HEPATOPROTECTIVE ACTIVITY OF PURIFIED ANTHOCYANIN FROM TEAK LEAVES AGAINST CCL4-INDUCED HEPATOTOXICITY IN WISTAR ALBINO RATS AND ITS ANALYSIS BY LC-MS/MS

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ARTICLE INFO	ABSTRACT
Key Words Teak, purified anthocyanin, *In vitro* callus culture, hepatoprotection, antioxidant, biochemical parameters.	Teak a tropical woody plant cultivated for its wood. The present study was carried to evaluate the hepatoprotective potential of the purified anthocyanin from teak leaves elucidated from the *in vitro* callus culture and its fractionation by LC-MS/MS. Hepatoprotectiveefficacy of purified anthocyanin was tested at the doses of 200 and 400 mg/kg, per (p.o.), against CCl₄ induced toxicity on Wistar albino rats. The purified anthocyanin and silymarin treated animal groups showed significant recoupment in the activities of different biochemical parameters like serum aspartate, alanine aminotransferase, alkaline phosphatase and lactic dehydrogenase, which were elevated by the toxicant carbon tetrachloride (CCl₄). The levels of total bilirubin and total protein along with the liver weight were also restored to normal by the administration of purified anthocyanin and silymarin. Similarly, the decreased level of succinic dehydrogenase in CCl₄ treated rats was regained by anthocyanin treatment. Administration of CCl₄resulted in the reduction of hepatic antioxidants such as glutathione, superoxide dismutase and catalase with concomitant increase in hepatic lipid peroxidation level. The expression of hepatic antioxidants was restored to normal by the purified anthocyanin and silymarin administrated rats. Kidney functional test in terms of urea, uric acid and creatinine levels were regained to normalcy by anthocyanin. Histological data of liver substantiate the biochemical results. The purified anthocyanin was fractionated by LC-MS/MS revealed the presence of 5major anthocyanidins such as peonidin, petunidin, malvidin, cyanidin and pelargonidin. The purified anthocyanin was found to be effective in mitigating CCl₄-induced hepatotoxicity in rats.

INTRODUCTION:

Liver, a pivotal organ found in vertebrates with multiple functions such as detoxification of various metabolites, protein synthesis and enhance biochemical efficacy for digestion. It is an accessory digestive gland responsible for the secretion of bile, which helps in emulsification of fat[1]. Hence, liver is prone to various disorders because of its strategic location and multidimensional functions. Hepatic disease indicates damage to the cells architecture or functions of liver. This derailment may be caused by biological

factors or autoimmune diseases or different types of synthetic drugs such as paracetamol, carbon tetrachloride, thioacetamide, dimethylnitrosamine and even excessive consumption of alcohol [2]. Among these chemicals, carbon tetrachloride (CCl4) is a classic liver toxicant that have an easy access to the digestive and respiratory tract and converted into trichloromethyl radical (CCl3.) through cytochrome oxidase system [3].Hence, it is necessary to find an alternative natural molecule for the treatment of liver prone diseases, which should be effective and less toxic. Many studies correlated ROSs with liver disorders, especially the synthetic toxicants like alcohol and drugs[4]. Natural antioxidants such as green tea have been reported to elevate the antioxidant compounds or enzyme level in rat's liver cytoplasm which consequently minimizes the injury rate to normal[5]. Similarly, many secondary metabolites such as polyphenols, flavonoids and anthocyanins proved to be potent antioxidants that contribute to protect the liver cells from the injuries induced by drugs. Teak (*Tectonagrandis*), a hardwood tropical timber yielding tree species placed in the family Lamiaceae. Interestingly, most of the tribes used the red coloured extract of young teak leaves, bark and fruits for curing many ailments and used for the preparation of diversetherapeutic formulations. Previously, GreeshmaMurukan and Murugan K (2017) [6] reported that the young tender leaf of teak is a rich source of anthocyanin with potent antioxidant efficacy. The leaves of teak showed many other pharmacological potentialities like antibacterial, anticancerous and antioxidant properties[7]. In this scenario, the present study focused on*in vitro* callus culture, elucidation of anthocyanin, purification, fractionation by LC-MS/MS from teak leaves and its*in vivo* hepatoprotective

properties against carbon tetra-chloride induced hepatotoxicity in Wistar albino rats.

MATERIALS AND METHODS

Plant material: The healthy fresh leaves of*Tectonagrandis* were collected from wild teak plantations of Nilambur hills, Kerala, which is a place lying between $11^0 16'$ N and $76^0 13'$E.

Callus induction: For the initial establishment of callus culture,leaves were used as the source of explant. The explantwas surface sterilized using 0.01% mercuric chloride for 3 min and washed thoroughly with double distilled water for callus induction. The explants were then inoculated on the MS media supplemented with various concentrations and combinations of 2,4-dichlorophenoxy acetic acid (2,4-D), naphthalene acetic Acid (NAA), Kinetin and 6-benzylaminopurine (BAP) alone or in combinations for callus induction.. The *in vitro* cultureswere maintained in the culture room at 22± 4ºC with a 16h photoperiod. Each vial contained a single explant.

Sub-culturing: Observations were made till one month of inoculation. After successful callus initiation 3 calliof 5-mm size was taken from for subculturing on 20 ml MS media with the same successful combination of phytohormones[8]. For each media there were five replicates. Observations were recorded every alternate day.

Anthocyanin induction: The different hormones play an important role in anthocyanin production. In this study different hormonal combinations were used for elicitation of maximum anthocyanin production.

Determination of anthocyanin content in fresh callus: Fresh calli (0.5 g) was mixed with five milliliters of methanol containing

1% concentrated HCl at 4°C. Subsequently, the samples were centrifuged at 15000 g for 20 min at 4°C. Absorbance of the clear supernatant was measured at 528 nm. Anthocyanin content was calculated according to the method described by Mori *et al.*, (1993)[9]. Total anthocyanincontent was expressed as µg g^{-1} fresh weight of callus.

