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Effect of *Phaseolous trilobus* Seeds on the Paracetamol Induced Liver Damage in Rats

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Abstract: The present study was designed to investigate the role of methanol extract of *Phaseolous trilobus* (*P. trilobus*) seeds (Fabaceae) in regulating the sodium pump, in hepatic injury induced by paracetamol. Alteration in sodium pump was induced by chronic administration of paracetamol, at the dose of 500 mg kg^{-1} b.wt. p.o. for 28 days. Serum marker enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), Bilirubin, liver glutathione, Na^+-K^+ ATPase estimation and histopathology of liver were studied in Wistar albino rats. Chronic administration of paracetamol for four weeks to rats, increased levels of all marker enzymes and decreased glutathione and Na^+-K^+ ATPase level, indicating the hepatocellular damage. Histopathological evaluation supported this change with evidence of swelling, hydrophobic degeneration and necrosis of hepatocytes. These changes were reversed with simultaneous administration of paracetamol and different doses of methanol extract of *P. trilobus* seeds at the dose 125, 250, 500 mg kg^{-1} b.wt. p.o., for 28 days. Results were comparable with reference standard drug Silymarin. Reversal of level of serum marker enzymes, Na^+-K^+ ATPase, glutathione and restricted hepatic damage in simultaneously administered methanol extract of *Phaseolous trilobus* seeds in Wistar albino rats confirmed the hepatoprotective effect of methanol extract of *P. trilobus* seeds.

Key words: Hepatoprotective, paracetamol, antioxidant, *Phaseolous trilobus*, Glutathione

INTRODUCTION

The Liver in vertebrate body performs many vital functions, including metabolic and detoxification activities. A number of chemical agents and routine drugs produce cellular as well as metabolic injury. Paracetamol induced hepatotoxicity could be due to depression of (Na^+-K^+ ATPase) enzyme (Kulkarni *et al*., 2002). The altered activity (Na^+-K^+ ATPase) with toxic hepatic injury indicates the role of Na^+-K^+ ATPase at cellular level, with altered function of enzymes such as ALT and AST (Reddipali, 2008). There is an ever increasing need of an agent which could protect it from such damage. Therefore, many herbal and other indigenous sources have been adequately explored for safe and effective hepatoprotective action.

Phaseolous trilobus (*P. trilobus*) Ait, family Fabaceae (commonly known as Ranmoog or Mudgparni) is found in dry deciduous forest of Satpuda hills of Maharashtra, India and is used by local folklore in the treatment of hepatic disorders. Leaves of the plant are

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traditionally used as sedative, cooling, febrifuge, antibilious and tonic. The paste prepared from the leaves is used in hemorrhoids. The fruit contains proteins, minerals and vitamin K. Other constituents present in root, seed, leaf and seed coat are dalbergioidin, kievitone, phaseollidin, flavonoid glycosides viz., quercitin, kaempferol, vitexin, isovitexin (Kirtikar and Basu, 1995; Chopra *et al*., 1980).

Water-soluble extract of beans reported to have strong antioxidant property (Tsuda *et al*., 1994). Whole plant (panchang i.e., five parts- root, stem, leaves, fruit and seeds) of *P. trilobus* is an important constituent of Chyavanprash, is one of the most popular Ayurvedic preparations placed under Rasayana group of drugs, used widely as a health promotive and disease preventive tonic. The word Rasayana' literally means the path that Rasa takes (Rasa: plasma; Ayana: path). Rasayana drugs are very rich in powerful antioxidants, good hepatoprotective and immunomodulating agents (Govindarajan *et al*., 2007). The present study was taken up to evaluate hepatoprotective and antioxidant activity of methanol extract of seeds of *P. trilobus* in paracetamol induced liver damage of rats and to verify the ethnomedicinal use of *P. trilobus* in the treatment of hepatic disorders by the tribal communities of Nandurbar District, Maharashtra, India.

MATERIALS AND METHODS

The seeds of *P. trilobus* were collected from Mahatma Phule Agriculture University Campus, Rahuri, Maharashtra, in the month of November, 2007. The seeds were botanically authenticated by taxonomist Dr A. M. Mujumdar and voucher specimens AHMA S-070 was deposited in the herbarium of Agharkar Research Institute, Pune, Maharashtra, India. Glutathione reduced (49750), 5, 5-Dithiobis (2-nitrobenzoic acid) (43760), Silymarin (S0292), Ascorbic acid (A0278), were obtained from Sigma Chemicals, USA. All other chemicals and solvents used were of analytical grade.

