Ameliorative effects of phyllanthin on carbon tetrachloride-induced hepatic oxidative damage in mice

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Objective: To evaluate the liver protecting efficacy of phyllanthin, a lignin, isolated from the leaves of Phyllanthus amarus using mice model.

Methods: Phyllanthin was orally administered with or without CCl₄ for 30 d. Serum levels of hepatic marker enzymes namely alanine transaminase and aspartate transaminase were evaluated. Oxidative stress was ascertained by measuring hepatic lipid peroxidation levels and by estimating non-enzymatic antioxidants such as glutathione, total ascorbic acid, enzymatic antioxidants namely catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase, and glutathione transferase. Histopathological and ultramicroscopic analyses were also carried out.

Results: Oral administration of CCl₄ caused significant increase in lipid peroxidation. The hepatic levels of both non-enzymatic antioxidants and enzymatic antioxidants were significantly lowered in CCl₄-treated mice as compared to control. Treatment with phyllanthin significantly mitigated these changes in the CCl₄-treated mice. Histopathological and ultramicroscopic studies correlated well with the biochemical findings, as phyllanthin treatment reversed the alterations induced by the toxin and the subcellular features of phyllanthin treated mice were similar to those present in the normal mouse liver.

Conclusions: This study reports the in vivo anti-hepatotoxic potential of this isolated molecule phyllanthin, which may be responsible for the liver protecting property of Phyllanthus amarus.

Keywords
Phyllanthus amarus, Phyllanthin, Hepatoprotection, Oxidative stress, Glutathione

1. Introduction

The use of carbon tetrachloride (CCl₄) to induce experimental oxidative stress and liver damage is quite common[1]. It is also well known that CCl₄ rapidly metabolizes to free radical products in the hepatic tissue with subsequent initiation of lipid peroxidation (LPO)[2]. Repeated exposure with this hepatotoxin is known to exhaust the endogenous antioxidant defense pool of biological systems. To correct this oxidative imbalance, antioxidative principles derived from varied sources are utilized. Some naturally occurring antioxidative principles originating from plant resources have exhibited enormous potential in resolving this oxidative stress which is also considered as the root cause for many pathogenic disorders affecting humans.

The Indian sub-continent through ancient ages had followed a rich tradition of employing medicinal plants for treatment of various ailments. Various experiments have been carried out using plants and their products as liver protecting alternatives. The genus Phyllanthus is represented by nearly 1 200 plant species which are distributed in tropical and subtropical countries and used in traditional medicine to treat chronic liver
towards antioxidative efficacy of phyllanthin against the standard and well established hepatotoxin CCl₄ in mice model. The fibroblasts and keratinocytes and its growth inhibitory activity potential of phyllanthin containing microcapsules on human countries for the treatment of various diseases such as jaundice, liver protecting effect of phyllanthin, one of the major lignins from P. amarus responsible for its antihepatotoxic property. Some in vitro data on the hepatoprotective potential of phyllanthin have been previously published[14-16]. Similarly, the in vitro antioxidative potential of phyllanthin containing microcapsules on human fibroblasts and keratinocytes and its growth inhibitory activity towards Staphylococcus aureus has also been demonstrated by Lam et al[17].

In this study, we have assessed the hepatoprotective and antioxidative efficacy of phyllanthin against the standard and well established hepatotoxin CCl₄ in mice model. The in vivo liver protecting effect of phyllanthin, one of the major lignin and lead molecule of P. amarus is determined.

2. Materials and methods

2.1. Chemicals

All chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA). CCl₄ (HPLC grade) and all other solvents used in the study were procured from Merck Specialties Pvt. Ltd., Mumbai, India.

2.2. Phyllanthin isolation

Silica gel column chromatography using gradient elution with hexane–ethyl acetate solvent mixture was used for the isolation of phyllanthin as reported elsewhere[15]. The purity of the isolated compound was confirmed by reversed–phase HPLC analysis. This isolated compound was used in the present study.