Purification of anthocyanin: The crude anthocyanin extract contains other molecules such as chlorophylls, stilbenoids, less polar flavonoids and other non polar compoundswas removed by liquid-liquid partition using ethyl acetate/chloroform combination. The extract was further purified by an adsorption chromatography contain Amberlite XAD column with acidified ethanol as the mobile solvent[10].

LC-MS/MS analysis: The column eluted fraction with highest anthocynin content was used for the LC- MS/MS analysis. The experiment was performed on a Thermo Scientific DionexUltiMate® 3000 RSLC system with chromatographic separation achieved on a Thermo Scientific Acclaim® RSLC 120 C18 reversed-phase column (2.1 × 100 mm, 2.2 µm) operated at 40 °C with gradient elution at 0.5 mL/min. The mobile phase consisted of three components: A) acetonitrile, B) deionized (DI) water, and C) 20% formic acid. Mobile phase C was held constant at 10% to provide 2% total formic acid in the mobile phase throughout the run. Mobile phase A was ramped from 0% to 8% from 11 to 42 min, then held for 13 min before returning to the initial composition from 55 to 60 min. Electrospray ionization (ESI) was used as the interface and was operated in positive selected ion monitoring (SIM) mode. The probe temperature was set at 500 °C and needle voltage was set at 2000 V. The cone voltage was set at 50 V for all SIM scans with a span of 0.3 amu for each SIM.

Hepato-protective analysis

Acute oral Toxicity test: The Acute oral toxicity test was studied using the protocol described by the OECD guideline 408 for testing chemicals [11, 12]. The experiment was designed in such a way that the animal model, Wistar albino rats were randomly distributed into 5 groups: a control group and drug 5 g/kg treatment groups. Anthocyanin extract purified from the callus culture were dissolved in 10% Tween 20 and administered orally on daily basis for two weeks at single doses of 5 g/kg, meanwhile the control group were treated with only 10% Tween 20 in distilled water. During the test period, the rats were weighed and visual observations for mortality, behavioral pattern such as salivation, fur, lethargy, and sleep, physical appearance changes, injury, pain and signs of illness were recorded. Wistar albino rats were divided into five groups (n = 6). Group I (normal control) comprises of rats given with a single dose of water (25 mL/kg, p.o.) for 7 days daily and a single dose intraperitoneal (i.p.) injection was given on 7th day with 8 mL/kg olive oil.Group II (negative control) animals were given 0.2% CCl_4 in olive oil (8 mL/kg, i.p.) on 7th day intraperitoneally and were also treated with a single dose of water (25 mL/kg, p.o.) for 7 days. Group III & IV animals were administered withpurified anthocyanin at doses of 200 and 400 mg/kg, p.o., dissolved in 2% gum acacia for 7 days respectivelywhereas for animals in groups V (positive control) were administered with silymarin, the standard drug (50 mg/kg, p.o.) for 7 days.Parallelly, on 7th day after 1 h of administration of silymarin and purified anthocyanin animals in groups III to V were treated with 0.2% CCl_4 in olive oil (8 mL/kg, i.p.). By retro-orbital puncture blood samples from all the experimental animal groups were collected after 24 h treatment. Subsequently the animals were sacrificed. Further, the blood was allowed to clot and

was centrifuged at 3500 rpm for 15 min at 4 °C to separate the serum which was used for biochemical marker enzyme assays.Moreover, the liver tissue samples were taken from the left liver lobe and cut into two pieces. One piece was used for pathological examination and was fixed in 10% formalin solution for 24 h meanwhile the other piece was used for the assessment of lipid peroxidation and other parameters.

Biochemical parameters: Biochemical parameters like serum aspartate (AST) and alanine aminotransferase and (ALT), alkaline phosphatase (ALP) activity, total bilirubin and total protein were determined with the serum separated by centrifugation using commercially available kits (Span Diagnostics Ltd., Surat, India).Liver weight was also evaluated by weighing the weight of liver with respect to body weight per 100 g [13].

Hematological parameters: At the end of the experimental period blood was drawn through cardiac puncture and was collected in EDTA (anticoagulant) coated test tubes that prevent coagulation. Total white blood cells counts, hemoglobin, platelets count, Red blood cells count and Hematocrit (HCT/PCV), MCHC, MCH, and MCV were determined by using automatic analyzer (using Beckman Coulter HMX analyzer, USA) [14]. Urea, creatinine, cholesterol, triglycerides, uric acid and blood glucose was determined for analyzing the kidney function by using automatic analyzer at 37ºC by using standard reagent kits (Merck Germany) [15].

Assay for hepatic antioxidant activities

Lipid peroxidation (LPX): From each experimental animal 900 mg of liver tissue was taken and then washed with normal saline and soaked in filter paper.The tissue was homogenized using 3.0 mL of 0.15 MmTris-HCl buffer (pH 7.4) and then

centrifuged for 1 h at 3000 rpm at 4 °C. The resultant supernatant was used for the estimation of LPX. The level of malondialdehyde (MDA) produced primarily was used for determining the LPX content, which was expressed as nmol/g liver tissue homogenate [16].

Glutathione (GSH) content: Liver homogenate was prepared with 0.15 M Tris-HCl buffer of pH 7.4 and by adding trichloroacetic acid proteins were precipitated. The samples were centrifuged at 15000 rpm at 4 °C for 1 h. The resultant supernatant was used for the analysis of GSH content and was expressed in U/g liver tissue homogenate [17].

Catalase (CAT) activity: Catalase enzyme was isolated and assayed as per the method of Mahmoud and Hussein, (2015)[18].One unit (U) of CAT activity was defined as the amount of extract needed to decompose 1 µmol of H_2O_2 per min.

Superoxide dismutase (SOD) assay: The activity of superoxide dismutase (SOD) was determined by the measuring the inhibition of nitrobluetetrazolium (NBT) by the enzyme [19].

Lactate dehydrogenase (LDH) and Succinate dehydrogenase (SDH) assay: The levels of LDH in samples of cell lysate were measured with a commercial LDH assay kit (CytoTox96@l; Promega Corporation, Madison, WI). The absorbance of the formazan is measured at 492 nm. Formazan concentrations are directly proportional to the concentration of LDH in the sample[20]. The activity of succinate dehydrogenase (SDH) was assayed according to the method of Slater and Bonner, (1952)[21]. The change in OD was recorded at 15 sec interval for 5 min at 420 nm. The succinate dehydrogenase activity was expressed as U/L.