Preparation of Plant Extracts

The seeds were dried in shade and coarsely powdered. The powdered seeds (500 g) were defatted with petroleum ether (60-80°C); dried marc obtained was subjected to successive soxhlet extraction with methanol. The methanol extract was concentrated under reduced pressure and dried in air. The yield obtained for methanol extract was 5% w/w. Solution of extract was prepared in distilled water (100 mg mL^{-1} for toxicity study and 20 mg mL^{-1} for hepatoprotective studies) for administration to animals.

Animals Used

Thirty six three months old Wistar albino rats (150-240 g), of both sexes were procured from National Toxicology Center, Pune. The animals were kept in polypropylene cages and maintained at 24±2°C and relative humidity of 30-70%. A 12 h dark and light cycle was followed. The animals were provided with standard pelleted diet (Amrut laboratory animal feeds, Chakan, Pune, India) and water *ad libitum*. The study was conducted in accordance with the guidelines of the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) and was approved by Institutional Animal Ethics Committee (IAEC).

Toxicity Study (OECD 425, 2001)

Toxicity study was performed as per OECD guideline number 425. Limit test was performed initially. Swiss albino mice weighing 20-25 g were used in the toxicity study. Six mice were serially administered with 5000 mg kg^{-1} dose of extract prepared in water as recommended in the guideline. (Using the default progression factor (3.2), doses would be

selected from the sequence 1.75, 5.5, 17.5, 55, 175, 550, 1750, 5000 mg kg^{-1}). After dose administration, individual animal was observed after every hour for signs of toxicity and abnormality in the behavior up to the 48th h, followed by daily observations for toxicity and mortality up to the 14 days. Body weights of the animal were recorded on every third day. On 14th day post dosing, all the mice were sacrificed and processed for gross necropsy. (OECD, 425, 2001).

Experimental Procedure

Paracetamol Induced Liver Damage

The hepatoprotective activity was investigated using paracetamol induced liver damage (Venkatesha *et al*., 2000). The rats were divided into six groups of six animals each. Group I served as normal control which received vehicle (Distilled water p.o.) only, for 28 days, Group II received only paracetamol (500 mg kg^{-1} p.o.) for same duration, Group III received standard drug (Silymarin 50 mg kg^{-1} p.o.) simultaneously with paracetamol (500 mg kg^{-1} p.o.) and Group IV, V, VI received different doses methanol extract of *P. trilobus* at the dose of (125, 250, 500 mg kg^{-1} p.o.) simultaneously with same dose of paracetamol for 28 days. On 29th day, blood was drawn by retro-orbital puncture method under ether anesthesia and centrifuged at 3500 rpm for 10 min at 4°C to separate the serum. The animals were sacrificed; livers were removed immediately and processed for further investigations.

Assessment of Serum Enzyme Activities and Bilirubin

The serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were estimated using commercially available kits (Ranbaxy Diagnostics Ltd., Baddi. H.P. India). The alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) was estimated using standard kits (Biolab Diagnostics Pvt. Ltd., Boisar M.S. India) whereas serum bilirubin was estimated by Malloy and Evelyn method. The liver was dissected out immediately, rinsed with cold phosphate buffer and subjected for estimation of Na^{+}-K^{+}-ATPase.

***In-vivo* Antioxidant Activity**

Estimation of Glutathione (Bentler *et al.*, 1963)

Liver glutathione was estimated using Ellmans reagent. The sulfydryl groups present in glutathione forms a colored complex with DTNB; absorbance was measured at 412 nm (96 well microplate reader Bio-tek).

To 1 mL of 10% liver homogenate in 0.25 M sucrose in phosphate buffer, 1.8 mL of water and 2 mL of phosphate buffer (pH 7) were added and absorbance was measured at 412 nm to detect sample blank. To above reaction mixture 200 μL of DTNB reagent was added and absorbance was measured at 412 nm after 5 min. A parallel blank without liver homogenate was also done. The amount of glutathione was determined using its molar extinction co-efficient of 13,600/m/cm and expressed in terms of μmol mg^{-1} of protein.

