Nanoemulsified ethanolic extract of *Phyllanthus amarus* Schum & Thonn ameliorates CCl₄ induced hepatotoxicity in Wistar rats

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*Phyllanthus amarus* (PA) is commonly used in traditional medicine for hepatoprotectivity. The major limitation is that, treatment requires a large quantity of herbal extract for a longer duration. Aim of the present study was to encapsulate ethanolic plant extract for sustained release of constituents in intestine and facilitate maximum absorption. The efficacy was compared for the hepatoprotective activity of nanoencapsulated ethanolic extract of *P. amarus* (NPA) and PA in carbon tetrachloride (CCl₄) induced hepatotoxic male rats. Based on total phenol content (TPC), the loading efficiency of nanocapsules was 89% (pH 7.0) and optimum concentration was 2:18 (mg/mL) for plant extract: olive oil. Scanning electron microscopy (SEM) showed a spherical morphology, photon correlation spectroscopy (PCS) identified mean particle diameter as 213 nm and Fourier transform infrared spectroscopy (FT-IR) revealed that the phytoconstituents were stable. An oral dose of NPA (20 mg/kg body wt.) showed a better hepatoprotective activity than PA (100 mg/kg body wt.) and also repeated dose oral toxicity proved to be safe. These biochemical assessments were supported by rat biopsy examinations. In conclusion, the nanoemulsification method may be applied for poor water-soluble ethanolic herbal extracts to reduce the dosage and time.

**Keywords:** Hepatoprotective activity, Nanoemulsion, *Phyllanthus amarus*, Sodium alginate

*Phyllanthus amarus* is a herb used in Ayurvedic medicine for liver disorders¹. It is a well-known antiviral agent², has antiseptic properties, and is also used to treat gonorrhea, jaundice and mammary abscesses³. In addition, *P. amarus* has been reported to possess antidiabetic, anticancer and anti-inflammatory activities⁴-⁶. It contains many types of phenolic compounds; lignans such as phyllanthin and hypophyllanthin, flavonoids such as quercetin and astragalin and ellagitannins such as amaromic acid, amarin and phyllanthin D⁷-¹⁰. These phenolic compounds were related to *P. amarus* antioxidant activity¹¹.

Biochemical constituents of plants are important sources of natural antioxidants and efficacy of plant extract is more when they are consumed as a crude extract¹². However, a major limitation is that the quantity of herbal extract required for treatment is more and a long duration of treatment is required due to the degradation of various plant constituents such as alkaloids, amides, prophenylphenols, steroids, hydrocinnamic acid and oxalic acid in gastrointestinal tract, in addition to poor absorption of these constituents in intestine¹³.

In this study, nanocapsules of *P. amarus* were prepared by emulsion-coacervation method and characterized them by using scanning electron microscopy (SEM), photon correlation spectroscopy (PCS) and Fourier transform infra-red spectroscopy (FT-IR). Finally, the efficacy of nanoemulsified ethanolic extract of *P. amarus* was studied for their hepatoprotective activity by reducing the treatment dosage and period.

**Materials and Methods**

*Extraction and preparation of nanoemulsified ethanolic extracts using sodium alginate*—Leaves and berries of *P. amarus* were collected from International Institute of Biotechnology and Toxicology, Padappai, during the period 2009. They were washed thoroughly with several changes of sterile water followed by 70% ethanol, blotted between fields of filter paper...
(Whatman No.1), shade dried and homogenized in a mixer. Homogenate was extracted in 99% ethanol for 8 h in Soxhlet distillation and the solvent was evaporated at room temperature (28-30°C). The residue was lyophilized (Chistlyophilizer, German) and stored at -20 °C until further use and the yield of the plant extract was 11%. Plant specimen was authenticated by Botanical Survey of India (Coimbatore, India) and sample was preserved in International Institute of Biotechnology and Toxicology (IIBAT).