Histological observation: The liver tissue was fixed using formaldehyde fixative and it was further dehydrated using alcohol and embedded in paraffin wax. The liver tissue were cut in a size ranging of 3–5 µm and stained by haematoxylin and eosin (HE). Subsequently, the sections were examined under photomicroscope and the morphological changes like necrosis of hepatic cell, ballooning degeneration, fatty changes or inflammation caused by cell infiltration and congestion were noticed[22].

Statistical analysis: The entire data was tabulated as the mean ±SD for nine parallel measurements using Windows Office package 2014. The analysis part includes Student's *t*-test, and $p < 0.05$ was considered as significant.

RESULTS AND DISCUSSION

***In vitro* callus culture:** The major hurdle accounted with teak culture was related with its high endogenous fungal contaminations. Effective surface sterilization was carried out by initial treatment with ethanol followed by 0.1% $HgCl_2$ solution for 5 min and washed repeatedly with sterilized water. These treated explants were further immersed in 3% sodium hypochlorite solution + 2 drops of Tween 20 + 0.05% fungicide for 20 min and then washed thrice with sterile deionized water. This protocol yielded 95% contamination free cultures with 90% survival cultures. Longer duration of sterilization treatments resulted browning and explant death.

The callus could be induced on MS media from young fresh leaves nearer to terminal buds as well as nodal explants (Figure 1a and 1b).Various kinds of phytohormones and their combinations resulted different levels of callus formation. Optimal callus induction was noticed with leaf when compared to nodal explants irrespective of media or hormones employed. This may be due to the fact that the immature leaves are highly meristematic.Maximum callus biomass was obtained on MS medium supplemented with BAP (1 mg/L) + NAA (0.2 mg/L) combination. In the present analysis, fast initiation of callus was recorded on 21st day using MS medium with leaf explants. The other hormonal treatments MS medium with 2 mg /L 2,4-D + 0.5 mg/L KIN and MS with NAA (2.5 mg/L) + BAP (0.5 mg/L) could induce callus with in a mean period of 28th and 30th days, respectively (Table1). As the second phase, sub culturing of calli was attempted with 2,4-D, KIN, BAP, NAA, coconut water either singly or in combinations. Interestingly, 2,4-D+KIN combination was not effective in callus induction but, the combination began to alter the colour from 30th day of inoculation from brownish to pink pigmentation (Figure 2 & Table 2).The results tempt to state that BAP + NAA produced optimal callus growth whereas 2,4-D (1 mg/L) +KIN (2 mg/L) yielded remarkable colouredanthocyanin content.

Purification and fractionation of anthocyanin by LCMS/MS analysis: Anthocyanin was extracted from the callus cultures using acidified methanol (1% HCl). Other molecules present in the crude anthocyanin extract such as chlorophyll, stilbenoids, flavonoids were removed by liquid-liquid partition method using ethyl acetate:choloform combination. The separated fraction was subjected to silica gel column chromatography using acidified methanol as the eluent. The fraction with rich amount of anthocyanin was pooled and further purified using Amberlite-XAD column, an adsorbent column with an adsorption capacity of 92.9%. It is also found that acidified ethanol above 50% (v/v) can effectively elute anthocyanin from the adsorbents.

LCMS/MS analysis was successful for fractionating the major anthocyanin components present in *Tectonagrandis*. The major anthocyanin fractions were eluted between 2.28 and 11.94 min time period. The anthocyanins identified were Peonidn-3-O-glucoside (463.1014), Pelargonidin-3-O-rutinoside (579.3155), Petunidin-3-O-arabinoside(449.0533), Malvidin-3,5-O-diglucoside(653.90), Cyanidin-3-O-glucosyl rutinoside(755.977), Pelargonidin-3-O-sophoroside (594.9994), Malvidin-3-O-6"aceyl galactoside (533.9968), 4'-O-methyl delphinidin 3-O-rutinoside (623.8522), Isopeonidin-3-O-glucoside(461.5581) and Cyanidin 3-O-(3",6"-O-dimalonyl glucoside)(621.4772) (Figure 3 & Table 2).

Acute oral toxicity studies: Acute oral toxicity analysis was important to evaluate the safety of herbal extracts and also to justify the tested concentrations. Purified anthocyanin from teak leaves was found to be safe to a concentration of 5 g/kg during the 21 days study period of single oral administration. No symptoms of mortality / morbidity or behavioral changes were noticed in any of the treated Wistar albino rats during the entire period of studies. Morphological features such as fur, skin, eyes and nose were normal. Similarly, no sign of tremors, diarrhoea, lethargy or abnormal characters like posture, reactivity to sensory stimuli were noticed. No remarkable variations in body mass between control and treated groups. No sound variations were observed in food and water intake among the experimental groups as compared to the control. Thus, the safety sign displayed by the purified anthocyanin from teak leaves is commendable for further biological evaluations.

Activity of serum marker enzymes (AST, ALT and ALP): As displayed in the table 4, CCl_4 intoxicated rats showed a profound increase in the activities of liver marker enzymes such as AST, ALT and ALP as compared to the normal control group. However, pre-administration of Wistar albino rats with purified anthocyanin from teak leaves at the doses of 200 and 400 mg/kg, p.o., or silymarin 50 mg/kg, p.o.,followed by CCl_4 (30% in liquid paraffin (1ml/kg b.w,i.p) remarkably recouped the levels of AST, ALT and ALP enzymes when compared to the CCl_4 intoxicated rats. The present results were in agreements with the animal study of data hydroethanolic stem bark extract from *Oroxylumindicum*, beetroot methanolic extract and *Andrographispaniculata* aqueous leaf extracts [23-25].

Total protein and albumin content: Contrarily, the total protein and albumin content were reduced drastically in the CCl_4 dispensed group, which was restored significantly to normal when the rats were delivered with purified anthocyanin at the concentrations of 200 and 400 mg/kg, p.o., or silymarin 50 mg/kg, p.o., (Fig.4).

