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Pharmacognostic, Phytochemical and *In Vivo* Hepatoprotective Activity on *Pongamia Pinnata* Linn Bark

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Research Article

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Abstract

Pongamia pinnata Linnn (Fabaceae) is a tree/shrub with a broad distribution from India, south-eastern Asia, Indonesia and Australia. Different parts of the plant have been used in traditional medicine for bronchitis, whooping cough, rheumatic and diabetes. Transverse section and powder microscopy study of the bark showed phelloderms, cork cells, cortex cells, medullary rays, stone cells, calcium oxalate crystals and starch grains. The total ash 10.64% w/w. The acid insoluble ash 50.4% w/w, water soluble ash 49.1% w/w, The loss on dried 8% w/w, swelling index 5.4% w/w and foaming index was <100. The extractive values of bark powder of *P. pinnata* Linn was found to be more for ethanol solvent followed by distilled water, chloroform, ethyl acetate, petroleum ether. Preliminary phytochemical screening of hydro-alcoholic bark extract of *P. pinnata* Linn revealed the presence of flavonoids, phenolic compounds, tannins, alkaloids, carbohydrates, glycosides, and sterols gave positive reaction. The total phenolic, alkaloid and flavonoid contents of the hydro-alcoholic bark extract of *P.pinnata* Linn was found to be 68.96 ± 0.376 (mg GA/g). 52.06 ± 0.31 (mg AE/g) and 71.06 ± 0.11 (mg QE/g). From the column chromatography3-methoxy-(3´´,4´´-dihydro-3´´-hydroxy-4´´-acetoxy)-2´´,2´´-dimethylpyrano-(7,8:5´´,6´´)-flavone and Caryophyllene oxide compounds were isolated. The hydroalcoholic bark extract of *P. pinnata* Linn significantly decreases all the elevated levels of SGOT, SGPT, ALKP, TBL, CHL and increases TPTN and ALB levels at 400 and 800 mg/kg in rats when compared to standard drug silymarin.

Keywords: *Pongamia Pinnata; Phytochemical; Hepato Protective*

Introduction

Plant Name

Pongamia pinnata Linn and its belongs to the Fabaceae family

Synonym Names

Derris indica (Lam.) Bennett, Pongamia glabra Vent and Pongamia pinnata Merry [1].

Introduction to Fabaceae Family

The Fabaceae or Leguminosae, commonly known as the legume, pea or bean family, are a large and important economically family of flowering plants.

Fabaceae is the most common family found in tropical rain forests and in dry forests in the America and Africa. The group is widely distributed and is the third-largest land plant family in terms of number of species, behind only the Orchidaceae and Asteraceae, with 630 genera and over 18,860 species (Figure 1).

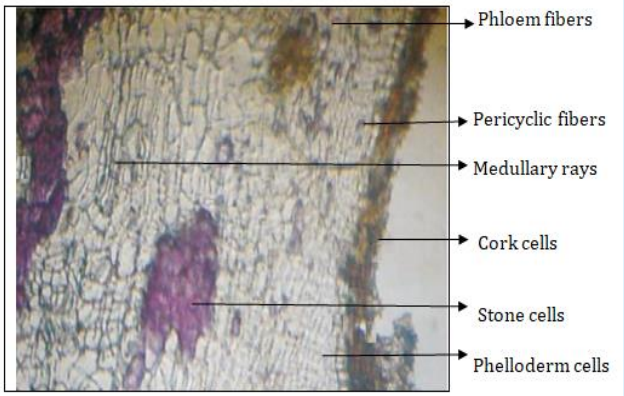


Figure 1: T.S. of P. pinnata Linn. Bark.

Plant Description

A medium sized semi evergreen glabrous tree with a short bole and spreading crown upto 18 m or more in height. *P. pinnata* Linn is a fast-growing tree which reaches 40 feet in height and spread, forming a broad, spreading canopy casting moderate shade.

Distribution and Habitat

P. pinnata Linn is a tree/shrub with a broad distribution from India, through central and south-eastern Asia, Indonesia and into northern Australia. Habitats include coastal and riverine habitats, primarily in humid tropical and subtropical areas (500–2500 mm rainfall per annum).