Nanoencapsulation was performed using a three step procedure - emulsification, cross-linking with calcium chloride (CaCl₂) and solvent removal⁴,¹⁵. Dispersion of ethanolic plant extract in aqueous sodium alginate (molecular weight of 80,000-120,000 kDa, Sigma Chemicals, St Louis, MO, USA) containing Tween 80® solution caused immediate formation of micelles with an oil core. Alginate shell was then solidified by cross-linking with analytical grade CaCl₂ and the solvent was removed by evaporation under pressure. Nanocapsules containing plant extract were obtained as dispersion in aqueous phase.

Variation in proportion of plant extract and olive oil—Optimal plant extract : olive oil mass ratio for nanocapsule preparation was determined using different ratios (0.005:0.195, 0.01:0.190, 0.02:0.180, 0.03:0.17, 0.06:0.14 and 0.15:0.05), dissolved in 10 mL of ethanol to obtain a final concentration of 2% and was mixed with 10 mL of aqueous alginate (0.6 mg/mL) (1:1 ratio) at pH 7.0 containing 1% (w/v) Tween 80®. After sonication for 15 min, the emulsion was combined with 4 mL of 0.67 mg/mL CaCl₂ solution and was continuously stirred for 30 min. The plant extract-loaded alginate nanocapsule suspension was then equilibrated overnight prior to removal of ethanol by rotary evaporation at 40 °C for 20 min. Finally, the alginate nanocapsules containing plant extract were obtained as dispersion in aqueous phase.

Determination of particle size—Particle size was determined using photon correlation spectroscopy (PCS), Malvern S4700 PCS System, UK. For particle size analysis, the nanoemulsified plant extract was first suspended in 100 mL of filtered water (0.2 µm filter, Ministart, Germany), subjected to sonication for 30s and then vortexed for 10s before analysis.

Characterization of nanocapsules—Shape and surface morphology of nanoemulsions were examined using scanning electron microscopy (SEM) (JSM-T20, Tokyo, Japan). An appropriate sample of polymeric nanoparticles was mounted on metal stubs, using double-sided adhesive tapes. Samples were gold coated and observed for morphology at an acceleration voltage of 15 kV.

Chemical stability of nanoemulsified ethanolic extracts—Lyophilized samples mixed with potassium bromide at a ratio of 1:100 (w/w) were laminated using a pellet mold in Fourier transform infrared spectrometer (FT-IR). Spectra of ethanolic extract, encapsulated extract and extract released from alginate beads were obtained using FT-IR spectrometer (Shimadzu, Japan) by scanning between 4000–400/cm.

Storage Stability of nanoemulsified ethanolic extracts—Physical stability of the plant extract loaded nanocapsules was determined by the assessment of average size after storage at 4 °C and 28 °C for 2 months. Based on the total phenol content, the loading efficiency was calculated for the release profile of the phytoconstituents at 28 and 4 °C for the nanoemulsified ethanolic herbal extracts.

In vitro release of nanoemulsified extracts at acidic pH (1.2) and alkaline pH (7.2)—To study the release kinetics, the nanocapsules were treated separately with two solutions; an acidic simulated gastric fluid,
pH 1.2 and simulated intestinal fluid, pH 7.2 at 37 °C. The buffers used simulate the physiological microenvironment in the stomach and intestinal tract respectively. Release profile of the phytoconstituents from the nanoemulsion was calculated based on total phenol content (TPC) (before and after filtration) and converted into the loading efficiency as mentioned above.

Test animals and treatment pattern—Male albino rats of Wistar strain (*Rattus norvegicus*) weighing 100-120 g were used for the study. The animals were housed in a group of 3 rats per cage under well-controlled conditions of temperature (21±2 °C) and humidity (50-60%) in 12/12 h light-dark cycle. Animals had free access to diet (Tetragon Chemie Pvt. Ltd., Bangalore, India) and water *ad libitum*. Hepatotoxicity was induced with CCl₄ (Sigma Ltd, USA) (1 mL/kg body wt.) for 7 days by intraperitoneal injection. Animals were checked for AST, ALP and ALT levels 7 days after CCl₄ injection. Hepatotoxic rats were divided into 5 groups, with each group containing 6 animals. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC), IIBAT.