Succinic dehydrogenase (SDH) and lactic dehydrogenase (LDH): Administration of CCl_4 resulted in to a significant decrease in the hepatic enzyme succinic dehydrogenase activity and an increased profile of LDH activity (Table 5). Purified anthocyanin (at the doses of 200 and 400 mg/kg, p.o.), or silymarin (50 mg/kg, p.o.,) treated rats showed significant enhancement in the SDH and also retained the LDH activity. The values were significant at 5% level ($P<$ 0.05). Ferreira et al., (2010) [26]proved the hepatoprotective potential of phloroacetophenone from *Myrciamultiflora* against CCl_4 induced toxicity.

Level of total bilirubin, glucose, cholesterol and triglycerides: Similarly, CCl_4 treated rats displayed an increased profile of total bilirubin (1.38 mg/dl), glucose (192.5 mg/dl), cholesterol (137.1mg/dl) and triglycerides (140.5

mg/dl) levels in the CCl_4 treated rats. Meanwhile, the rats treated with purified anthocyanin at the doses of 200 and 400 mg/kg, p.o., or silymarin 50 mg/kg, p.o., soundly regained the levels of the above parameters to normal i.e., more or less at par with that of control rat group (Table 6). Liver weight was also increased in CCl_4 intoxicated group $(5.77 \pm 0.13$ g) when compared to control rats $(4.3 \pm 0.09$ g). Meanwhile, the liver weight was restored its normal size when the rats were administered with purified anthocyanin at the doses of 200 and 400 mg/kg, p.o., or silymarin 50 mg/kg, p.o.

Activity of antioxidant enzymes (LPX, GSH, SOD and CAT): Dramatic increased LPX level (MDA) was noticed in CCl_4 intoxicated rats. Treatment of the rats with purified anthocyanin at the doses of 200 and 400 mg/kg, p.o., or silymarin (50 mg/kg, p.o.) remarkably ($P< 0.01$) reverted the level of LPX as compared to CCl_4 treated rats (Table 7). Similarly, the activities of serum and liver GSH, SOD and CAT enzymes were decreased in CCl_4 intoxicated rats as compared to control group. In fact, after the administration of rats with purified anthocyanin (200 and 400 mg/kg, p.o.) or silymarin (50 mg/kg, p.o.) remarkably elevated the activities of GSH, SOD and CAT when compared to CCl_4 treated rats (Table 7).

Urea, creatinine, and uric acid level: Fig 6 reveals the profound rise in the urea, creatinine, and uric acid levels after CCl_4 toxicity ($P \leq 0.05$). Purified anthocyanin at the doses of 200 and 400 mg/kg, p.o., or silymarin (50 mg/kg, p.o.) treated rats showed the regained level of all the above parameters. Analysis of variance showed significant recoupment at 5% level in the indices.

Hematological profile: Blood parameters enumerate the impact of the toxicant and its recovery by anthocyanin among the treated rats. The data reflects the mean values of parameters that were decreased in the rats treated with CCl_4, indicative of the toxic syndrome i.e., Hb (g/dl) was 13.9 ± 0.78 for control, 10.2 ± 0.04 for CCl_4 treated group, 12.49 ± 0.11 and 13.67 ± 0.91 for purified anthocyanin at doses of 200 and400 mg/kg, p.o., treated groups. RBC Count ($\times10^6$ mm^3) was $5,221\pm0.43$ for control, $3,230\pm0.18$, 4230 ± 0.41, 5189 ± 0.07 for CCl4, anthocyanin 200, 400 treated rats respectively. Hematocrit (HCT/PCV) % was 47.38 ± 0.20 for control, 32.9 ± 0.06 for CCl_4 group, 39.4 ± 0.04 and 42.14 ± 0.35 for anthocyanin 200 mg and 400 mg treated group. MCV (fl) $87.81+2.3$ for control, 99.4 ± 0.75 for CCl_4 treated group, 75.6 ± 0.48 for anthocyanin 200 mg treated group and 84.4 ± 0.95 for anthocyanin 400 mg treated group. MCH (pg) was 28.54 ± 0.35 for control, 32.6 ± 0.03 for CCl_4 treated rats, 24.6 ± 0.06 for anthocyanin 200 mg treated group and 27.5 ± 0.08 for anthocyanin 400 mg treated group. MCHC (g/l) was 32 ± 0.05 for control, 36.4 ± 0.038 for CCl_4 treated group, 30 ± 0.14 for anthocyanin 200 mg treated group and 32.82 ± 0.07 for anthocyanin 400 mg treated group. Total WBC Count (mm3) was 8456 ± 7.2 for control, 6234 ± 6.1 for CCl_4 treated group, 7862 ± 4.52 for anthocyanin 200 mg treated group and 8231 ± 7.5 for anthocyanin 400 mg treated group. Platelet Count ($\times10^9$/L) was 193.2 ± 0.988 for control, 70.4 ± 0.233 CCl_4 treated group,123.4 ± 0.676 for anthocyanin 200 mg treated group and 191.2 ± 5.5 for anthocyanin 400 mg treated group (Table 8).

Histo-pathological observations: In control group, no histopathological anomaly or changes were noticed in the liver (Fig. 6 a) In fact, CCl_4 administrated rats displayed severe hepatic irregularities in the liver like necrosis, ballooning, disintegration, fat