Calculation

The glutathione content of liver sample, in μmol mg^{-1} of protein was given by the following expression:

$$\mu\text{mol GSH / mg of protien} = \frac{E \times \text{final O.D.} - \text{Initial O.D.}}{13.600}$$

Where:

E = 0.542

OD = Absorbance reading

Histopathological Studies

The liver tissue was collected and immediately fixed in 10% formalin, dehydrated in gradual ethanol (50-100%), cleaned in xylene and embedded in paraffin. Sections of 4-5 µm were prepared (Besto, India) and then stained with hematoxylin and eosin dye for photomicroscopic observations.

Statistical Analysis

The mean±SEM were calculated for each parameter. The statistical analysis of results was carried out with graph pad {(prism 4 software) (http://www.graphpad.com/manuals/Prism4/PrismUsersGuide.pdf)} and based on an Analysis of Variance (ANOVA) followed by the Dunnetts test.

RESULTS AND DISCUSSION

Acute Toxicity Study

No deleterious effects were observed in the animals up to the dose 5000 mg kg^{-1}.

Paracetamol Induced Hepatotoxicity

The findings of ALT, AST, ALP, LDH, Na^+-K^+ ATPase, glutathione have been summarized in the Table 1. It is seen that administration of paracetamol at a dose of 500 mg kg^{-1} for 28 days caused significant rise ($p<0.01$) in level of serum marker enzymes such as AST, ALT, ALP, LDH, total bilirubin and direct bilirubin. Silymarin ($p<0.01$) significantly reduced these levels near to normal. A significant ($p<0.01$) decrease was observed in the serum level of AST, ALT, ALP, LDH, total bilirubin and direct bilirubin, in the animals treated with different doses (125, 250 500 mg kg^{-1}) of methanol extract of *P. trilobus* seeds and showed dose dependant activity. At the dose of 500 mg kg^{-1} methanol extract of *P. trilobus* showed comparable activity with the standard drug Silymarin ($p<0.01$).

Increase in liver weight, liver volume, but no significant change in body weight, was observed in paracetamol treated group; whereas liver weight and liver volume decreased in rats receiving simultaneous administration of methanol extract of *P. trilobus* along with paracetamol (Table 2). This was further confirmed by histopathological examination.

In vivo antioxidant activity showed significant decrease in glutathione level, in paracetamol treated groups, which was found to be increased, significantly in extract treated group (Table 1).

Table 1: Effects of methanol extract of *P. trilobus* seeds on biochemical parameters in various groups in paracetamol induced hepatotoxicity

Group	AST	ALT	ALP	LDH	TB	DB	GSH	(Na^+-K^+
	----------(IUL^{-1})----------				----------(%mg)----------		(µmol mg^{-1})	ATPase)
Normal	58.19±3.8	43.09±3.8	170.1±3.5	133.3±8.6	0.14±0.012	0.17±0.004	1.8±0.02	10.00±0.814
Paracetamol Treated	130.90±7.9**	120.80±4.8**	340.5±12*	273.5±8.9*	1.08±0.1*	0.60±0.016**	0.81±0.01	7.604±0.35
Silymarin +PR	50.91±4.6**	43.03±6.8**	170.3±7.5*	177.3±9.5*	0.35±0.01*	0.24±0.004**	1.70±0.01**	9.036±0.297**
PT 125 mg kg^{-1}+	65.84±14.0*	70.10±4.6*	226.3±1.2*	256.8±9.4*	0.46±0.03**	0.50±0.01*	1.50±0.01**	9.49±0.28**
PR PT250 mg kg^{-1}+	42.90±9.0**	63.86±2.2**	218.9±2.4*	203.7±16*	0.41±0.02**	0.42±0.01*	1.50±0.01**	9.673±0.50**
PR PT500 mg kg^{-1}+PR	40.80±6.5**	61.88±0.3**	198.7±2.6*	179.6±16*	0.38±0.01**	0.32±0.01*	1.50±0.01**	9.99±0.287**

PR: Paracetamol, Values are expressed as Mean±SEM; (n = 6). *$p<0.05$, when compared with the paracetamol treated groups (one-way ANOVA followed by Dunnetts test). **$p<0.01$, when compared with the paracetamol treated groups (one-way ANOVA followed by Dunnetts test)

Table 2: Effects of methanol extract of P. *trilobus* seeds on liver weight and liver volume in paracetamol induced hepatotoxicity