Traditional Uses

Traditionally the plant is useful for bleeding piles, beriberi, bronchitis, whooping cough, rheumatic, anti-diabetes, anti-hepato-protective, hypertension, hyperlipidia, skin infections wounds, ulcers, diarrhoea and antimicrobial activity [2].

Taxonomical Classification

- Kingdom - Plantae
- Subkingdom - Tracheobionta
- Super division - Spermatophyta
- Division - Magnoliophyta
- Class - Magnoliopsida
- Subclass - Rosidae
- Order - Fabales
- Family - Fabaceae
- Genus - *Pongamia*
- Species - *pinnata*

Vernacular Names of *Pongamia Pinnata* Linn.

- Hindi, Marathi and Guajarati - Karanj, Karanja
- Sanskrit - Ghrtakarauja, Karanjaka, Naktahva, Naktamala
- English - Indian beech
- Telugu - Pungu, Gaanuga
- Tamil - Ponga, Pongam
- Malayalam - Pungu, Punnu
- Oriya - Koranjo
- Punjabi - Sukhehein, Karanj, Paphri
- Assam - Karchuw
- Bengali - Dahara karanja, Karanja, Natakaranja
- Kannada - Honge, Hulagilu

Material and Methods

Plant Material

The bark of *P. pinnata* Linn. were collected from Warangal in September 2007 (1.5kg) and was authenticated by Prof.V.S. Raju, Department of Botany, KaKatiya University, Warangal. A specimen was deposited in the Herbarium (Voucher specimen number (CO/07) leaves were collected from the plant and dried under shade.

Extraction Process

The freshly collected bark (4kg) material was shade dried and powdered. The coarsely powdered bark material was then extracted with hydro-alcohol 70:30 (70 % v/v methanol) using a Soxhlet apparatus at 50–60°C for 8 to

10h. After complete extraction, the filtrates were concentrated separately by rotary vacuum evaporation (>45°C) and then dried. It was weighed (64gm) and stored in a dessicator.

Chemicals for Microscopical Studies

Phluroglucinol, HCL, glycerine, methanol and other analytical grade chemicals.

Macro and Microscopic Analysis

Macroscopic features of the bark was analysed by the standard methods [3].

Powder Microscopy

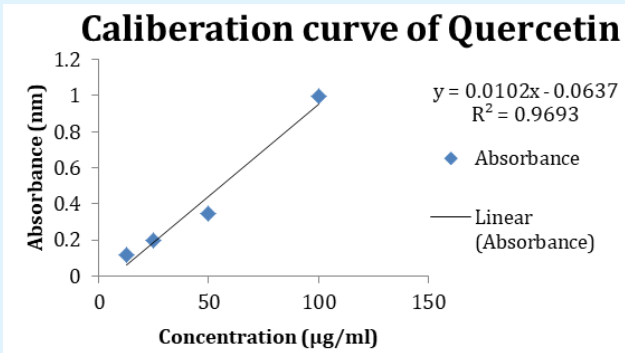
The air dried bark was powdered and studied under microscope. Different staining reagents (such as iodine for detection of starch grains and phloroglucinol for detection of lignified components) were used. To a little quantity of aerial parts powder taken over a microscopic slide, 1-2drops of 0.1%w/v phloroglucinol solution and a drop of concentrated hydrochloric acid were added and covered with a cover slip. The slide preparation was mounted in glycerol and examined under microscope. The characteristic structures and cell components were observed [4].

Preliminary Phytochemical Screening

The hydroalcoholic extract of *P. pinnata* Linn phytochemical analysis. A series of identification tests were performed to detect the presence of alkaloids, flavonoids, saponins, proteins and aminoacids, fixed oils and fats, glycosides, tannins and steroids [3-5].

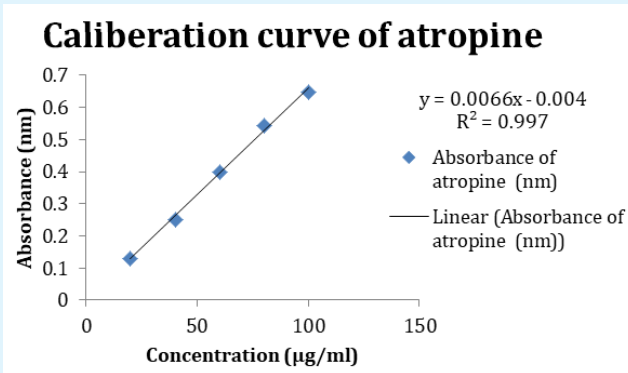
Quantitative Estimation of Flavonoid, Alkaloid and Phenolic Content of *P. pinnata* Linn