2.3. Animals

Swiss strain female mice (Mus musculus) weighing between 32–35 g were maintained under controlled conditions of (25±2 °C temperature and 12 h light/dark cycle in the animal house of the Department of Zoology, Gujarat University, Ahmedabad and used in the study. They were maintained on pelleted rodent (Amrut Feeds) obtained from Pranav Agro Industries Limited, Pune, India. All experimental procedures were assessed and approved by the Committee for the Purpose of Control and Supervision of Experiment on Animals (Reg–167/1999/CPCSEA), New Delhi, India.

2.4. Experimental design

The mice were separated into four different groups consisting of 10 mice in each group. Group I received 0.2 mL of olive oil which was used as the vehicle to dissolve CCl₄ and phyllanthin. Group II was marked as the phyllanthin-treated group and mice were administered 10 mg/kg body weight per day of phyllanthin[18]. The mice in Group III were administered CCl₄ (826 mg /kg body weight)[19] and Group IV mice received CCl₄ along with 10 mg/kg body weight per day of phyllanthin. Treatments in all the groups were continued daily for 30 d. On Day 31, blood was collected by cardiac puncture in clean sterilized tubes and serum was separated by centrifugation at 2800 r/min for 10 min. Liver tissue was dissected out and used for further biochemical, histopathological and ultramicroscopic analysis.

2.5. Biochemical estimations

The LPO was measured by the method of Ohkawa et al.[20] which was based on the formation of red chromophore that absorbed light at 532 nm due to the reaction of thiobarbituric acid with products of LPO like malondialdehyde (MDA) and others which were collectively called thiobarbituric acid reactive substances. The tissue glutathione (GSH) was determined by the method of Grunert and Philips in which a saturated alkaline medium was used and the GSH present in the tissues was made to react with sodium nitroprusside to give a red coloured complex which was measured at 520 nm[21]. The method of Roe and Kuether was used to estimate the liver total ascorbic acid (TAA)[22], which was oxidized to dehydroascorbic acid by in the presence of trichloroacetic acid. This couples with 2, 4-dinitrophenyl hydrazine in the presence of thiourea and sulphuric acid yielded a red coloured complex which was read at 540 nm against blank.

Activities of enzymes such as catalase (CAT)[23], superoxide dismutase (SOD)[24], glutathione peroxidase (GPx)[25], glutathione reductase (GR)[26], and glutathione transferase (GST)[27] were measured using standard reported protocols. The protein content was estimated using bovine serum albumin as the standard[28]. Activities of serum transaminases [alanine transaminase (ALT) and aspartate transaminase (AST)] were measured using kits supplied by Agappe Diagnostics, Ernakulam, Kerala, India.

2.6. Histopathological examination

Tissues for histopathological examination were preserved in 10% neutral buffered formalin immediately after autopsy. Standard technique for hematoxylin and eosin (H & E) staining was followed. The tissues were dehydrated by passing through ascending grades of alcohol, cleared in xylene and embedded in paraffin wax (58–60 °C m.p.) About 5 µm thick sections were cut on a rotary microtome and stained in H & E,
dehydrated in alcohol, cleared in xylene and mounted in di-n-butylphthalate-polystyrene-xylene and examined under a light microscope.

2.7. Ultrastructural studies

Primary fixation was carried out using 3% glutaraldehyde in 0.2 mol/L phosphate buffer for 2 h at 4 °C and then post-fixed with 1% OsO4 in the same buffer at 4 °C for 2 h, and later on embedded in Epon. Ultrathin sections were stained with uranyl acetate and counter stained in lead citrate. The sections were later examined using a Zeiss EM 900 transmission electron microscope.

2.8. Statistical analysis

The data were expressed as mean±SD and results were analyzed by one-way analysis of variance (ANOVA) followed by least significant difference (LSD) multiple comparison tests using the 19th version of SPSS software. The values were significant when P<0.05.

3. Results

3.1. Biochemical analysis

Figure 1 shows the results of the marker enzymes, ALT and AST in the serum of control and experimental mice. Results indicated that in CCl4-treated mice, the levels of ALT and AST (P<0.05) were significantly increased when compared to vehicle control group. Oral administration of phyllanthin resulted in restoration of these marker enzymes to near normal levels in the CCl4-treated mice.