Group	Liver weight (g)	Liver volume (mL)
Normal	9.0±0.36	7.5±0.09
Paracetamol treated	12.0±0.95	10.5±0.06
Silymarin+Paracetamol	9.0±0.86**	7.0±0.13**
PT.125 mg kg^{-1}+Paracetamol	10.5±0.76*	10.0±0.35
PT.250 mg kg^{-1}+Paracetamol	10.5±0.56*	9.0±0.25*
PT.500 mg kg^{-1}+Paracetamol	10.0±0.09**	9.5±0.26*

Values are expressed as Mean±SEM; (n = 6). *p<0.05, when compared with the paracetamol treated groups (one-way ANOVA followed by Dunnetts test). **p<0.01, when compared with the paracetamol treated groups (one-way ANOVA followed by Dunnetts test)

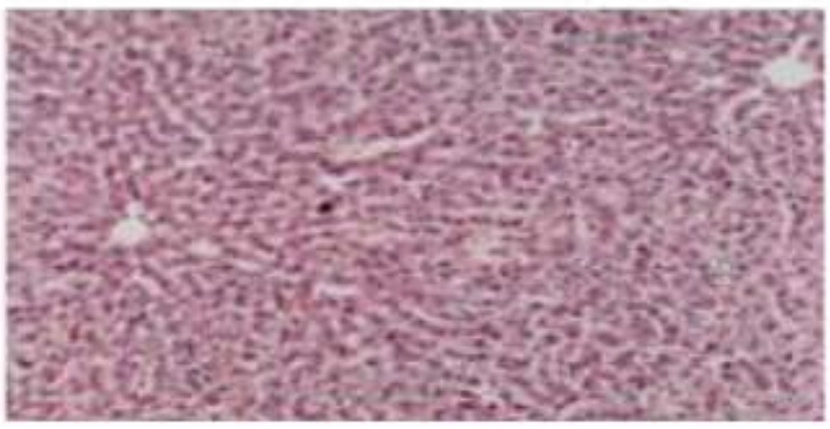

Fig. 1: Photomicrograph of normal liver showing prominent central vein, portal triads and normal hepatocytes (HE X100)

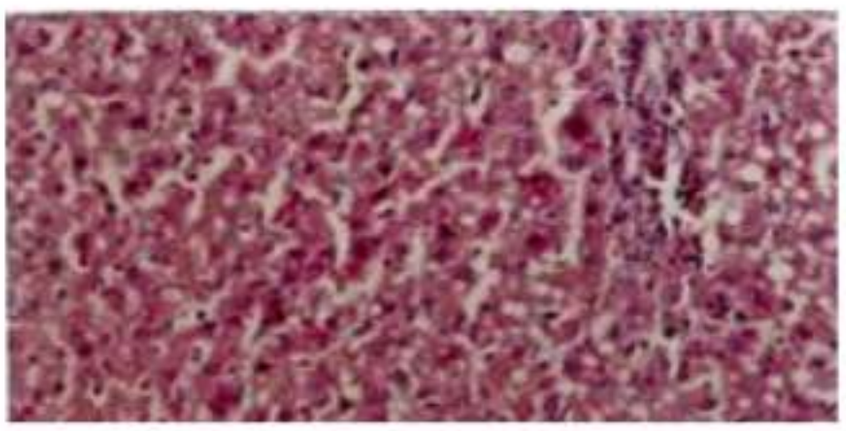

Fig. 2: Photomicrograph of liver showing cell swelling and loss of intadness with paracetamol of 500 mg kg^{-1} (HE X100)

Histopathological studies revealed that paracetamol caused focal necrosis, fatty changes, ballooning degeneration and infiltration of lymphocyte around the central vein, as compared to normal, saline treated rats which shows normal central vein, portal triad and hepatocytes as shown in Fig. 1 and 2. Necrosis induced by paracetamol was markedly prevented by pretreatment with variable doses of methanol extract of *P. trilobus* seeds in dose dependant manner i.e., at the dose of 125 mg kg^{-1} (Fig. 3) and 250 mg kg^{-1} (Fig. 4), rats showed little infiltrations of lymphocytes and small areas of degeneration, where as at the dose 500 mg kg^{-1} (Fig. 5) and 50 g kg^{-1} dose (Fig. 6) of Silymarin showed occasional area of cell degenerations with normal central vein and portal triad around the central vein with no fatty changes. Histogram shows methanol extract of *P. trilobus* seeds at the dose of 500 mg kg^{-1} b.wt. shows comparable result with the standard drug Silymarin.