accumulation or infiltration of inflammatory cells (Fig.6 b). Administration with silymarin almost regained the functional perfection of the liver (Fig. 6 c). Interestingly, the rats treated with purified anthocyanin marginalized the hepatic anomaly caused by the toxicant remarkably and retrived the liver in a dose dependant manner (Figs. 6 d & e). CCl_4 is a hepatotoxin used generally for triggering liver damages in different experimental animals to elucidate the mode underlying hepatotoxicity. The mechanism of CCl_4 includes transformation of the toxicant by cytochrome P450 2E1 to trichloromethyl - • CCl_3 and which inturn converted in to a more reactive species (CCl_3O_2•) by oxygen. Trichloromethylperoxy free radical covalently conjugates to any biomolecules and leads to a series of reaction like oxidising polyunsaturated fatty acids in the membrane phospholipids, causing physiological and morphological anomalies to the membrane includingcell necrosis[27]. Hepatocytic damage is evaluated by hepatic indicator enzymes like AST, ALT and ALP, total bilirubin and total protein levels. During the damages of hepatic cells, these liver bound enzymes leach into the blood resulting into its elevated level in the serum. AST and ALT enzymes abode mostly in the parenchymal cells of liver. AST is dominant in the liver but low in heart, kidneys and muscles.Meanwhile, ALT is common in liver, cardiac tissues, muscles, brain, kidney and RBCs. So, AST is a specific marker of liver toxicity and its elevation results in to plausible damages in other organs [28]. Meanwhile, ALP and bilirubin profiles are related to functionality of hepatic cell. Increased level of serum ALP is due to its increased production as a consequence of biliary pressure. The present data showed a liver necrosis in the rats intoxicated with CCl_4 which was substantiated (i) by the profound activities of hepatic AST, ALT,

ALP enzymes and total bilirubin level (ii) decreased levelof total protein content. Interestingly, after the administration with purified anthocyanin from teak leaves the serum marker enzymes activities, total bilirubin and total protein were recalled to normal levels, thus suggesting the efficacy of the molecules to retain the morphological integrity of hepatocellular structure of liver. Similarly, the purified anthocyanin regained the impairment caused by the CCl_4 on hepatic antioxidant enzymes like LPX, SOD, SDH, LDH, GSH and CAT. The probable mode of action of anthocyanin involved in safe guarding the CCl_4-induced liver damage is via scavenging the excessive ROS synthesized in the cells.Greeshma and Murugan[29] reported the antioxidant potentiality of purified anthocyanin from in vitro callus culture of *Tectonagrandis*. MDA is commonly employed as lipid peroxidation marker and a parameter for judging the oxidative stress [30]. The enhanced MDA level under the oxidative stress in rats treated with CCl_4 as compared to control group reflects the toxicity felt in the cells. Treatment with purified anthocyanin remarkably reduced the induced hepatic MDA rise i.e., the purified anthocyanin from teak leaves protected CCl_4-induced liver damage via preventing lipid peroxide synthesis and inhibiting oxidative chain of reactions. The antioxidant defence system also includes molecules like GSH. The activities of antioxidants GSH and CAT were decreased drastically by CCl_4 treated rats. Here the present study, treatment with purified anthocyanin from teak leaves mitigated the impaired defence system in liver cells, as indicated by the increased enzymatic activities. The obtained results were further supported by histopathological data. Histopathological figures of CCl_4 administration reflects the severe liver damage in rats such as necrosis, ballooning degeneration, cell infiltration and other manifestations.

Table 1: Hormonal combinations and percentage of callus induction in teak using Murashie&Skoog (MS) medium and Woody Plant Medium (WPM).

Leaf as explant	Concentration (mg/l)	Callus induction (%)	Callus growth grade
Hormonal combinations			
MS medium	0	1.8	-
MS + 2,4-D	0.5	4	+
MS + 2,4-D	1	13	+
MS + 2,4-D	2	43.3	+
MS + 2,4-D	3	25	+
MS + 2,4-D	4	29	+
MS + 2,4-D	5	31	+
BAP+NAA	0.5+0.2	57	++
BAP+NAA	1+0.2	92.5	++++
BAP+NAA	0.5+1	50	++
BAP+NAA	0.5+2	60	+++
2,4-D+KIN	2+0.5	73	++++
2,4-D+KIN	1+1	60	+++
2,4-D+KIN	1+2	52	++
2,4-D+KIN	2+1	48	+
2,4-D+KIN	3+1	41	+
2,4-D+KIN	2+2	5	-
2,4-D+KIN	2+3	32	+
2,4-D+KIN	3+4	29	+
2,4-D+KIN	4+5	18	+
2,4-D+KIN	5+6	13.2	+
MS+CW(ml/l)	5	23	+
MS+CW(ml/l)	10	25	+
MS+CW(ml/l)	15	28	+
MS+CW(ml/l)	20	30	+
WPM	0	2.2	-
BAP+NAA	0.5+1	29.6	+
BAP+NAA	0.5+2	44.8	+
BAP+NAA	0.5+2.5	51.4	++
2,4-D+KIN	1+0.5	31	+
2,4-D+KIN	2+0.5	60.2	+++
2,4-D+KIN	4+0.5	16	+

Table 2: Sub-culturing of calli in MS media supplemented with different hormonal combinations and respective anthocyanin content in teak.

Leaf as explant	Concentration (mg/l)	Anthocyanin content (mg/g callus)
Hormonal combinations		
MS medium	0	0
MS + 2,4-D	0.5	0.06
MS + 2,4-D	1	0.09
MS + 2,4-D	2	0.12

MS + 2,4-D	3	0.19
MS + 2,4-D	4	0.23
MS + 2,4-D	5	0.24
BAP+NAA	0.5+0.2	0.27
BAP+NAA	1+0.2	6.84
BAP+NAA	0.5+1	3.3
BAP+NAA	0.5+2	4.2
2,4-D+KIN	1+1	8.92
2,4-D+KIN	1+2	15.23
2,4-D+KIN	2+1	9.8
2,4-D+KIN	3+1	7.6
2,4-D+KIN	2+2	4.2
2,4-D+KIN	2+3	3.0
2,4-D+KIN	3+2	2.6
2,4-D+KIN	3+4	3.8
2,4-D+KIN	4+5	4.9
2,4-D+KIN	5+6	2.59
MS+CW(ml/l)	5	0.38
MS+CW(ml/l)	10	0.41
MS+CW(ml/l)	15	0.42
MS+CW(ml/l)	20	0.28
WPM	0	0
BAP+NAA	0.5+0.5	0.24
BAP+NAA	1+0.5	0.19
BAP+NAA	2+0.5	0.16
2,4-D+KIN	1+0.5	0.20
2,4-D+KIN	2+0.5	0.17
2,4-D+KIN	4+0.5	0.08