Treatment protocol—Group 1: (Negative control) Sterile water was orally administered during the entire period of study. Group 2: (Hepatotoxic control) CCl₄ induced hepatotoxic rats were orally administered with sterile water throughout the study period. Group 3: (Plant extract, 100 mg/kg body wt.) CCl₄ induced hepatotoxic rats were orally administered with plant extract for 7 days, which has already been proved in the literature for hepatoprotective activity. Group 4: CCl₄ induced hepatotoxic rats were orally administered with unmanotized plant extract (20 mg/kg body wt.) for 7 days. Group 5: CCl₄ induced hepatotoxic rats were orally administered with nanoemulsified plant extract (20 mg/kg body wt.) for 7 days. Group 6: CCl₄ induced hepatotoxic rats were orally administered with reference drug, Liv 52 tablets (500 mg/kg body wt.) for 7 days. Group 7: Plant extract (100 mg/kg body wt.) was administered orally for 7 days. Group 8: Nanoemulsified plant extract of 20 mg/kg body wt. was administered orally for 7 days.

All the animals in the study were observed twice daily for overt signs of toxicity, morbidity and mortality during the entire study period. The body wt. was measured on the 0, 7 & 14th day. Blood was collected from overnight fasted animals on day 8, 13 and 16, from the orbital sinus of all animals. Heparinized plasma used for biochemical analysis was obtained by centrifugation at 1500 g for 20 min. Biochemical parameters viz. glucose, total cholesterol, triglycerides, creatinine, aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) were analyzed by automated biochemistry analyzer (Humastar 300, Human GmbH, Germany). All the animals were sacrificed at the end of the treatment period (Day 16) using CO₂ and a detailed gross necropsy was performed, which includes careful examination of the external surface of the body, all orifices, the cranial, thoracic, and abdominal cavities and their contents. A piece of the liver and kidney of each animal was fixed with 10% neutral buffered formalin, embedded in paraffin wax, sectioned at a thickness of approximately 3-5 micron and stained with hematoxylin-eosin. Detailed histopathological examination of liver sections from all the animals of G1 to G8 was performed and the following grading system was used; 1: Minimal, 2: Mild, 3: Moderate, 4: Marked, 5: Severe.

Statistical analysis—Data is expressed as mean ± standard deviation. For statistical analysis, experimental values were compared with negative control. Data was analyzed by Modified Leven’s test for homogeneity and subjected to one-way analysis of variance (ANOVA). The alpha level at which all tests were conducted is 0.05 and NCSS 2007 software was used for the analysis.

Results

Nanotization of plant extracts using sodium alginate with variation in parameters — Total phenol content of the original plant extract was 0.18 ± 0.02 mg GEA/mg of ethanolic extract of *P. amarus*. Optimum encapsulation efficiency was attained at 0.02: 0.180 mg/10 mL of ethanol and the loading efficiency was 89% for NPA at pH 7.0 (Fig. 1A and B). This was significantly different from other concentrations tested. Different concentrations were tried for calculating the encapsulation efficiency; in some concentrations the encapsulation efficiency was less due to aggregation, which will not form the nanocapsules. At lower concentration of the extract, there was no difference in the total phenol content before and after filtration. However, at higher concentrations of the extract, the phenol content is more in the filtrate showing the possible loss of phenols, indicating the reduction in the encapsulation efficiency.
Polydispersity index value less than 0.3 are of narrow distribution and more than 0.3 are of broad distribution. Mean particle diameter is 213 ± 4.4 nm, which indicated that the NPA showed an excellent narrow distribution (Fig. 1C) and microscopically, the nanocapsules were spherical in shape (Fig. 2). The FT-IR spectrum of sodium alginate nanocapsules cross-linked with CaCl₂, plant extract of P. amarus without nanoencapsulation and with nanoencapsulation is shown in (Fig. 3). Fingerprint characteristic vibration bands of sodium alginate nanocapsules appear at 2924 (C-H stretching), 2852 (C-H stretching), 2362 (C-H stretching), 1747 (C=O streching), 1103 (ester C-O group) and 952 (aromatic CH bending). FT-IR of P. amarus showed peaks at 2924 (C-H stretching), 2854 (C-H stretching), 1745 (C=O streching), 1462 (C-O streching), 1249 (C-O streching), 1107 (ester C-O group), 952 (aromatic CH bending) and 721 (aromatic CH bending). The common peaks that appear both in sodium alginate nanocapsules and plant extract P. amarus were; 2924, 2852, 1747, 1103 and 952 and the common peaks that appeared between plant extract P. amarus and the nanoemulsified P. amarus were; 2924, 2854, 1745, 1462, 1249, 1107 and 950. From the FT-IR profile it is confirmed that all functional groups of P. amarus were present after interaction with the polymer. FT-IR determined for the chemical stability of the original plant extract, nanocapsules with plant extract and for sodium alginate nanocapsules without plant extract showed that the characteristic bands of chemical groups remain unchanged for P. amarus.