Table 1 depicts the results of hepatic LPO, GSH and TAA levels and Table 2 presents the activities of various hepatic antioxidative defense parameters of the control and treated animals. Results indicated no significant difference among the observed values of vehicle control and phyllanthin–treated mice. It was found that CCl4 treatment significantly (P<0.05) increased LPO levels and decreased the various antioxidative indices, i.e., GSH, TAA, CAT, SOD, GPx, GR and GST (P<0.05). Co-treatment with phyllanthin resulted in significant (P<0.05) mitigation of CCl4–induced changes in the hepatic tissue.

Table 1

| Experimental groups | LPO (nmol MDA/mg protein/60 min) | GSH (µg/100 mg tissue weight) | TAA (mg/g tissue weight) |
|---------------------|---------------------------------|------------------------------|--------------------------|
| Vehicle control     | 1.70±0.20                       | 10.39±1.67                   | 4.53±0.99                |
| Phyllanthin         | 1.73±0.56                       | 10.50±2.09                   | 4.38±0.87                |
| CCl4                | 4.53±0.99*                      | 18.76±1.21*                  | 4.12±0.87*               |
| CCl4+Phyllanthin    | 1.75±0.58                      | 42.39±2.08*                  | 5.09±0.82                |

Results are expressed as mean±SD, n=10, a as compared between vehicle control (Group I) and CCl4–treated (Group III), b as compared between CCl4–treated (Group III) and CCl4+phyllanthin–treated (Group IV). P<0.05.

Table 2

| Experimental groups | CAT (µM H2O2 consumed/mg protein/min) | SOD (µM NADPH consumed/mg protein/min) | GPx (units/mg protein) | GR (µmol GSH consumed/mg protein/min) | GST (µmol CDNB conjugate formed/mg protein/min) |
|---------------------|--------------------------------------|----------------------------------------|------------------------|---------------------------------------|-----------------------------------------------|
| Vehicle control     | 10.59±1.67                           | 3.89±0.56                              | 2.45±0.67              | 2.78±0.84                             | 2.89±0.77                                     |
| Phyllanthin         | 10.50±2.09                           | 3.76±0.20                              | 2.38±0.78              | 2.56±0.59                             | 2.52±0.98                                     |
| CCl4                | 3.78±0.09*                           | 1.09±0.15*                            | 0.92±0.02*             | 0.98±0.12*                            | 1.56±0.09*                                   |
| CCl4+Phyllanthin    | 10.39±2.33*                          | 3.61±0.65*                            | 2.33±0.61*             | 2.37±0.55*                            | 2.20±0.98*                                   |

Results are expressed as mean±SD, n=10, a as compared between vehicle control (Group I) and CCl4–treated (Group III), b as compared between CCl4–treated (Group III) and CCl4+phyllanthin–treated (Group IV). P<0.05.

3.2. Effect of phyllanthin on histological change in the liver

Light microscope of liver of H & E stained, vehicle control mice (Figure 2A) and phyllanthin treated mice (Figure 2B) showed a normal arrangement of hepatocytes, with clearly visible nucleus, sinusoids and central vein. Treatment with CCl4 resulted in extensive centrilobular (zone 3) necrosis and inflammatory cell infiltration in the sinusoidal region. Excessive

Figure 1. Protective effect of phyllanthin on CCl4–induced changes in serum marker enzymes for liver function.

A: ALT, B: AST. Results are expressed as mean±SD, n=10, a as compared between vehicle control (Group I) and CCl4–treated (Group III), b as compared between CCl4–treated (Group III) and CCl4+phyllanthin–treated (Group IV). P<0.05.
Figure 2. Light micrograph (H & E staining) of liver in different groups.
A: vehicle control mice (400×), B: phyllanthin–treated mice showing normal architecture and compactness with clearly defined central vein (400×), C: CCl₄-treated mice – extensive centrilobular necrosis and excessive accumulation of connective tissue and feathery regeneration of nucleus are observed (400×), D: CCl₄ treated mice – excessive accumulation of connective tissue formation and centrilobular necrosis observed (400×), E: phyllanthin and CCl₄–treated mice showing normal architecture (100×), F: phyllanthin plus CCl₄–treated mice showing normal arrangement of hepatocytes, with clearly visible nucleus, sinusoids and central vein. Cytoplasmic vacuolization and necrosis are absent (400×).