Table 3: Anthocyanin compounds fractionated and identified by LCMS/MS analysis

Ion Mode	[M+H]/[M-H]	Peak Retention time	Compounds
Positive	301.155	8.47	Peonidin
	463.1014	3.11	Peonidn-3-O-glucoside
	579.3155	8.47	Pelargonidin-3-O-rutinoside
	449.0533	2.28	Petunidin-3-O-arabinoside
Negative	653.90	3.028	Malvidin-3,5-O-diglucoside
	755.977	4.92	Cyanidin-3-O-glucosyl rutinoside
	594.9994	11.164	Pelargonidin-3-O-sophoroside
	533.9968	11.198	Malvidin-3-O-6"aceyl galactoside
	623.8522	3.112	4'-O-methyl delphinidin 3-O-rutinoside
	461.5581	3.079	Isopeonidin-3-O-glucoside
	621.4772	7.476	Cyanidin 3-O-(3",6"-O-dimalonyl glucoside)

Table 4. Mitigating the effect of treating CCl4 induced hepatotoxicity in rats by purified anthocyanin from teak leaves in terms of liver marker enzymes

	G I	G II	G III	G IV	G V
ALT (U/L)	32.67±2.64	69.12±1.97***	54.09±0.80***	40.23±0.93***	36.49±1.10***
AST (U/L)	36.25±1.50	65.24±1.23***	59.42±0.94***	41.37±0.64***	40.82±1.27***
ALP(U/L)	60.80±2.78	114.0±1.35***	98.23±1.62***	61.00±2.06***	64.87±2.17***

Table 5: The effect of treating CCl4 induced hepatotoxicity in male rats with purified anthocyanin from teak leaves for 4 weeks on liver enzymes SDH and LDH

Enzymes	G I	G II	G III	G IV	G V
Succinic dehydrogenase (SDH) (U/L)	3.26	1.44	2.76	3.0	3.11
Lactic dehydrogenase (LDH) (U/L)	237.2	541.4	301.3	272.6	297

Table 6. The positive effect of purified anthocyanin against CCl4 induced hepatotoxicity in rat in terms of glucose, triglycerides, cholesterol and total bilirubin

	G I	G II	G III	G IV	G V
GLU (mg/dl)	86.9 ±2.5	192.5±10.2	100.6 ±6.4	90.5 ±1.7	93.06 ±2.4
TG (mg/dl)	80.32±5.8	137.1 ±9.3	97.3 ±4.5	86.2 ±6.3	84.1 ±7.56
CHOL (mg/dl)	90.32±7.2	140.5 ±12.2	107.4 ±8.3	98.3±5.2	91.7 ±9.3
TBR mg/dl	0.42 ±0.03	1.38 ±0.14	0.72±0.11	0.50±0.09	0.48±0.01

Table 7. The effect of purified anthocyanin against CCl4 induced hepatotoxicity in rats on the activity of antioxidant enzymes

	G I	G II	G III	G IV	G V
Serum Catalase (S.CAT) U/I	2.81 ±0.22	0.14±0.009	2.34±0.33	4.87±0.07	4.92±0.22
Serum Superoxide dismutase (S.SOD) U/ml	543.2 ±6.09	197.4±2.7	612.5±10	709.2±2.7	698.4±1.3
Serum Glutathione reductase (S.GSST) U/ml	675.3 ±7.2	211.3±3.1	753.1±8.9	844±10.4	809.2±0.45
CAT U/g. Liver tissue	5.98 ±0.77	0.55±0.04	6.77±0.99	9.34±0.06	8.79±0.90
SOD U/g. Liver tissue	787 ±11.2	197.4±13	822±11.7	890.5±12.3	832±0.82
GSH U/g. Liver tissue	709 ±9.7	312.1±11.8	766.7±4.6	808.2±0.96	790.6±5.2
MDA nmol/ g. Liver tissue	1.78 ±0.15	20.09±1.4	8.3±0.87	2.33±0.13	2.0±0.21

Table 8. Hematological parameters in rats treated with purified anthocyanin and CCl₄

	G I	G II	G III	G IV	G V
Hb (g/dL)	13.9±0.78	10.2±0.04	12.49±0.11	13.67±0.91	13.7±0.12
RBC Count($\times10^6$ mm^3)	5,221±0.43	3,230±0.18	4230±0.41	5189 ± 0.07	5195±0.4
WBC Count (mm^3)	8456±7.2	6234±6.1	7862±4.52	8231±7.5	8345±4.8
HCT/PCV	47.38±0.20	32.9±0.06	39.4±0.04	42.14±0.35	46.2±0.17
MCV (fl)	87.81±2.3	99.4±0.75	75.6±0.48	84.4±0.95	86.3±3.2
MCH (pg)	28.54±0.35	32.6±0.03	24.6±0.06	27.5±0.08	27.7±1.4
MCHC (g/L)	32±0.05	36.4±0.038	30±0.14	32.82±0.07	31.4±0.12
Platelet Count(10^9/L)	193.2± 0.988	70.4±0.233	123.4 ±0.676	191.2±5.5	191.6±0.17
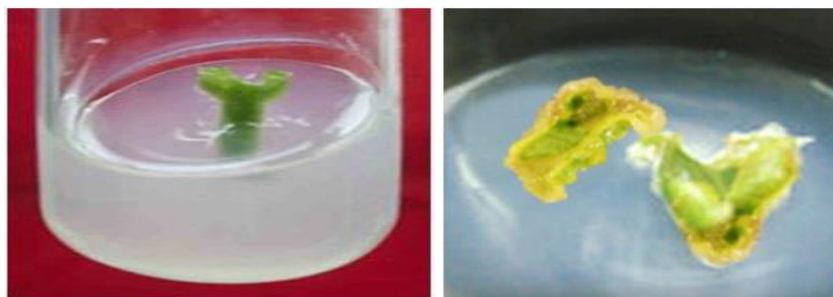
Figure 1a& b: *In vitro* culture initiation from nodal region and leaves of teak
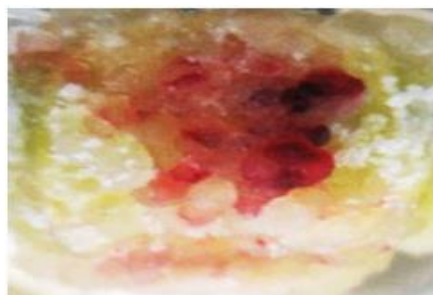
Figure 2: Induction of pink colouration from leaf callus.
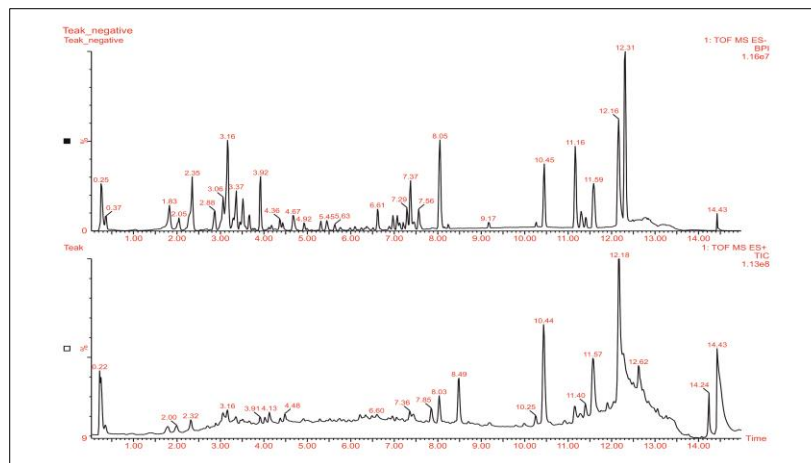
Figure 3: LCMS/MS spectrum of purified anthocyanin from *in vitro* callus culture of teak