Storage stability of nanoemulsion—Effect of storage temperature (4 ºC and 28 ºC) on total phenol content and loading efficiency revealed that the nanocapsules were fairly stable when stored for 60 days at 4 ºC. The loading efficiency was significantly similar (P>0.05) until 8th weeks of storage for P. amarus, which was different after 8th week of storage at 4 ºC (P<0.05). However, nanocapsules stored at 28 ºC released the total phenol content within a period of 3 weeks for P. amarus (Fig. 4).

In vitro release of nanoemulsion at alkaline and acidic pH—When studied in in vitro conditions using simulated gastric fluid (pH 1.2), the total phenol content was released slowly till 4 h of incubation, and maximum release was observed after 4 h of incubation. The loading efficiency was calculated for different hours of incubation at acidic pH (1.2) which is not released until 3rd hour of incubation at pH (1.2), so there is no statistically significant difference in the loading efficiency until 3rd h of incubation. The loading efficiency for 4th-8th h is statistically significant from 1st h * (P<0.05, ANOVA) for P. amarus. At alkaline pH (7.2), 98% of nanocapsules were released within 4 h of incubation which is statistically significant from 1st h *(P<0.05, ANOVA) for P. amarus (Fig. 5).

Morbidity/mortality and clinical signs—No morbidity/mortality and any clinical signs were observed in the animals from G1-G8.

Body weight—All the animals in G1, G3, G4, G5, G6, G7 and G8 showed constant body wt. gain throughout the observation period. However, mean body wt. of animals in G2 on 7th and 14th day showed significant decrease when compared to G1. There was no statistically significant difference in mean body wt. of animals in G3, G5 and G6 when compared with G1. This suggests that there was constant body wt. gain in hepatotoxic induced animals (G3, G4, G5...
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...treated with PA and NPA when compared with non-hepatotoxic control (G1) throughout the observation period. No significant changes were observed in feed consumption of (G2-G8) groups when compared with the control group.

**Biochemistry profile**—Significant changes were observed in biochemical parameters such as ALT (Fig. 6A), AST (Fig. 6B), ALP (Fig. 6C) and total protein (Fig. 6D) in animals belonging to G2, G3, G4, G5 and G6 on 8th day compared to control (G1). The group G5 showed a decrease in the AST, ALT, ALP and total protein on 13th day when compared with other treated groups; G3, G4 and G6. Blood urea nitrogen (BUN) (Fig. 6E), cholesterol (Fig. 6F), creatinine (Fig. 6G), and triglycerides (Fig. 6H) showed no significant difference in the hepatotoxic control.

**Gross pathology**—Gross pathology examination of the animals revealed CCl4 related gross pathological findings in kidney (enlarged bilateral, pale mottled) and liver (pale and diaphagmatic nodule).

![Fig. 2—Nanoemulsified ethanolic plant extract of P. amarus: (A)-light microscope, 100 × (B, C and D)-scanning electron microscope.](image)

![Fig. 3—FT-IR profile for identification of functional groups: (A)-calcium alginate beads (B)-crude plant extract of P. amarus and (C)-nanoemulsified P. amarus extract with sodium alginate cross linked with CaCl2.](image)
Organ weight—There were no statistically significant changes in the organ weight (kidney and liver) of G2, G3, G4, G5, G6, G7 and G8 when compared with G1.

