Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Disease

journal homepage: www.elsevier.com/locate/apjtd

Document heading doi: 10.1016/S2222-1808(14)60416-3

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

Rajesh Krithika, Ramtej Jayaram Verma*

Department of Zoology, University School of Sciences, Gujarat University, Ahmedabad-380 009, India

PEER REVIEW

Peer reviewer

Dr. Komal Shah, Research Associate, Indian Council of Medical Research (ICMR), Department of Zoology, University School of Sciences, Gujarat University, Ahmedabad-380009, India.
Tel: +91-79-2630 2362
Fax: +91-79-26308545
E-mail: komal_12885@yahoo.com

Comments

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.
Details on Page S69

ABSTRACT

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 CCl_4 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 CCl_4 caused significant increase in lipid peroxidation. The hepatic levels of both non-enzymatic antioxidants and enzymatic antioxidants were significantly lowered in CCl_4-treated mice as compared to control. Treatment with phyllanthin significantly mitigated these changes in the CCl_4-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 (CCl_4) to induce experimental oxidative stress and liver damage is quite common[1]. It is also well known that CCl_4 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

*Corresponding author: Prof. Ramtej J. Verma, Department of Zoology, School of Sciences, Gujarat University, Navrangpura, Ahmedabad-380 009, India.
Tel: +91-79-2630 2362
Fax: +91-79-26308545
E-mail: ramtejverma@gmail.com

Article history:
Received 15 Dec 2013
Received in revised form 29 Dec, 2nd revised form 2 Jan, 3rd revised form 9 Jan 2014
Accepted 23 Jan 2014
Available online 28 Jan 2014

disease[3-5]. The aerial parts of *Phyllanthus amarus* (*P. amarus*) has been widely used in folklore in India and other tropical countries for the treatment of various diseases such as jaundice, diarrhea, kidney ailments, malaria, genitourinary infections[6]. *P. amarus* is one of the extensively studied and reported plants for its hepatoprotective property against various toxins[7]. The crude extract of *P. amarus* has been found to attenuate paracetamol[8], ethanol[9], aflatoxin B1[10] and galactosamine-induced hepatotoxicity[11].

Many high performance thin layer chromatography and high performance liquid chromatography (HPLC) methods have been standardized for the quantification of phyllanthin and hypophyllanthin[12,13], 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 CCl_4 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). CCl_4 (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 CCl_4 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 CCl_4 (826 mg /kg body weight)[19] and Group IV mice received CCl_4 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% OsO_4 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 CCl_4-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 CCl_4-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 CCl_4 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 CCl_4-induced changes in the hepatic tissue.

Table 1

Effect of phyllanthin on CCl_4-induced changes in liver non-enzymatic antioxidant levels and LPO.

Experimental groups	LPO[1]	GSH[2]	TAA[3]
Vehicle control	1.70±0.20	40.38±2.20	5.16±1.02
Phyllanthin	1.73±0.56	40.96±2.43	5.19±0.87
CCl_4	4.53±0.99[a*]	18.76±1.21[a*]	4.12±0.87[a*]
CCl_4+Phyllanthin	1.75±0.58[b*]	42.39±2.08[b*]	5.09±0.82[b*]

Results are expressed as mean±SD, n=10, a as compared between vehicle control (Group I) and CCl_4-treated (Group III), b as compared between CCl_4-treated (Group III) and CCl_4+phyllanthin-treated (Group IV). *$P<0.05$.
LPO[1]: nmol MDA/mg protein/60 min, GSH[2]: μg/100 mg tissue weight, TAA[3]: mg/g tissue weight.

Table 2

Effect of phyllanthin on CCl_4-induced changes in liver enzymatic antioxidants.

Experimental groups	CAT[1]	SOD[2]	GPx[3]	GR[4]	GST[5]
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±1.20	2.38±0.78	2.56±0.59	2.52±0.98
CCl_4	3.78±1.09[a*]	1.09±0.11[a*]	0.92±0.02[a*]	0.98±0.12[a*]	1.56±0.09[a*]
CCl_4+phyllanthin	10.39±2.33[b*]	3.61±0.67[b*]	2.33±0.61[b*]	2.37±0.55[b*]	2.20±0.98[b*]

Results are expressed as mean±SD, n=10, a as compared between vehicle control (Group I) and CCl_4-treated (Group III), b as compared between CCl_4-treated (Group III) and CCl_4+phyllanthin-treated (Group IV). *$P<0.05$.
CAT[1]: μmol H_2O_2 consumed/mg protein/min, SOD[2]: units/mg protein, GPx[3]: nmol NADPH consumed/mg protein/min, GR[4]: nmol NADPH consumed/mg protein/min, GST[5]: μmol of CDNB conjugate formed/mg protein/min.