This study provides a scientific evidence for hepatoprotective activity of *P.trilobus* traditionally used in the treatment of liver disorder by the tribal community of Nandurbar

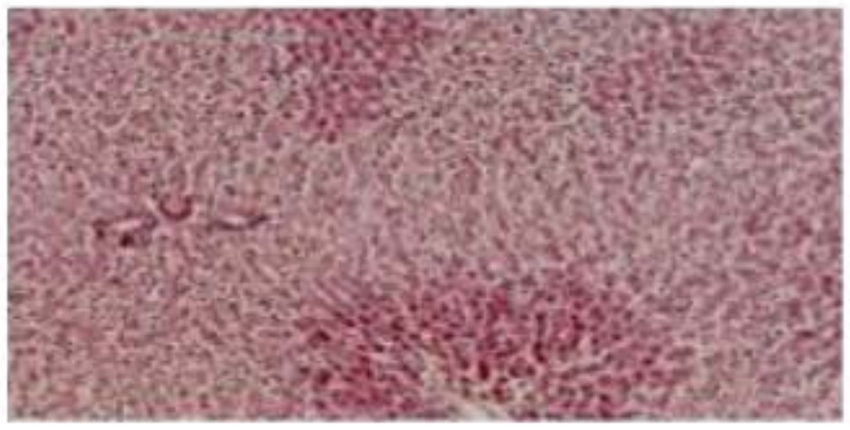

Fig. 3: Photomicrograph of liver showing little infillteration of lymphocytic and small area of degeneration in paracetamol 500 mg kg^{-1}+125 mg kg^{-1} *P. trilobus* extract (HE X100)

Fig. 4: Photomicrograph of liver showing small area of degeneration and lymphocytic interaction (HE X100) in paracetamol 500 mg kg^{-1}+250 mg kg^{-1} *P. trilobus* extract (HE X 100)

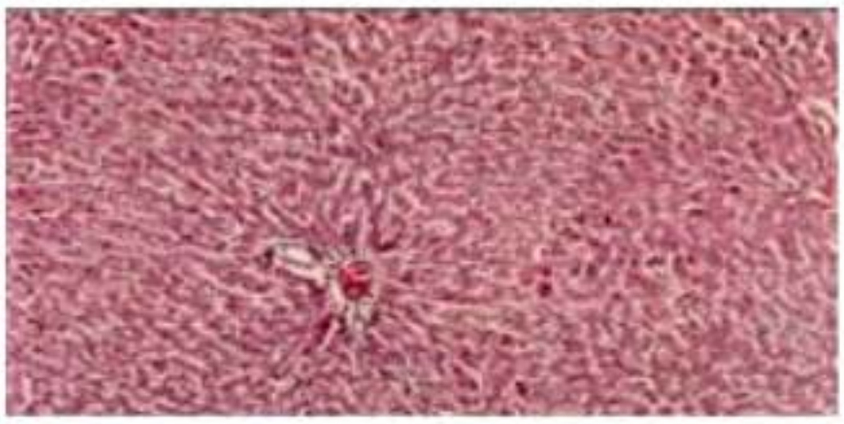

Fig. 5: Photomicrograph of liver showing preservation of structure and architecture in paracetamol 500 mg kg^{-1} *P. trilobus* extract (HE X100)

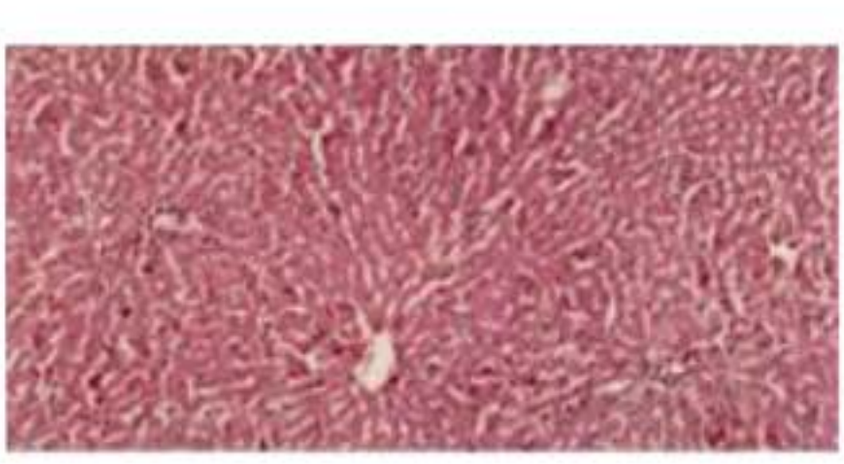

Fig. 6: Photomicrograph of liver showing small area of degeneration and almost normal architecture (HE X100)