Histopathology for CCl₄ induced hepatotoxicity treated with PA and NPA—Histological profile of the normal liver sections showed normal hepatic cells with well preserved cytoplasm, prominent nucleus, nucleolus, central vein and a compact arrangement of hepatocyte (Fig. 7A). In contrast to this, the liver sections of CCl₄ treated animals revealed hepatocyte degeneration/regeneration, vacuolar degeneration and single cell necrosis (Fig. 7B, C & D). These lesions were improved / observed with lesser severity in PA (100 mg/kg body wt.), Liv 52 (500 mg/kg body wt.) and NPA (20 mg/kg body wt.) treated livers. The kidney was more prominent and a histological architecture was restored and found to be on par with the control animal.

Discussion

Sodium alginate is used for nanoemulsion due to its non-toxic and non-immunogenic activity and slow release in the simulated gastric fluid pH (1.2)¹⁸. Slow release is accredited to ion exchange, chelation and reduction reactions¹⁹,²⁰. Owing to the advantages mentioned above, emulsion method was followed for nanoencapsulation of ethanolic plant extracts. This method also enhanced the solubility of ethanolic extracts. In this study, P. amarus and P. amarus ethanolic extract was dissolved in ethanol and emulsified using Tween 80® with sodium alginate as polymer. The loading efficiency using this method was 89-90% and the size of nanocapsules ranged from 200-220 nm in diameter. This method has been used earlier for turmeric oil, however the loading efficiency was very less (5.47%) due to the instability of the oil¹⁵. The method was found to be efficient for the ethanolic plant extract used in this study. Micro, sub micro and nanoemulsion of herbal formulations (active principles and plant extracts) include; triptolide microemulsion²¹, berberine nanoemulsion²², silybin nanoemulsion²³, caffeic acid nanoemulsion²⁴ and tea extract nanoemulsion²⁵. Water-in-oil emulsion reduces the secondary oxidation due to its physical stability²⁶. These results suggest that the polyphenol is not oxidized in emulsions due to the protective role towards lipid auto-oxidation²⁷.

Smaller sized particles show interaction with local tissues and provoke dysfunction of the organs²⁸. Hence, toxicity study was carried out using nanoemulsion formulation for behavioral changes, and biochemical
Fig. 6—Effect of nanoemulsified ethanolic extract of P. amarus and P. amarus treatment on (A)-alkaline transferase (ALT); (B)-aspartate transferase (AST); (C)-alanine phosphatase (ALP); (D)-blood urea nitrogen (BUN); (E)-total protein content (TPC); (F)-cholesterol (Chl); (G)-creatinine (cre); (H)-triglycerides (trig) in CCl₄ induced hepatotoxic rats during different days of blood collection. [Each bar represents mean ± S.E.M. of 6 animals in each group. G1: non hepatotoxic control, G2: CCl₄ induced hepatoprotective control, G3: ethanolic plant extract of PA (100 mg/kg body wt.), G4: unnanotized plant extract (PA) (20 mg/kg body wt.), G5: nanotized plant extract, (NPA) (20 mg/kg body wt.), G6: Liv 52 tablets (500 mg/kg), G7: treated only with plant extract (2000 mg/kg), G8: treated only with nanotized plant extract (20 mg/kg). Blood was collected from G1-G8 on day 8 after inducing hepatotoxicity with CCl₄ and also on 13 and 16 day after various treatments. *P<0.05, ANOVA is significantly different from non-diabetic control].
parameters. The biochemical and pathological examinations revealed that, there are no noticeable changes in all the parameters of the nanoparticle treated group when compared with the control.