Figure 3. T. E. micrograph of liver in different groups.
A: vehicle control mice – the liver shows normal architecture (3000×), B: phyllanthin–treated mice – the liver resembles vehicle control mice with normal nucleus and mitochondria (3000×), C: liver treated with CCl₄ – excessive cytoplasmic vacuolization is observed which indicates an empty appearance in the cell (3000×), D: CCl₄ treated mice liver – mitochondria and rough endoplasmic reticulum appears disorganized (4400×), E: phyllanthin–treated mice along with CCl₄ – the liver shows total recovery (3000×), F: phyllanthin plus CCl₄–treated mice – mitochondria and rough endoplasmic reticulum appear normal (4400×).
extracellular matrix formation linking central and portal regions were apparently evident (Figure 2C and 2D). Intense fatty degeneration, necrosis, vacuolization and feathery regeneration of hepatocytes were noted in all CCl₄-treated animals (Figure 2C and 2D). Results revealed complete restoration of the normal architecture and arrangement of hepatocytes in mice administered with CCl₄ along with phyllanthin (Figure 2E and 2F). The inflammatory and fatty degenerative changes were completely absent.

### 3.3. Ultrastructural results

Transmission electron micrographs of liver from vehicle control mice showed normal architecture (Figure 3A). Nucleus contained nuclear chromatin material concentrated at the nuclear periphery. Cytoplasm was rich in mitochondria. Similar profile was obtained for liver of phyllanthin alone treated animals (Figure 3B). Electron micrographs of liver treated with CCl₄ (Figure 3C) revealed excessive cytoplasmic vacuolization and an empty appearance in the cells were noted. Nucleus was found to be vacuolated. The hepatic nuclear chromatin material was found disorganized. Mitochondria appeared disorganized and swollen and rough endoplasmic reticulum showed structural changes (Figure 3D). The ultrastructure of hepatocytes of mice administered with phyllanthin plus CCl₄ (Figure 3E) revealed complete recovery from the CCl₄-induced damage with clear internal structures. Nucleus showed well organized nuclear membrane. Vacuolization was found to be completely absent and mitochondria and rough endoplasmic reticulum appeared completely normal (Figure 3F).

### 4. Discussion

Oxidative stress plays a key role in disease pathogenesis and antioxidants have emerged as therapeutic agents for the management of these disorders. The enzymatic and non–enzymatic antioxidants are considered as natural protectors against oxidative assaults frequently encountered within living systems[19]. A number of active compounds such as phyllanthin and hypophyllanthin[29], quercetin and rutin[7], amarinic acid[30], and amarin[31] have been isolated from P. amarus. Syamasundar et al. have studied the in vitro hepatoprotective property of phyllanthin against CCl₄ and galactosamine–induced toxicity[16]. Similarly, Yadav et al. have reported the combined hepatoprotective effects of silymarin and standardized ethanolic extract of P. amarus in CCl₄-intoxicated rats[32].

The findings of the present study demonstrated that co-treatment of phyllanthin along with the hepatotoxin ameliorated the changes induced by the toxin alone. The increase in the activities of intracellular marker liver enzymes ALT and AST in CCl₄–treated mice indicated leakage due to membrane damage[33], and the reduction observed after phyllanthin treatment suggests recovery towards normalization due to the recouperation of the cell membrane.

In the present study, oral administration of CCl₄, as compared to vehicle control, significantly reduced the GSH and TAA contents, while it significantly increased the levels of LPO. Similarly, treatment with the hepatotoxin for 30 d caused a significant decrease in the activities of CAT, SOD, GPX, GR, and GST. The marked reduction seen in the oxidative defense parameters with a concomitant increase in LPO after a 30–day treatment with CCl₄ suggested oxidative stress. Our results demonstrated the significant protective effects of phyllanthin against CCl₄–induced changes in LPO level by increasing non–enzymatic and enzymatic antioxidative defense parameters in liver of mice. LPO degradation of cell membranes is considered as one of the principal causes of hepatotoxicity mediated by CCl₄[34]. The free radicals produced as a consequence of CCl₄ activation triggers LPO which causes alterations in structure and function of liver cell membrane[35]. Near normal levels of hepatic MDA, a frequently used indicator of oxidative damage was maintained in the phyllanthin treated mice. Similarly, the levels of enzymatic and non–enzymatic parameters were elevated after treatment with the liver protecting molecule. This establishes the strong antioxidative potential of phyllanthin.