3.2. Effect of phyllanthin on histological change in the liver

Light micrograph 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 CCl_4 resulted in extensive centrilobular (zone 3) necrosis and inflammatory cell infiltration in the sinusoidal region. Excessive

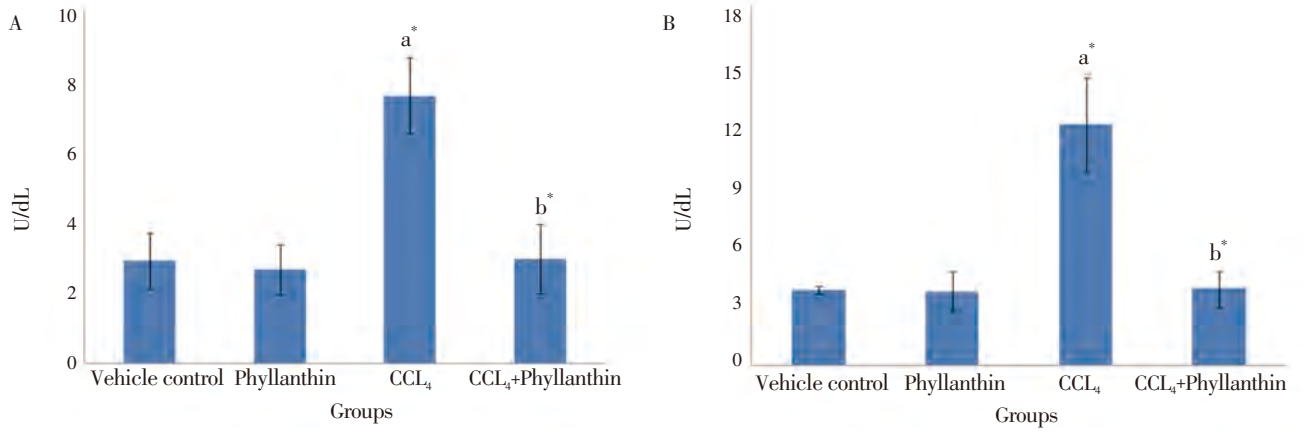

Figure 1. Protective effect of phyllanthin on CCl_4-induced changes in serum marker enzymes for liver function.
A: ALT, B: AST. Results are expressed as mean±SD, n=10, a as compared between vehicle control (Group I) and CCl_4-treated (Group III), b as compared between CCl_4-treated (Group III) and CCl_4+phyllanthin-treated (Group IV). *$P<0.05$.