Determination of Total Flavonoid content: Total flavonoids content in the plant extract in brief 1000 µg/ml of sample and add methanol of 3ml followed by 200µl of 10% $AlCl_3$ solution, 200µl of 1M potassium acetate solution and 5.6ml of distilled water was added [6]. The absorbance at 420nm monitored (biotek instrument, instrumentinc Winooski, VT, USA) after 30min incubation at room temperature. Total flavonoids content was calculated with respect to the standard curve of the flavonoid. Querectien dehydrated quantification was performed with respect to the standard curve of Querectien result were expressed in micrograms (mg) of Querectien dehydrated equivalent (QE) per ml of the extract. All tests were performed in triplicate (Graph 1).



Graph 1: Caliberation curve of Quercetin.

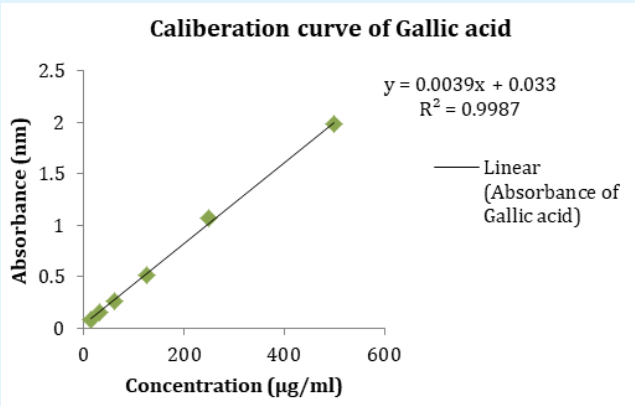
Determination of Total Alkaloid content: The plant extract (1mg/ml) was dissolved in 2 N HCl and then filtered. The pH of phosphate buffer solution was adjusted to neutral with 0.1 N NaOH. One ml of this solution was transferred to a separating funnel and then 5 ml of bromocresol green solution solution along with 5 ml of phosphate buffer were added. The mixture was shaken and the complex formed was extracted with chloroform by vigorous shaking. The extracts were collected in a 10 ml volumetric flask and diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm. By using standard atropine calibration curve, measured the concentration of alkaloid content in atropine equivalents using unit's mg/gm. (GAE) [7] (Graph 2).



Graph 2: Calibration curve of atropine for alkaloid content.

Determination of Total Phenolic content: The amount of total phenolic content in extract was determined according to Folin-Ciocalteu method. 0.2 µL of sample solution (1mg/mL) were introduced into test tube containing 2 mL

of Folin-Ciocalteu's reagent and 5 mL of Na_2CO_3 (7.5%) and methanol. The final volume was brought up to 7 mL with deionized water. After 2 h incubation at room temperature, the absorbance was measured at 765 nm with spectrophotometer (Shimadzu, UV-1800). The total phenolic content was expressed as gallic acid equivalents (GAE) in milligram per gram of extract (mg GAE/g extract). All tests were performed in triplicate (Graph 3).

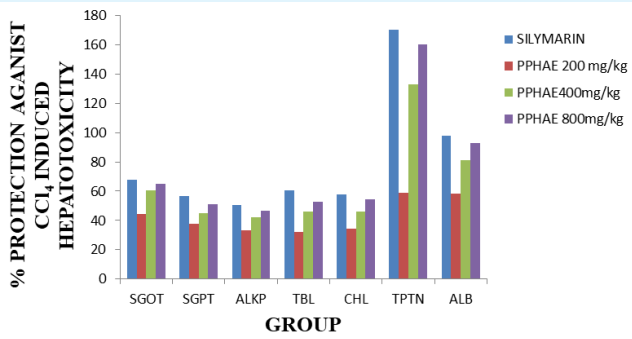


Graph 3: Caliberation curve of Gallic acid for phenolic content.