In in vivo studies, carbon tetrachloride (CCl₄) induced formation of lipid peroxides in the rat liver and the raised serum enzymes, glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) were inhibited by pretreatment with aqueous and methonolic extract at a dose of 100 mg/kg body wt. demonstrating the hepatoprotective action of *P. niruri*^{31}. The aqueous extract at doses equivalent to 2 g/kg body wt. of dried plant were not toxic in hamster liver. This confirmed the non-toxic plant doses in humans^{32,33}. Gallotannins which are the major constituents of the water extract are at least in part responsible for the antimutagenic and anticarinogenic effects of *P. amarus*^{34}. Phyllanthin and hypophyllanthin of *P. amarus* are said to be used as hepatoprotective agents and also protect hepatocytes against carbon tetrachloride (CCl₄) and galactosamine induced cytotoxicity in rats^{35}. The ethanolic extract of *P. amarus* (100 mg/kg body wt.) showed better activity for hepatoprotection in CCl₄ induced hepatotoxic rats, due to the higher concentration of phyllanthinin in the ethanolic extract in comparison to aqueous extract of *P. amarus*^{36}.

Nanoemulsified plant extracts for hepatoprotective activity were prepared by different methods such as liposomal encapsulated silymarin^{37}, nanoparticles of *Cuscuta chinensis*^{38}, gingoselect phytosome ROS^{39} and silybin phytosome^{40}. The chief problem in using plant extracts for treatment is the high dose that has to be administered in order to deliver the desired therapeutic potential. This issue can be potentially resolved using the nanotization method. In addition to

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**Fig. 7**—Histopathology of (A)-control rat liver showing normal central portal triad and central vein H&E, 200×; (B)-liver of CCl₄ treated rats showing vacuolation and inflammatory cells around portal triad, H&E, 200×; (C)-liver of CCl₄ treated rats showing vacuolation and inflammatory cells around central vein, H&E, 100×; (D)-liver of CCl₄ treated rats showing vacuolation and inflammatory, H&E, 100×.
using a low dose, the nanotized extract lowered the ALT, AST and ALP levels in a short span of 4 days, which encourages the use of nanotized preparations for therapy. The histological profile of the normal liver sections showed normal hepatic cells with well preserved cytoplasm, prominent nucleus, nucleolus, central vein and a compact arrangement of hepatocyte. In contrast to this, the liver sections of CCl₄ treated animals revealed hepatocyte degeneration/regeneration, vacuolar degeneration and single cell necrosis. These changes are associated with or without inflammatory cell infiltration. Congestion of the central vein and sinusoids were also seen with inflammatory cells infiltrating sinusoids. These lesions were improved with lesser severity in PA (100 mg/kg body wt.) treated group and the NPA (20 mg/kg body wt.) group showed significant improvement as that of Liv. 52 treated groups.

In the present study, CCl₄ injected to adult rats produced hepatotoxin which is believed to require activation by hepatic microsomal mixed function oxidase to trichloromethyl free radical carbon tetrachloride and is more reactive in the presence of oxygen trichloro methyl peroxyl radical. Surprisingly, we found that the required dose of the nanotized extract was 5 times lower (20 mg/kg body wt.) than that of unnanotized plant extract (100 mg/kg body wt.) which was able to decrease the ALT, AST and ALP and other biochemical parameters within a period of 4 days and significantly increase the body wt. of the CCl₄ treated animals. This effect can be attributed to the characteristic of nanocapsules due to sustained release. The nanocapsules of P. amarus prepared in this experiment released the phytoconstituents slowly at acidic pH (1.2–stomach) and rapidly at the alkaline pH (7.2–intestine). This release is due to the inclusion of sodium alginate as a polymer in the preparation of the nanocapsules. In addition, the droplets of oil-in-water emulsion are phagocytosed by the macrophage and reached a high concentration in the liver, spleen, and kidney in which the amount of the dissolved drug is very large. Thus, it helps to strengthen the stability of the hydrolyzed materials, improve the penetrability of drugs to the skin and mucous, and reduce the drug stimulus to tissues. While the nanotized extract delivered the hepatoprotective effect at a dose of 20 mg/kg body wt. within 4 days, the same dose of unnanotized preparation did not exhibit similar response.

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