Figure 4: The effect of pretreatment with purified anthocyanin on CCl₄ induced hepatotoxicity in rats on total protein and albumin
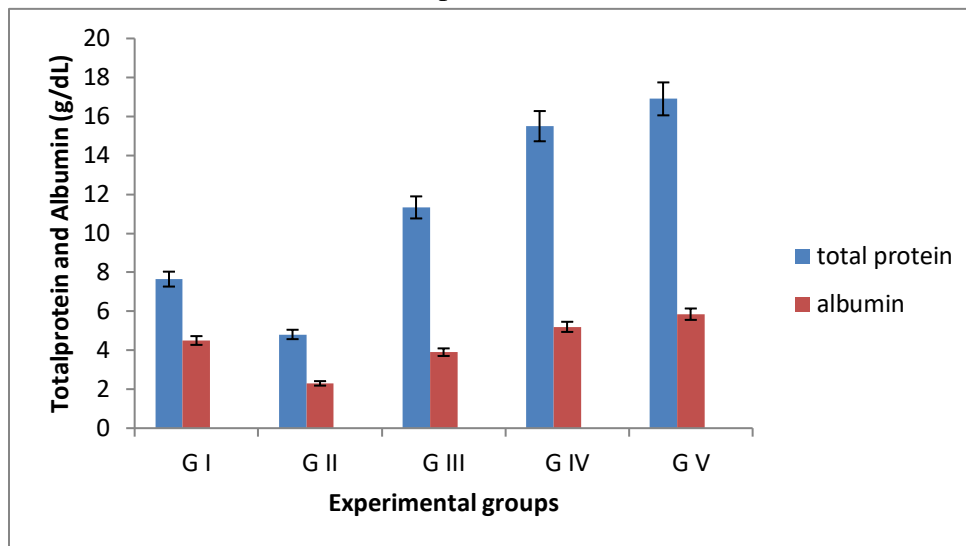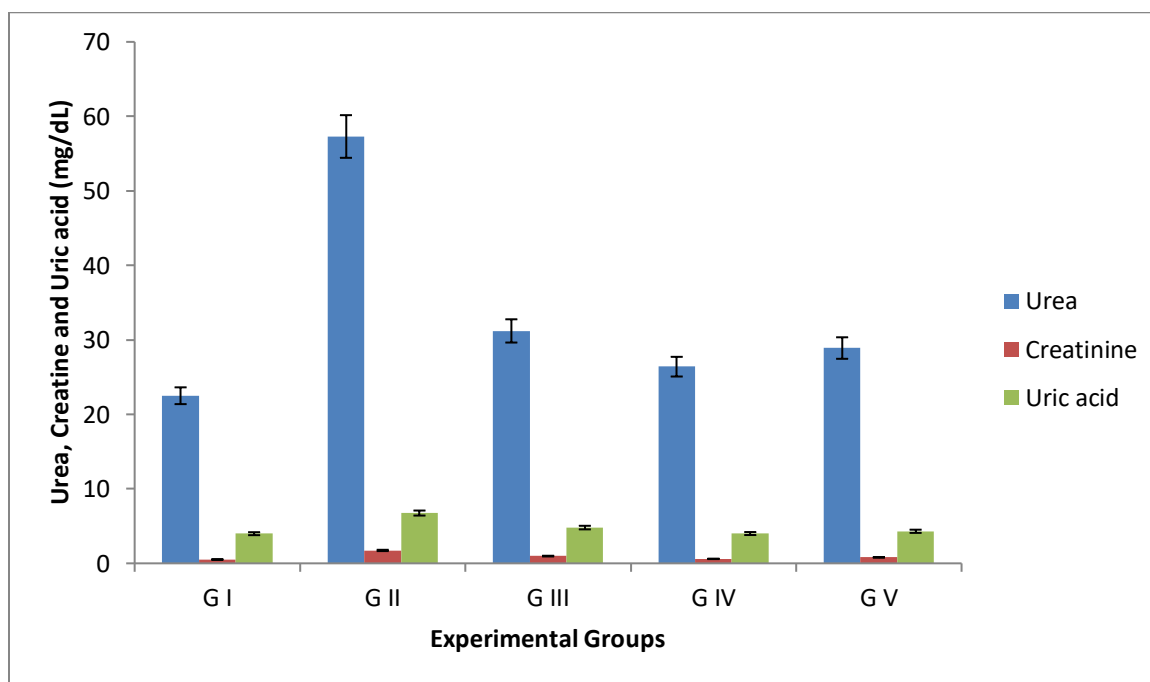