Column chromatography: As TLC of the crude hydroalcoholic extract showed few spots, on column chromatography (Merck), and eluted with a stepwise gradient using hexane-EtOAc followed by EtOAc- MeOH mixtures. A total of 427 fractions were collected. Fractions were combined on the basis of their TLC profiles, and subjected to further silica gel chromatography, eluting with hexane, hexane-$CHCl_3$ and $CHCl_3$-MeOH mixtures to afford 2 major fractions (1-2), each 200 ml. On crystallization using chloroform, fraction A afforded 1 (15 mg), while fraction Byielded 2 (20 mg).

Materials and Methods for Effect of *P. pinnata* Linn hydro alcoholic extract on CCl_4-induced hepatotoxicity in rat [6].

Wister albino rats of either sex were obtained from National Institute of Nutrition, Hyderabad, Andhra Pradesh, India as per rules and regulations of Institutional Animal Ethics committee and by regulatory body of government (Regd no: 812/01/A/CPCSEA) (Graph 4).



Graph 4: Effect of crude bark extract of PPHAE 200, 400, 800 mg/kg along with silymarin 25 mg/kg body wt. on percentage protection of various biochemical parameters against CCl4¬ induced hepatotoxicity.

Hepatoprotective- CCl_4 model induced Hepatotoxicity

Wistar albino rats of either sex (150-200g, n=5), CCl_4, Standard and test drug were used the hydroalcoholic extract of *P. pinnata* Linn at different doses, silymarin and drug vehicle were administered p.o in sodium carboxy methyl cellulose suspension as described above. The serum was used for the estimation of various biochemical parameters like SGOT, SGPT, ALKP, TBL, CHL, TPTN and ALB [6] (Table 1).

Group	0h	24h	48h	72h
Group I -Control	Vehicle	Vehicle	Vehicle	Withdrawl of blood
Group II-CCl_4	Vehicle	Vehicle+CCl_4	Vehicle	
GroupIII-Silymarin	Silymarin	Silymarin+CCl_4	Silymarin	
Group-IV - Test 200mg/kg	Extract	Extract+ CCl_4	Extract	
Group-V Test 400mg/kg	Extract	Extract+ CCl_4	Extract	
Group-VI (Test 800mg/kg)	Extract	Extract+ CCl_4	Extract	

Table 1: The protocol for CCl_4 induced hepatotoxicity.

Results and Discussion

Morphological Examination

P. pinnata Linn bark consists of channelled, recurved, slightly quilled, usually 0.2-1cm thick, lenticellate pieces, more or less smooth outer surface ash-grey to greyish-brown and internal surface yellowish-white to cream coloured.

Transverse Section and Powder Microscopy

Study of the bark shows 5-20 or more layers of cork (phellem), composed of rectangular, thick-walled cells, and filled with reddish-brown content, at some places lenticels also appear. Phellogen (cork cambium) is 2-3 layered having polygonal, tangentially elongated, thin-walled, parenchymatous cells whereas phelloderm (secondary cortex) is 10-15 layered having oval to polygonal, tangentially elongated, thin-walled, parenchymatous cells. Beneath secondary cortex, a large group of oval to elongated stone cells, arranged in a tangential manner, occurs forming a continuous or discontinuous band. Secondary phloem is composed of phloem parenchyma, phloem fiber and stone cells, traversed by medullary rays. Phloem parenchyma consists of rectangular to polygonal thin-walled cells, alternating with stone cells. Phloem fibre is small, polygonal, thin-walled and a few associated with stone cells and arranged radially. Medullary rays are wavy, usually 2-4cells wide, radially elongated and rounded to oval in shape. A few stone cells are found scattered in secondary cortex as in secondary phloem and it also contains starch grains (Figure 2).

