parameters ↓**	Normal control	Negative control (toxicant)	Silymarin (100 mg/kg)	Phyllanthin (30 mg/kg)	CP-PC (30 mg/kg)	CP-P-PC (30 mg/kg)
TBARS (nmol of MDA/g)	22.50 ± 0.43	40.67 ± 0.72[a]	20.50 ± 0.76[d]	32.17 ± 0.60[d]	28.50 ± 0.43[d]	21.67 ± 0.49[d]
GSH (µmol/g)	4.33 ± 0.42	2.67 ± 0.33[c]	4.50 ± 0.56[f]	2.83 ± 0.54[f]	3.00 ± 0.26[f]	3.83 ± 0.31[f]
SOD (unit/mg protein)	49.17 ± 1.01	8.83 ± 1.17[a]	45.67 ± 1.91[d]	40.50 ± 0.76[d]	43.50 ± 0.42[d]	46.00 ± 2.27[d]
CAT (µM/min/mg protein)	18.30 ± 0.54	11.67 ± 0.76[b]	18.16 ± 0.91[e]	13.33 ± 0.49[f]	16.00 ± 0.57[f]	17.50 ± 1.38[g]
GPX (µg/min/mg protein)	15.16 ± 1.30	6.67 ± 1.33[a]	13.00 ± 1.44[e]	9.50 ± 0.76[f]	10.83 ± 0.87[f]	12.33 ± 0.76[g]
GR (nmol/min/mg protein)	133.17 ± 2.46	8.00 ± 1.16[a]	131.27 ± 0.95[d]	118.05 ± 2.08[d]	122.13 ± 0.74[d]	129.45 ± 3.45[d]

Values are mean ± SEM; $n = 6$ in each group. [a]P value > 0.0001 when toxicant control compared with normal control.; [b]P value > 0.001 when toxicant control compared with normal control.; [c]P value < 0.05 when toxicant control compared with normal control.; [d]P value > 0.0001 experimental groups compared with toxicant control.; [e]P value> 0.001 experimental groups compared with toxicant control.; [f]P value < 0.05 experimental groups compared with toxicant control.; [g]P value > 0.05 experimental groups compared with toxicant control.

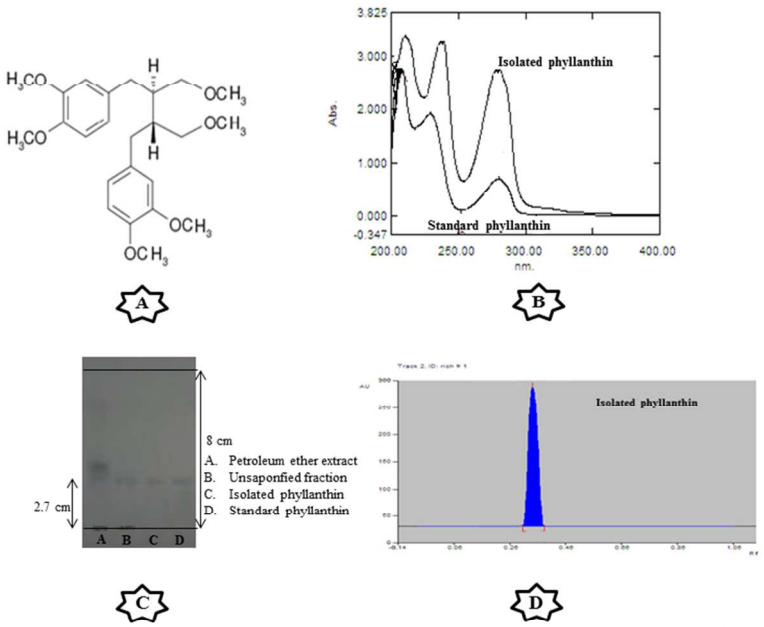

Fig. 1 (A) Chemical structure of phyllanthin; (B) UV overlain spectra of standard and isolated phyllanthin; (C) TLC photograph of extract, fraction, isolated and standard phyllanthin (R_F 0.33); (D) HPTLC chromatogram of isolated phyllanthin.