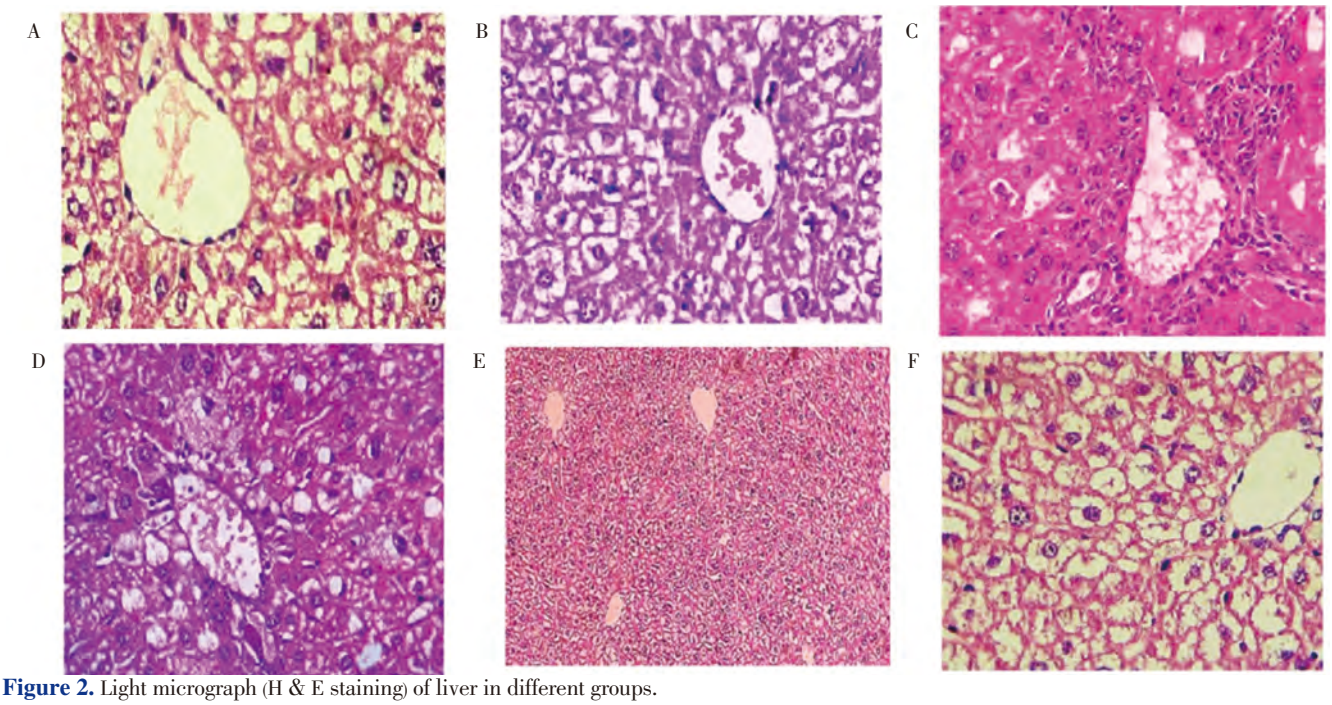

Figure 2. Light micrograph (H & E staining) of liver in different groups.
A: vehicle control mice (400×), B: phyllanthin-treated mice showing normal architecture and compactness with clearly defined central vein (400×), C: CCl_4-treated mice – extensive centrilobular necrosis and excessive accumulation of connective tissue and feathery regeneration of nucleus are observed (400×), D: CCl_4 treated mice – excessive accumulation of connective tissue formation and centrilobular necrosis observed (400×), E: phyllanthin and CCl_4-treated mice showing normal architecture (100×), F: phyllanthin plus CCl_4-treated mice showing normal arrangement of hepatocytes, with clearly visible nucleus, sinusoids and central vein. Cytoplasmic vacuolization and necrosis are absent (400×).