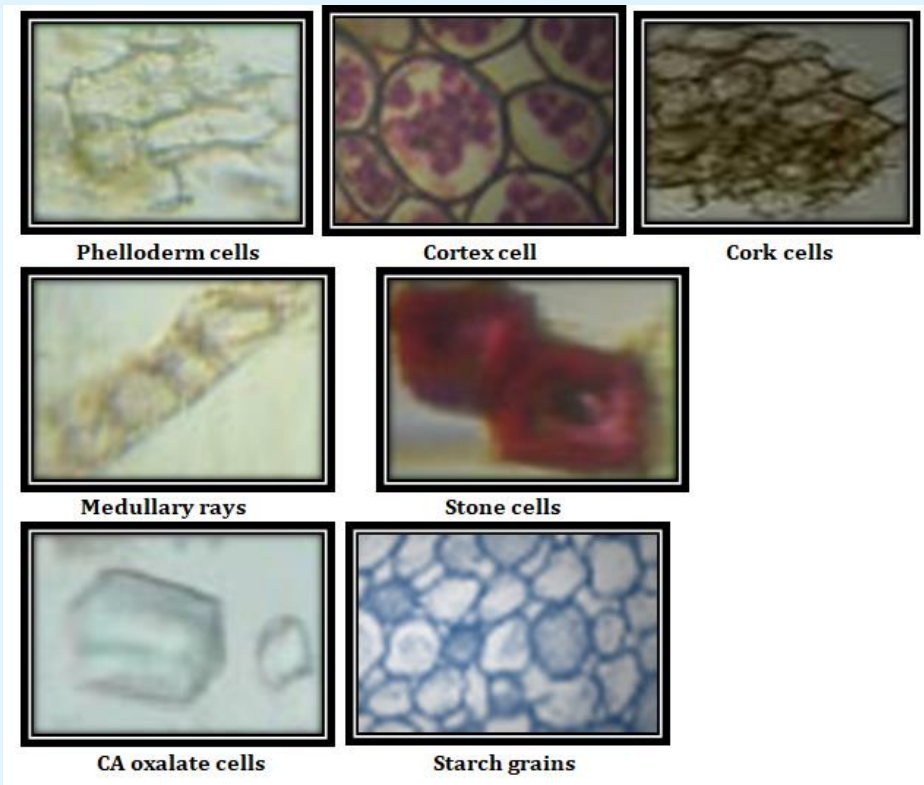


Figure 2: Powder Microscopy on P. pinnata Linn Bark.

Physicochemical Parameters

The total ash values of bark powder of *P. pinnata* Linn was found to be 10.64% w/w which indicates the presence of earthy matter. The acid insoluble ash was found to be more for bark powder i.e., 50.4% w/w than water soluble ash of the bark powder 49.1% w/w respectively. The loss on drying values of powder bark of *P. pinnata* Linn was found to be 8% w/w which indicates the presence of moisture content in the bark powder. The swelling index of bark powder of *P. pinnata* Linn was found to be 5.4% w/w. The foaming index of *P. pinnata* Linn bark powder was found to be <100 (Table 2).

Physicochemical parameters	Values obtained on dry weight basis (%w/w)
Total ash	10.64%
Acid-insoluble ash	50.4%
Water-soluble ash	49.1%
Loss on drying	8%
Swelling index	5.4%
Foaming index	<100

Table 2: Results for Physicochemical parameters of bark powder of *P. pinnata* Linn.

Extractive Values

The extractive values of bark powder of *P. pinnata* Linn was found to be more for methanol solvent followed by distilled water, chloroform, ethyl acetate, hexane (Table 3).

Extractive values	Values obtained on dry weight basis (gm)	Values obtained on dry weight basis (%w/w)
Ethyl acetate	0.161	8.10%
Hexane	0.137	6.85%
Chloroform	0.196	9.80%
Methanol	0.3	15%
Water	0.2	10.70%

Table 3: Results for extractive values of bark powder of *P. pinnata* Linn.

Preliminary Phytochemical Tests

The preliminary phytochemical screening of hydro-alcoholic bark extract of *P. pinnata* Linn revealed the presence of Flavonoids, Phenols, Alkaloids, Carbohydrates, Glycosides, Tannins, Sterols and Triterpenoids by giving positive reaction [3] (Table 4).

Phytochemical Analysis	Tests	Bark
Carbohydrates	Molish test	++
	Benedict tes	+
Glycosides	Foam test	+
	Na_2HCO_3	+
Amino acid and Protein	Biuret test (Proteins)	-
	Million's test (Amino acids)	-
Sterols and triterpenoids	Libermann-Burchard test	++
Tannins and Phenolic compounds	Lead acetate test (Tannins)	+
	Ferric chloride test (Phenolic compounds)	+++
Alkaloids	Dragendroff's test	++
	Mayer's test	+
Flavonoids	Shinoda test	+++

Table 4: Results for preliminary phytochemical analysis of hydroalcoholic bark extract of *P. pinnata* Linn. (+++ = more intense, ++ = intense, + = present, - = absent)