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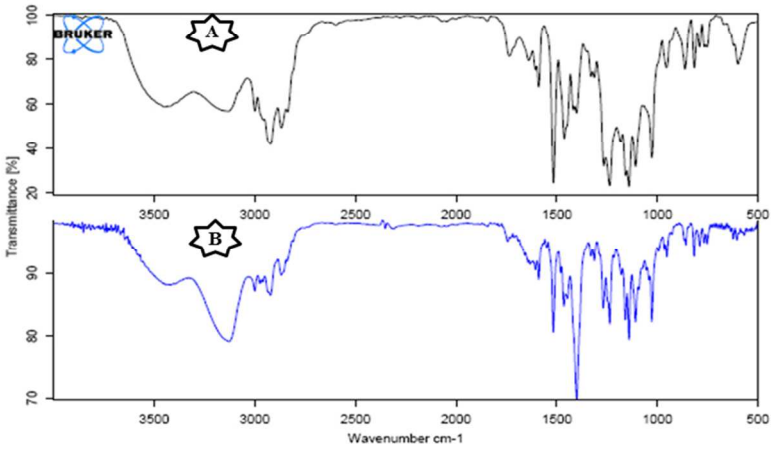

Fig. 2 FTIR spectra of (A) CP-PC; (B) Phyllanthin

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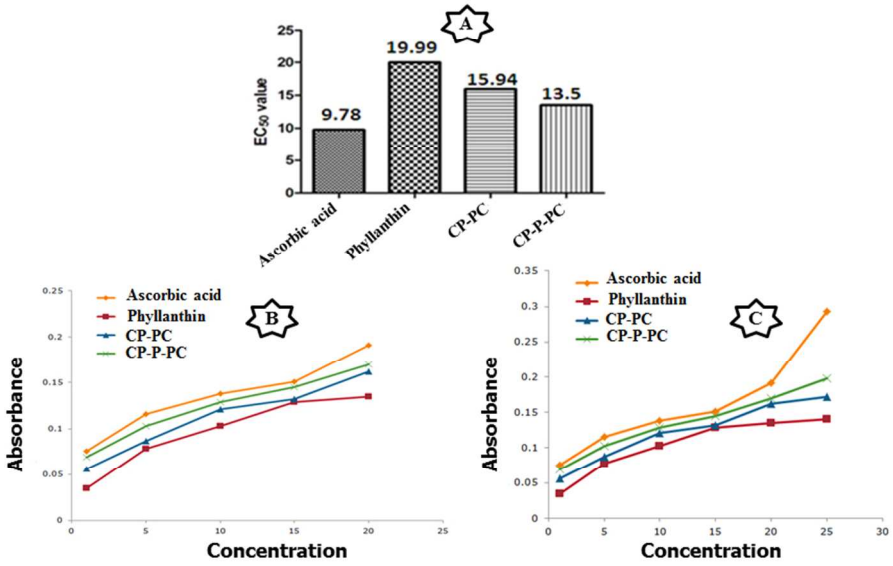

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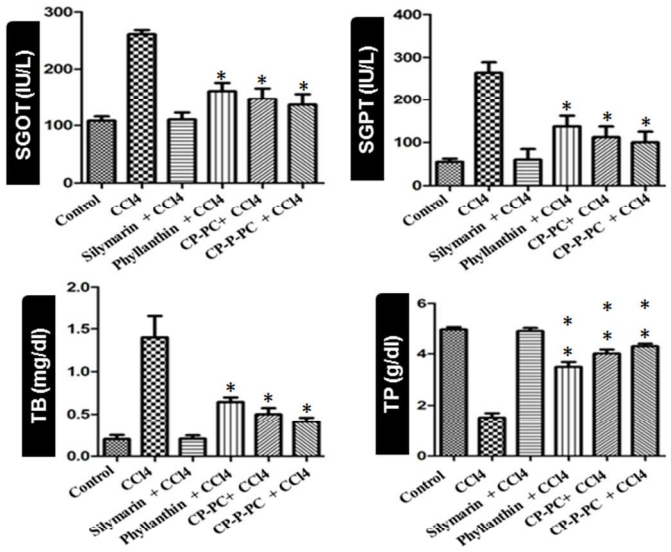

Fig. 4 Effect of phyllanthin, CP-PC and CP-P-PC on biochemical parameters percentage of CCl_4 damaged livers in rats

Values are expressed as mean ± S.E.M (n = 6)

Where, SGOT: serum glutamate oxaloacetate transaminase; SGPT: serum glutamate pyruvate transaminase; TB: total bilirubin and TP: total protein. * Significant compared to CCl_4 ($p<0.05$), ** significant compared to CCl_4 ($p<0.001$)