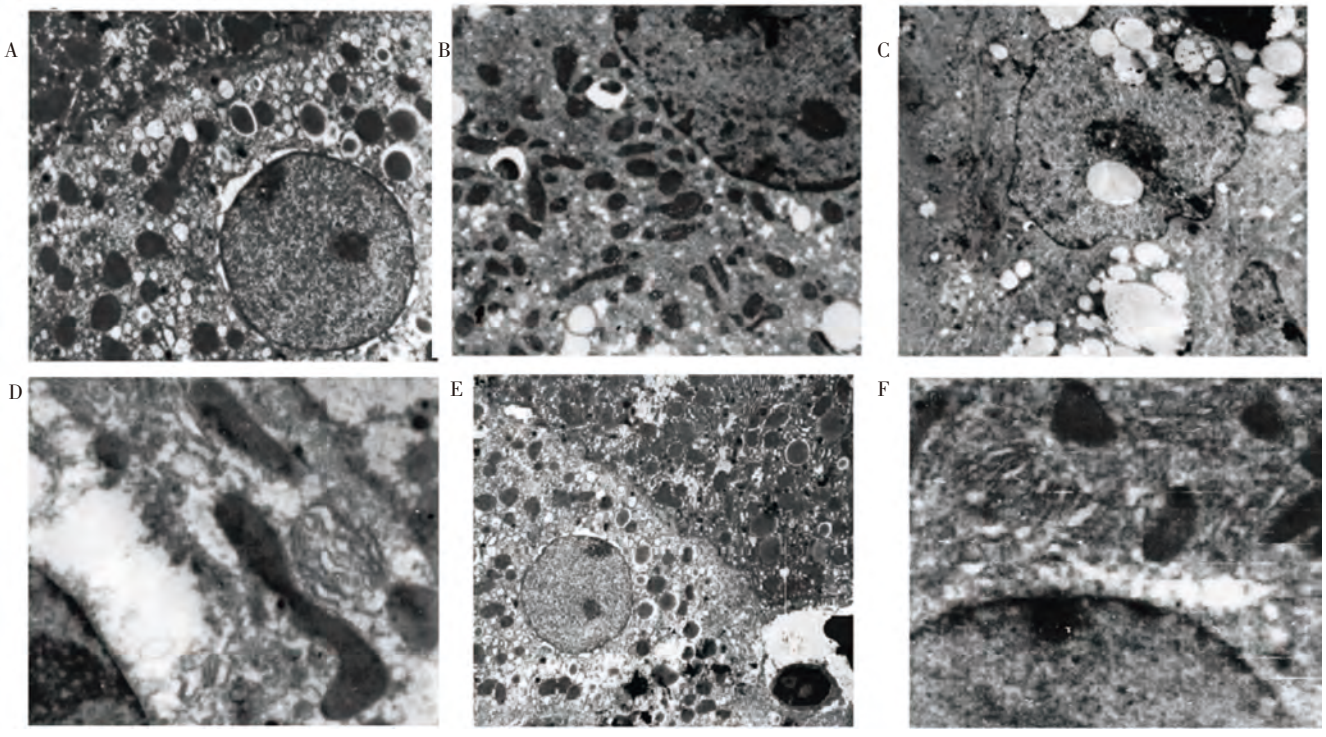

Figure 3. T. E. micrograph of liver in different groups.
A: vehicle control mice – the liver shows normal architecture (3 000×), B: phyllanthin-treated mice – the liver resembles vehicle control mice with normal nucleus and mitochondria (3 000×), C: liver treated with CCl_4 – excessive cytoplasmic vacuolization is observed which indicates an empty appearance in the cell (3 000×), D: CCl_4 treated mice liver – mitochondria and rough endoplasmic reticulum appears disorganized (4 400×), E: phyllanthin-treated mice along with CCl_4 – the liver shows total recovery (3 000×), F: phyllanthin plus CCl_4-treated mice – mitochondria and rough endoplasmic reticulum appear normal (4 400×).

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 CCl_4-treated animals (Figure 2C and 2D). Results revealed complete restoration of the normal architecture and arrangement of hepatocytes in mice administered with CCl_4 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 CCl_4 (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 CCl_4 (Figure 3E) revealed complete recovery from the CCl_4-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 CCl_4 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 CCl_4-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 CCl_4-treated mice indicated leakage due to membrane damage[33], and the reduction observed after phyllanthin treatment suggests recovery towards normalization due to the recoupment of the cell membrane.

In the present study, oral administration of CCl_4, 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 CCl_4 suggested oxidative stress. Our results demonstrated the significant protective effects of phyllanthin against CCl_4-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 CCl_4[34]. The free radicals produced as a consequence of CCl_4 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 CCl_4-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 CCl_4-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 CCl_4 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 CCl_4 toxicity.

Earlier electron microscopic studies on CCl_4 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 CCl_4 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 CCl_4 revealed several changes in cell organelles. The cytoplasm in the micrographs of the CCl_4-treated groups was cloudy, unlike that in the vehicle control group. Our results revealed that the ultramicroscopic features of the CCl_4 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 CCl_4-induced hepatic damage by enhancing the antioxidative defense mechanisms. Phyllanthin-mediated inhibition of hepatic damage caused by CCl_4 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.

Acknowledgements

The fellowship assistance to Dr. Krithika Rajesh (Research Associate, File No: 45/37/BMS-TRM)) from Indian Council of Medical Research, New Delhi, is acknowledged with thanks. The authors gratefully acknowledge the assistance of Ms. Bindu Kakkar, Technical Assistant, Department of Zoology, Gujarat University in transmission Electron microscopy studies. Authors are thankful to Mrs. Aishwariya Kumaran, for her valuable support in literature collection.

Comments

Background

The liver is the principal site for CCl_4-induced effects to manifest themselves. May pathological changes following CCl_4 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 CCl_4. 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 histopatological 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.

References

[1] Khan M, Younus T. Prevention of CCl_4-induced oxidative damage in adrenal gland by *Digera muricata* extract in rat. *Pak J Pharm Sci* 2011; **24**: 469-473.

[2] Boll M, Weber LW, Becker E, Stampfl A. Mechanism of carbon tetrachloride-induced hepatotoxicity. Hepatocellular damage by reactive carbon tetrachloride metabolites. *Z Naturforsch C* 2001; **56**: 649-659.