Quantitative Estimation of Flavonoid, Phenolic and Alkaloids of *P. pinnata* Linn

The total flavonoid content of the hydro-alcoholic bark extract of *P. pinnata* Linn was found to be 71.06 ± 0.11 (mg QE/g). The total phenolic content of the hydro-alcoholic bark extract of *P. pinnata* Linn was found to be 68.96 ± 0.376 (mg GA/g). The total alkaloid content of the hydro-alcoholic bark extract of *P. pinnata* Linn was found to be 52.06 ± 0.31 (mg AE/g). Thus the flavonoid content was found to be more in the hydro-alcoholic bark extract of *P. pinnata* Linn followed by phenolic content and alkaloid content (Table 5).

Test type	Bark
Total Flavonoid content	71.06 ± 0.11 (mg QE/g)
Total Phenolic content	68.96 ± 0.376 (mg GA/g)
Total Alkaloid content	52.06 ± 0.31 (mg AE/g)

Table 5: Results for the quantitative estimation of the hydro-alcoholic bark extract of *P. pinnata* Linn.

From the Column Chromatography

The flavone, 3-methoxy-(3´´,4´´-dihydro-3´´-hydroxy-4´´-acetoxy)-2´´,2´´-dimethylpyrano-(7,8:5´´,6´´)- flavones and Caryophyllene oxide were isolated.

Characterization of Isolated Compounds

Compound 1: The compound isolated from the fractions 36-55 (30% ethylacetate in hexane) as colourless fine needles, melting point is 200- 273.2ºC. This fraction was purified by CC of Pharmacia-Sephadex LH-20 with MeOH-H_2O (95: 5) and separated by reverse phase semi-preparative HPLC (ODS column, using MeOH: H_2O (66:34) 8 mL min^{-1}, flow rate, UV: 254 nm) to give compounds **1** (1.9 mg, t_R=16 min). The compound is soluble in petroleum ether, acetone, distilled water, and in methanol. It was identified as 3-methoxy-(3´´,4´´-dihydro-3´´-hydroxy-4´´-acetoxy)-2´´,2´´-dimethylpyrano-(7,8:5´´,6´´)- flavone. The identification was further conformed by comparison with authentic sample through co-TLC and mixed melting points (Figure 3).

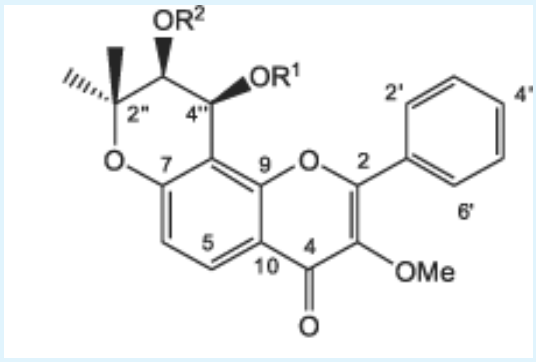


Figure 3: 3-methoxy-(3´´,4´´-dihydro-3´´-hydroxy-4´´-acetoxy)-2´´,2´´-dimethylpyrano-(7,8:5´´,6´´)- flavone.

It showed positive colour reaction with identification tests like shinoda gave pink colour, lead acetate produces yellow colour and ferric chloride test gave green colour.

Compound 2: The compound isolated from the fractions 184-192 (70% ethylacetate in hexane) as White amorphous, melting point is 59-61 ºC. This fraction was purified by CC of Pharmacia-Sephadex LH-20 with MeOH-H_2O (95:5) and separated by reverse phase semi-preparative HPLC (ODS column, using MeOH: H_2O (95:5) 4 mL min, flow rate, UV: 254 nm) to give compounds **2** (0.6 mg, t_R= 8.78 min). The compound is soluble in chloroform, ethyl acetate, n-hexane, distilled water, and in methanol. It was identified as Caryophyllene oxide. The identification was further conformed by comparison with authentic sample through co-TLC and mixed melting points (Figure 4).

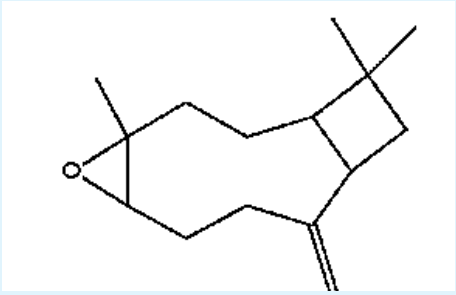


Figure 4: Caryophyllene oxide.