Figure 5: The effect of purified anthocyanin from teak leaves v/s CCl₄ induced hepatotoxicity in the rats
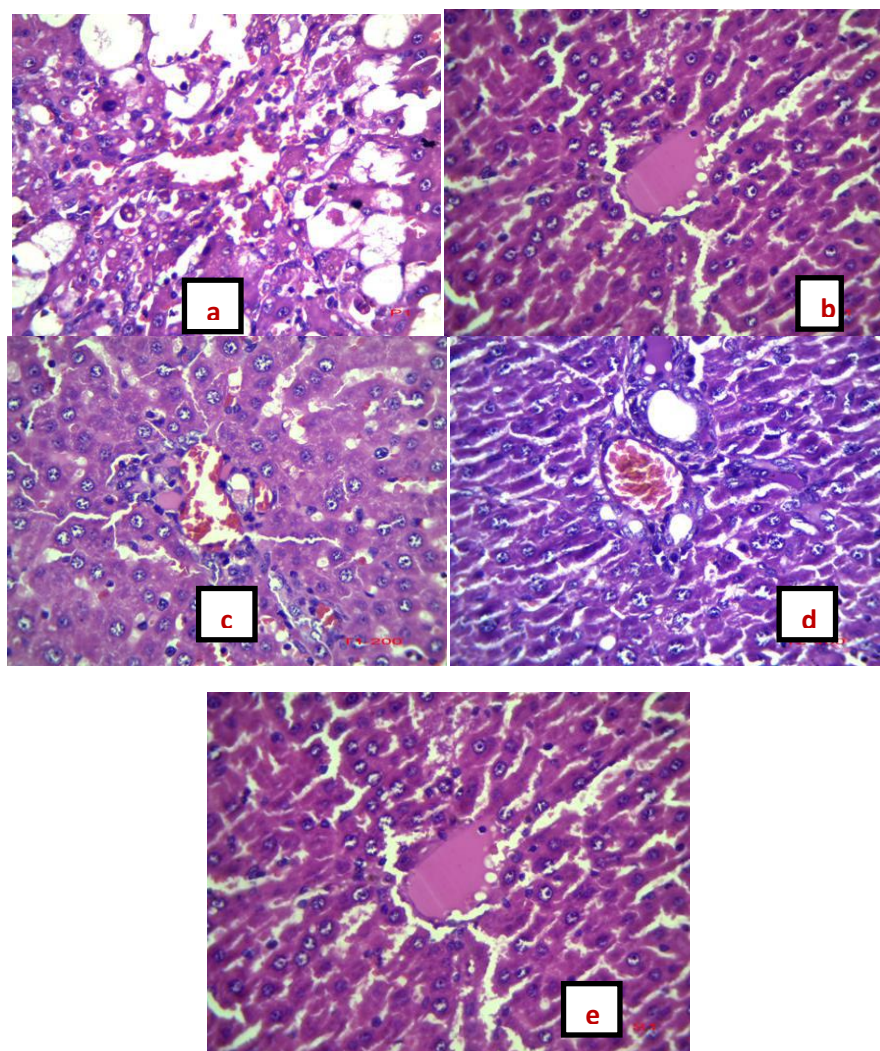
Figure 6: Histopathological changes noticed within hepatic cells with different experimental groups

a - Normal control, b –Negative control (CCl$_4$ treatment), c – Anthocyanin treated (200mg/kg,p.o), d- Anthocyanin treated (400mg/kg,p.o), e – Positive control (Silymarin treated)

After treatment with purified anthocyanin from teak leaves, the hepatic injuries were significantly inhibited and architecture of hepatocytes was almost brought to normal. The results of the various biochemical parameters, antioxidants and the histopathological figures articulate with each other, which strongly suggests that purified anthocyanin from teak leaves possesses significant hepatoprotective effect against CCl$_4$-intoxicated liver damages in rats. Gnanadesigan et al. [31] proved the hepatoprotective activity of *Ceriopsdecandra*against CCl$_4$ induced liver damage. Kamisan et al.[32] confirmed the efficacy of methanol i.e., leaf extract of *Melastomamalabathricum* against rat liver toxicity by CCl$_4$. Mondal et al.[33] validated hepatoprotective activity of *Macrothelypteristorresiana* against CCl$_4$-induced hepatotoxicity in rodents and analyzed polyphenolic compounds by HPTLC. The present results were supported by Rao et al.[34] using *Casuarinaequisetifolia*

against paracetamol; Garba et al. [35] using aqueous extract of *Kohautiagrandiflora* on paracetamol induced liver damage in albino rats and Gul et al. [36] using *Microcephalalamellata*, *Periplocaaphylla* and *Alhajimourarroum*. Uhunmwangho et al. [37] trailed hexane root extract of *Alchornealaxiflora* in sodium arsenate toxicity in Wistar Albino rats. Hepatoprotective activity of cinnamon ethanolic extract against CCl_4-induced liver injury in rats was analyzed by Eidiet al.[38]. Shah et al. [39] correlated punarnavashtakkwath, an Ayurvedic formulation, against CCl_4-induced hepatotoxicity in rats and on the HepG2 cell lines. Nchouwetet al.[40] validatedthat the stem barks methanolic and aqueous extracts of *Pseudocedrelakotschyi* on paracetamol-induced hepatic damage in rats. Ahmed et al.[41] proved the effect of lipoic acid in ethanol intoxicated chicks on liver biochemical parameters.

CONCLUSION

The overall results of the study indicate an ideal correlation between the natural biomolecule vs standard drug silymarin. The experimental results support the traditional therapeutic application of this coloured extract for liver ailments. Remarkable antioxidant enzyme level establishes the hepatoprotective effect of anthocyanins. Further study is warranted for the analysis of anthocyanin against other toxicants like paracetamol, ethanol induced hepatotoxicity and its mechanism of action. Clinical study is also warranted to support traditional use of this formulation in jaundice and other hepatic disorders.

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