The histopathological observations in the present study suggested fatty degenerative changes, necrosis, vacuolization and feathery regeneration of hepatocytes in CCl₄-intoxicated animals. Many earlier reports have confirmed the disruption of lattice nature of hepatocytes, degenerated nuclei, ballooning of liver cells, disintegrated central vein and necrotic changes in CCl₄–treated animals[36,37]. Attia and Ali have also reported similar histopathological changes, accompanied by fatty changes, hepatocellular ballooning, foamy appearance of cytoplasm with occasional hypertrophy in some nuclei and massive mononuclear inflammatory cells infiltration in the liver tissue of rats receiving CCl₄ orally for 4 weeks[38]. However, phyllanthin treatment was found to reverse all histopathological aberration caused by treatment with the toxin alone, which strongly supported our biochemical findings and confirmed the liver protecting role of phyllanthin as against CCl₄ toxicity.

Earlier electron microscopic studies on CCl₄ intoxicated animals had reported the presence of irregularly shaped mitochondria, with internal and external membrane breakage resulting in increased permeability and leakage of marker enzymes from the cytoplasm and increased vacuolization[39]. Likewise, small and large cytoplasmic vacuoles were also noted by Kurstak et al. after CCl₄ treatment[40].

In the present study, ultrastructural studies using transmission electron microscope was performed to evaluate the hepatoprotective potency of phyllanthin. Transmission electron micrographs of liver treated with CCl₄ revealed several changes in cell organelles. The cytoplasm in the micrographs of the CCl₄–treated groups was cloudy, unlike that in the vehicle control group. Our results revealed that the ultramicroscopic features of the CCl₄ and phyllanthin treated mice demonstrated no significant ultrastructural differences from those of the vehicle control. Their subcellular features were similar to those present in the normal mouse liver.
In conclusion, phyllanthin provided significant protection against CCl4–induced hepatic damage by enhancing the antioxidative defense mechanisms. Phyllanthin–mediated inhibition of hepatic damage caused by CCl4 treatment could form the basis of the indigenous medicinal use of the plant P. amarus which has been used as folklore against liver diseases in many parts of the world. The study had thus identified phyllanthin as a lead molecule for liver protection with enormous antioxidative potential.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

The liver is the principal site for CCl4–induced effects to manifest themselves. Many pathological changes following CCl4 intoxication have been identified and studied at the biochemical, histopathological and ultrastructural levels. Conventional medical therapies for many common liver disorders are at times show limited efficacy and may cause potentially life–threatening side effects. This has led to increased dependence on complementary and alternative medicines. The prime focus is on plant–derived active compounds as hepatoprotectives.

Research frontiers

The study reports the hepatoprotective property of phyllanthin, a lignin from P. amarus against well–established hepatotoxin CCl4. Many studies have been performed with the crude plant extract. Few in vitro studies have been performed with phyllanthin. The present study has evaluated the liver protecting property of this lignin in vivo and this is the first such elaborate report on this lignin elaborating its anti–hepatotoxic potential.

Related reports

This study is almost unique in the way that it reports the hepatoprotective property of phyllanthin in detail. The authors have studied histopathological and ultramicroscopic analysis of the condition for the first time and it is clearly evident from the results that the biochemical parameters corroborated well with the histopathological and ultramicroscopic finding.

Innovations & breakthroughs

This is the first complete in vivo report on the liver protecting property of phyllanthin which may be responsible for the hepatoprotective efficacy of the plant.

Applications

The study had identified phyllanthin as a hepatoprotective molecule with high potential. Molecular studied can be carried out further to assess the exact cause for such a property. After carrying out further studies in higher animals, clinical studies can be carried out which may benefit mankind if successful.

Peer review

This is a good paper describing the hepatoprotective property of phyllanthin. The authors have performed appreciable work. The authors can further extend their study to analyze the results on molecular level which will shed more light on the exact nature of protection conferred by this isolated molecule.

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