Histopathological Observation

The results of histopathological studies provided supportive evidence for biochemical analysis. Histology of liver section of normal control animal exhibit normal hepatic cells each with well-defined cytoplasm, prominent nucleus and nucleolus and well brought central vein (Figure 5).

Figure 5: Control.

The CCl_4 intoxicated group animals showed total loss of hepatic architecture with centrilobular hepatic necrosis, fatty changes vacuolization and congestion of sinusoids, kupffer cell hyperplasia, crowding of central vein and apoptosis (Figure 6) [8].

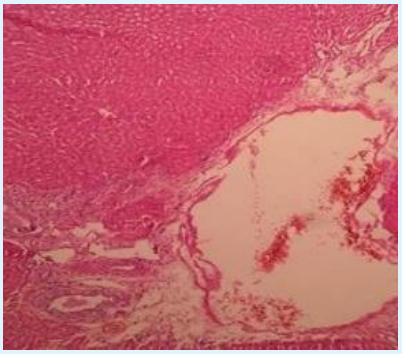


Figure 6: CCl_4 treated.

CCl_4 and silymarin treated animals showed protecting activity against CCl_4 injury (Figure 7).

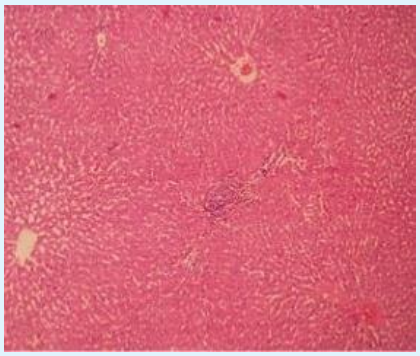


Figure 7: CCl_4+Silymarin 25mg/kg

The hydroalcoholic bark extract of *P. pinnata* Linn at medium dose of 400 mg/kg showed moderate or weak hepatoprotective activity for CCl_4 injury (Figure 8).

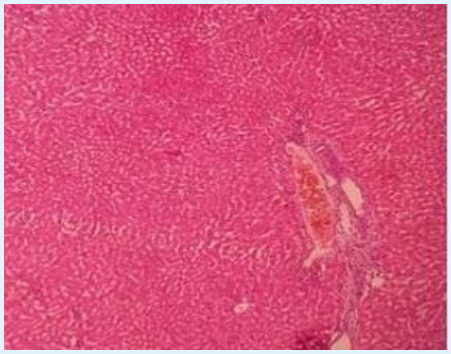


Figure 8: CCl_4+*Pongamia pinnata* L., 400mg/kg.

However the hydroalcoholic bark extract at the high dose of 800 mg/kg and silymarin had shown a potential hepato-protective activity and reduced the degenerative changes in liver (Figure 9).

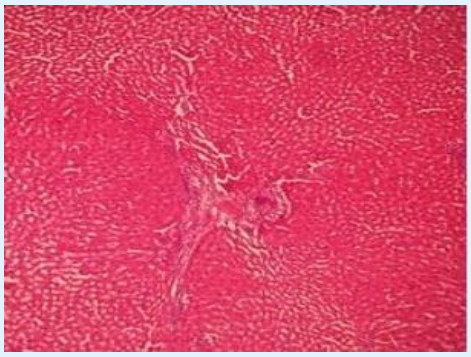


Figure 9: CCl_4 + Pongamia pinnata L., 800mg/kg.

Group	SGOT (IU/L)	SGPT (IU/L)	ALKP (IU/L)	TBL (mg/dl)	CHL (mg/dl)	TPTN (g/dl)	ALB (g/dl)
Control	108.4±2.13	95.92±3.23	218.60±1.68	2.10±0.18	110.6±2.48	6.21±0.12	3.90±0.94
CCl_4	315.4±12.0	241.4±4.58	428.1±24.47	3.48±0.05	270.7±11.19	2.58±0.82	1.98±0.21
Silymarin	102.2±1.71*	104.4±0.8*	212.6±1.68*	1.38±0.12*	114.1±0.42*	6.98±0.17*	3.92±0.18*
PPHAE 200mg/kg	188.6±3.03	160.7±1.91	280.4±3.86	2.68±0.12	165.5±2.92	5.43±0.82	2.58±0.91
PPHAE 400mg/kg	124.6±1.61*	132.4±1.6*	248.6±3.62*	1.88±0.51*	142.2±2.84*	6.01±0.23***	3.58±0.61**
PPHAE 800mg/kg	110.2±0.18*	118.2±1.88*	228.7±1.98*	1.65±0.22*	123.6±1.52*	6.72±0.73**	3.82±0.48***