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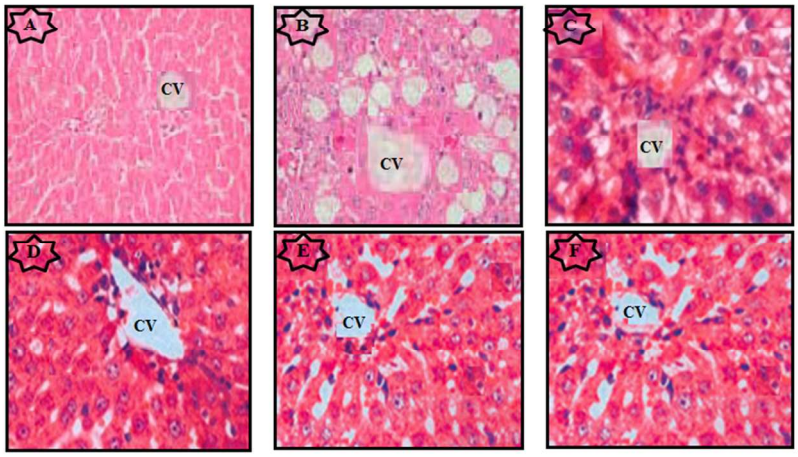

Fig. 5 Liver micrograph of phyllanthin, CP-PC, CP-P-PC and silymarin (100 mg/kg) treated rats: (A) vehicle control group; (B) CCl_4 control showing degeneration, massive fatty changes with inflammatory changes; (C) Silymarin and CCl_4 treated group (D) Phyllanthin and CCl_4 treated group; (E) CP-PC and CCl_4 treated group; (F) CP-P-PC and CCl_4 treated group.

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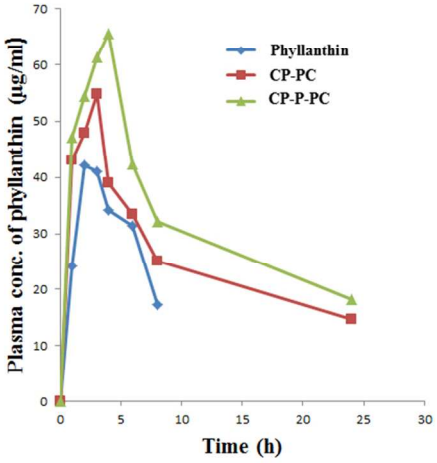

Fig. 6 Plasma concentration of phyllanthin, CP-PC and CP-P-PC in rats after oral administration. Values are mean ± SEM ($n = 6$ per group and time point).

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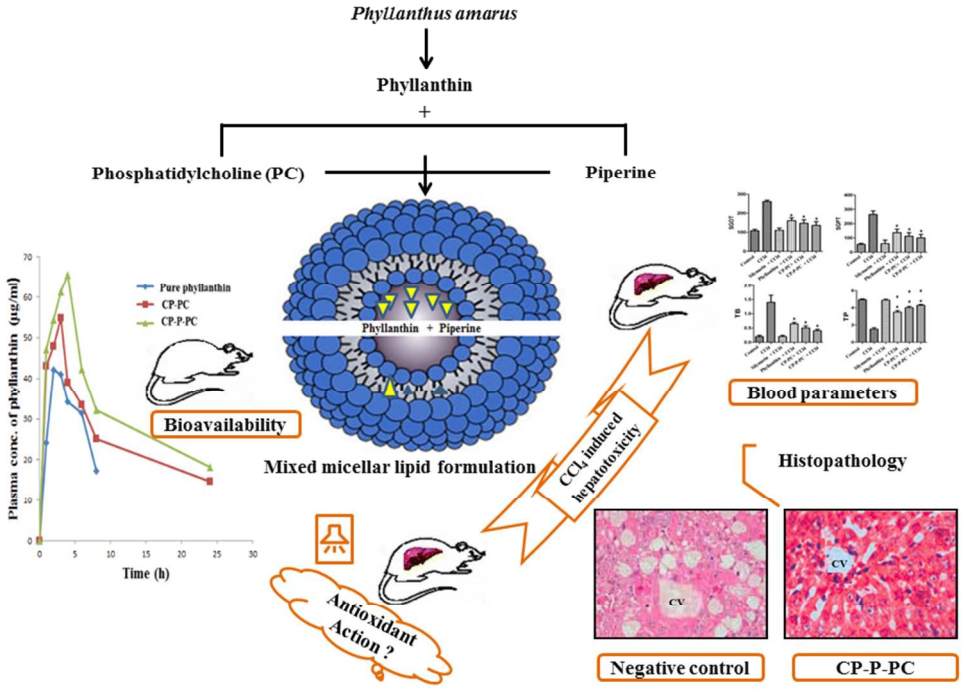

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