[3] Chatterjee M, Sil PC. Hepatoprotective effect of aqueous extract of *Phyllanthus niruri* on nimesulide-induced oxidative stress *in vivo*. *Indian J Biochem Biophys* 2006; **43**: 299-305.

[4] Das BK, Bepary S, Datta BK, Chowdhury AA, Ali MS, Rouf AS. Hepatoprotective activity of *Phyllanthus reticulatus*. *Pak J Pharm Sci* 2008; **21**: 333-337.

[5] Prakash A, Satyan KS, Wahi SP, Singh RP. Comparative hepatoprotective activity of three *Phyllanthus* species, *P. urinaria, P. niruri* and *P. simplex*, on carbon tetrachloride induced liver injury in the rat. *Phytother Res* 1995; **9**: 594-596.

[6] Unander DW, Webster GL, Blumberg BS. Uses and bioassays in *Phyllanthus* (Euphorbiaceae): a compilation II. The subgenus *Phyllanthus*. *J Ethnopharmacol* 1991; **34**: 97-133.

[7] Calixto JB, Santos AR, Filho VC, Yunes RA. A review of the plants of the genus *Phyllanthus*: their chemistry, pharmacology, and therapeutic potential. *Med Res Rev* 1998; **18**: 225-258.

[8] Wongnawa M, Thaina P, Bumrungwong N, Nitiruangjarat A, Muso A, Prasartthong V. Effect of *Phyllanthus amarus* Schum & Thonn.

and its protective mechanism on paracetamol hepatotoxicity in rats. *Acta Hort* 2005; **680**: 195-201.

[9] Pramyothin P, Ngamtin C, Poungshompoo S, Chaichantipyuth C. Hepatoprotective activity of *Phyllanthus amarus* Schum. et. Thonn. extract in ethanol-treated rats: *in vitro* and *in vivo* studies. *J Ethnopharmacol* 2007; **114**: 169-173.

[10] Naaz F, Javed S, Abdin MZ. Hepatoprotective effect of ethanolic extract of *Phyllanthus amarus* Schum. et Thonn. on aflatoxin B1-induced liver damage in mice. *J Ethnopharmacol* 2007; **113**: 503-509.

[11] Kongstan N. Effect of *Phyllanthus amarus* on hepatotoxicity induced by galactosamine in rats [dissertation]. Bangkok: Mahidol University; 2000.

[12] Tripathi AK, Verma RK, Gupta AK, Gupta MM, Khanuja SP. Quantitative determination of phyllanthin and hypophyllanthin in *Phyllanthus* species by high-performance thin layer chromatography. *Phytochem Anal* 2006; **17**: 394-397.

[13] Sharma A, Singh RT, Handa SS. Estimation of phyllanthin and hypophyllanthin by high performance liquid chromatography in *Phyllanthus amarus*. *Phytochem Anal* 1993; **4**: 226-229.

[14] Chirdchupunseree H, Pramyothin P. Protective activity of phyllanthin in ethanol-treated primary culture of rat hepatocytes. *J Ethnopharmacol* 2010; **128**: 172-176.

[15] Krithika R, Mohankumar R, Verma RJ, Shrivastav P, Illiyas ML, Gunasekaran P, et al. Isolation, characterization and antioxidative effect of phyllanthin against CCl_4-induced toxicity in HepG2 cell line. *Chem Biol Interact* 2009; **181**: 351-358.

[16] Syamasundar KV, Singh B, Thakur RS, Hussain A, Kiso Y, Hikino H. Antihepatoprotective principles of *Phyllanthus niruri* herbs. *J Ethnopharmacol* 1985; **14**: 41-44.

[17] Lam PL, Gambari R, Yip J, Yuen MC, Lam KH, Wong RS, et al. Development of phyllanthin containing microcapsules and their improved biological activity towards skin cells and *Staphylococcus aureus*. *Bioorg Med Chem Lett* 2012; **22**: 468-471.

[18] Venkatesan P, Satyan KS, Kumar MS, Prakash A. Protective effect of aqueous extract of *Phyllanthus amarus* Linn., phyllanthin and nirocil against carbon tetrachloride-induced liver and brain toxicity. *Indian J Pharm Sci* 2003; **65**: 309-312.