district. Results indicate that simultaneous administration of methanol extract of *P. trilobus* seeds at variable doses significantly attenuated the paracetamol induced hepatotoxicity. As extract didn't show any mortality at the dose of 5000 mg kg^{-1}, 1/10th of the dose, i.e., 500 mg kg^{-1} was considered as an effective dose for the hepatoprotective and antioxidant activity (Paget and Barnes, 1983).

Paracetamol (N-acetyl p-amino phenol) is a widely used analgesic and antipyretic drug, known to cause hepatotoxicity in experimental animals and humans at high doses. It is mainly metabolized in liver to excreatable glucuronide and sulphate conjugates. However, hepatotoxicity of paracetamol has been attributed to formation of toxic metabolites, when a part of paracetamol is activated by hepatic Cytochrome P450 to a highly reactive metabolite N-acetyl-p-benzoquinoneimine, which is normally conjugated with GSH and excreted in the urine as conjugates. Overdose of paracetamol leads to mitochondrial dysfunction followed by acute hepatic necrosis. Damage to the structural integrity of liver is reflected by an increase in level of serum transaminases and bilirubin; these are cytoplasmic in location and are released into circulation after cellular damage (Kumar *et al*., 2005a, b). Present study revealed a significant rise in the level of serum marker enzymes viz., AST, ALT, ALP, LDH and serum bilirubin level on exposure to paracetamol, indicating considerable hepatocellular injury. Administration of methanol extract of *P. trilobus* seeds at dose of 125, 250 and 500 mg kg^{-1} dose level attenuated the increased level of the serum enzymes, produced by paracetamol and caused a subsequent recovery towards normalization almost like that of Silymarin treatment.

The active transport of sodium-potassium across the cell membrane is controlled by sodium-potassium-adenosine triphosphatase (Na^+-K^+ATPase) enzyme, which is integral plasma membrane protein responsible for a large part of energy consumption constituting the cellular metabolic rate (Corcoram *et al*., 1987). (Na^+-K^+ ATPase) enzyme control cell volume, nerve and muscle signals. Drives the transport of amino acids and sugars. Paracetamol induced hepatotoxicity could be due to depression of Na^+-K^+ ATPase enzyme (Venkateswaran *et al*., 1997). The level of Na^+-K^+ ATPase enzyme was found to be increase in extract treated groups.

Hepatic GSH depletion or even extra hepatic GSH depletion can provide useful information on the protective role GSH against toxic foreign compounds. Thus GSH, be regarded as an endogenous protective agent against drugs (Kumar *et al*., 2005a, b). Paracetamol treated animals showed decrease in level of GSH than normal. While pretreatment of *P. trilobus* extract enhanced the GSH level significantly.

Liver weight was found to be increased in the paracetamol treated group as compare to normal, standard and methanol extracts of *P. trilobus* treated groups. Hepatomegaly occurred due to accumulation of lipids and proteins in hepatocytes, with an impaired protein secretion by hepatocytes. Water is retained in the cytoplasm of hepatocytes leading to enlargement of liver cells, resulting in increased total liver mass and volume (Kumar *et al*., 2007). Steatosis and Cholestasis increases the liver weight (Korourian *et al*., 1999). Pretreatment with methanol extracts of *P. trilobus* decreased the liver weight of rats. Result showed methanol extracts of *P. trilobus* has inhibitory effect on paracetamol induced hepatotoxicity.

In histopathological assessment, it was found that treatment with methanol extract of *P. trilobus* showed reduction in hepatotoxicity.

The protective action of methanol extract of *P. trilobus* seeds may be attributed to the presences of the flavonoids (Pourmorad *et al*., 2006). Which provides maximum conjugation with free radical species involved in paracetamol metabolism by microsomal enzymes, thus reducing the number of free radicals available and the extent of cellular damage. Decreasing

lipid peroxidation and maintains level of GSH, extract may enhance the resistance of the hepatocyte membrane damage and paracetamol medicated liver injury.

In conclusion, the result of the present study suggests that methanol extract of *P. trilobus* seeds could prevent oxidative liver damage by restoring enzyme levels to near normal.

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