Table 6: Effect of hydroalcoholic extracts of *P. pinnata* Linn (PPHA) on CCl_4 induced hepatotoxicity in rats.
Data expressed in means ± s.e.m, n=5
* Significant reduction compared to hepatotoxic group (P<0.05)
** Significant increase compared to hepatotoxic group (P<0.05)

Table 6 shows silymarin the standard drug at the dose of 25 mg/kg significantly reduced the increased levels of SGOT, SGPT, ALKP, TBL and CHL with the values 102.2±1.71, 104.4±0.8, 212.6±1.68, 1.38±0.12 and 114.1±0.42 respectively and increased the levels of TPTN and ALB 6.98±0.17 and 3.92±0.18 respectively. Hydroalcoholic bark extract of *P. pinnata* Linn at 400mg/kg produced 124.6±1.61, 132.4±1.6, 248.6±3.62, 1.88±0.51, 142.2±2.84, 6.01±0.23 and 3.58±0.61, whereas hydroalcoholic bark extract of *P.pinnata* Linn at 800 mg/kg produced 110.2±0.18, 118.2±1.88, 228.7±1.98, 1.65±0.22, 123.6±1.52, 6.72±0.73 and 3.82±0.48 respectively.

Table 7 shows Effect of hydroalcoholic bark extract of *P. pinnata* Linn on percentage protection against CCl_4 induced hepatotoxicity in rats.

Group	SGOT	SGPT	ALKP	TBL	CHL	TPTN	ALB
Silymarin	67.59	56.62	50.33	60.34	57.85	170.54	97.98
PPHAE 200mg/kg	44.52	37.89	33.4	32.36	34.36	58.94	58.29
PPHAE 400mg/kg	60.49	45.15	41.92	45.97	45.97	132.94	80.9
PPHAE 800mg/kg	65.06	51.03	46.57	52.58	54.34	160.46	92.76

Table 7: Effect of hydroalcoholic bark extract of *P. pinnata* Linn on percentage protection against CCl_4 induced hepatotoxicity in rats.

Conclusion

Secondary phloem is composed of phloem parenchyma, phloem fiber, medullary rays and stone cells were present in *P. pinnata* Linn, So many of the secondary metabolites are present in this plant [9]. The physicochemical parameters like total ash value, acid insoluble ash, water soluble ash, loss on drying, swelling index, foaming index and extractive values using solvents (methanol, distilled water, chloroform, ethyl acetate, petroleum ether). The extractive values determined the active constituents present in the drug. The extractive values of bark extract of *P. pinnata* Linn was found to be more for ethanol followed by distilled water, chloroform, ethyl acetate, petroleum ether. The foaming index and swelling index of bark extract of *P. pinnata* Linn was found to be less. The preliminary phytochemical screening of hydro-alcoholic bark extract of *P. pinnata* Linn. Revealed the presence of different phytochemical like Flavonoids, Phenols, Alkaloids, Carbohydrates, Glycosides, Tannins and Sterols. Quantitative estimation was performed for hydro-alcoholic bark extract of *P. pinnata* Linn the flavonoid content was found to be more for hydro-alcoholic bark extract of *P. pinnata* Linn followed by phenolic content and alkaloid content [10].

The results clearly depicted that CCl_4 intoxication in normal rats elevated the serum levels of SGOT, SGPT, ALKP, TBL, CHL whereas decreased the levels of TPTN, ALB significantly when compared to control indicating acute hepatocellular damage and biliary obstruction leading to necrosis [11]. The rats treated with the hydroalcoholic bark extract of *P. pinnata* Linn and silymarin showed a significant ($P<0.05$) decreases in all elevated SGOT, SGPT, ALKP, TBL, CHL and significant increase ($P<0.05$) in TPTN and ALB levels at 400 and 800 mg/kg [12].

Conflicts of Interest: None

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