[19] Krithika R, Verma RJ, Shrivastav PS, Suguna L. Phyllanthin of standardized *Phyllanthus amarus* extract attenuates liver oxidative stress and exerts cytoprotective activity on human hepatoma cell line. *J Clin Exp Hepatol* 2011; **1**: 57-67.

[20] Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; **95**: 351-358.

[21] Grunert RR, Philips PH. A modification of the nitroprusside method of analysis for glutathione. *Arch Biochem* 1951; **30**: 217-225.

[22] Roe JH, Kuether CA. The determination of ascorbic acid in whole blood and urine through the 2, 4-dinitrophenylhydrazine derivative of dehydroascorbic acid. *J Biol Chem* 1943; **147**: 399-407.

[23] Luck HA. Spectrophotometric method for the estimation of catalase. In: Bergmeyer HU, editor. *Methods of enzymatic analysis*. New York: Academic Press; 1963, p. 886-887.

[24] Kakkar P, Das B, Viswanathan PN. A modified spectrophotometric assay of superoxide dismutase. *Indian J Biochem Biophys* 1984; **21**: 130-132.

[25] Pagila DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte peroxidase. *J Lab Clin Med* 1967; **70**: 158-169.

[26] Mavis RD, Stellwagen E. Purification and subunit structure of glutathione reductase from bakers' yeast. *J Biol Chem* 1968; **243**: 809-814.

[27] Habig WH, Pabs MJ, Jakoby WB. Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. *J Biol Chem* 1974; **249**: 7130-7139.

[28] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with folin-phenol reagent. *J Biol Chem* 1951; **193**: 265-275.

[29] Somanabandhu A, Nitayangkura S, Mahidol C, Ruchirawat S, Likhitwitayawuid K, Shieh HL, et al. 1H and 13C-NMR assignments of phyllanthin and hypophyllanthin: lignans that enhance cytotoxic responses with cultured multi-drug resistant cells. *J Nat Prod* 1993; **56**: 233-239.

[30] Foo LY. Amarin, a di-dehydro hexahydroxy diphenoyl hydrolysable tannin from *Phyllanthus amarus*. *Phytochemistry* 1993; **33**: 487-491.

[31] Foo LY. Amarinic acid and related ellagitannins from *Phyllanthus amarus*. *Phytochemistrym* 1995; **39**: 217-224.

[32] Yadav NP, Pal A, Shanker K, Bawankule DU, Gupta AK, Darokar MP, et al. Synergistic effect of silymarin and standardized extract of *Phyllanthus amarus* against CCl_4-induced hepatotoxicity in *Rattus norvegicus*. *Phytomedicine* 2008; **15**: 1053-1061.

[33] Ahn TH, Yang YS, Lee JC, Moon CJ, Kin SH, Jum W, et al. Ameliorative effects of pycnogenol on carbon tetrachloride-induced hepatic oxidative damage in rats. *Phytother Res* 2007; **21**: 1015-1019.

[34] Weber LW, Boll M, Stampfl A. Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. *Crit Rev Toxicol* 2003; **33**: 105-136.

[35] Gupta AK, Chitme H, Dass SK, Misra N. Antioxidant activity of *Chamomile recutita* capitula methanolic extracts against CCl_4-induced liver injury in rats. *J Pharmacol Toxicol* 2006; **1**: 101-107.

[36] Dhanasekaran M, Ignacimuthu S, Agastian P. Potential hepatoprotective activity of ononitol monohydrate isolated from *Cassia tora* L. on carbon tetrachloride-induced hepatotoxicity in wistar rats. *Phytomedicine* 2009; **16**: 891-895.

[37] Sharma N, Shukla S. Hepatoprotective potential of aqueous extract of *Butea monosperma* against CCl_4 induced damage in rats. *Exp Toxicol Pathol* 2011; **63**: 671-676.

[38] Attia MN, Ali MA. Hepatoprotective activity of allicin against carbon tetrachloride induced hepatic injury in rats. *J Biol Sci* 2006; **6**: 457-468.

[39] Bassi M. Electron microscopy of rat liver after carbon tetrachloride poisoning. *Exp Cell Res* 1960; **20**: 313-323.

[40] Kurstak C, Cote MG, Plaa GL. Effect of hypothermia on carbon tetrachloride-induced changes in hepatic ultrastructure. *Toxicol Appl Pharmacol* 1973; **26**: 14-28.