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Evaluation of the protective effect of coded plant leaf ethanolic extract (222) against paracetamol-induced hepatotoxicity and oxidative stress in Wistar albino rats - Part III

N M Krishnakumar*, P G Latha, S Rajasekharan, S R Suja, Mathew Dan and M Navas
Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI),
Thiruvananthapuram - 695562, Kerala, India
krishnakumarnmohandas@gmail.com

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Abstract

In this part, the protective effect of the ethanolic leaf extract of coded plant (Code No. 222*) was evaluated against paracetamol (APAP)-induced hepatotoxicity and oxidative stress in Wistar rats. In toxin control paracetamol treated group, elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in the liver. But the elevated levels of the serum enzymes are decreased to near normal levels in the 222 extract treated groups. The alanine aminotransferase (ALT), aspartate aminotransferase (AST) and triglyceride levels are significantly ($P \leq 0.05$) decreased in 222 (125 mg/kg) to normal level comparable to the standard control, silymarin. The levels of cholesterol and triglyceride were significantly ($P \leq 0.05$) increased in paracetamol treated rats. Administration of 222 at 125 mg/kg reduces the cholesterol and triglyceride levels significantly ($P \leq 0.05$), comparable to the standard control silymarin. Further, there was a significant ($P \leq 0.05$) increase in the hepatic antioxidant enzyme status of 222 extract (125 mg/kg) treated animals compared to standard control group against the oxidative stress generated by paracetamol. Histopathological studies of the liver samples confirmed the hepatoprotective property of the coded drug 222. It was also seen that histopathological damage induced by paracetamol were improved in rat liver, treated with the extract. The results of the present study revealed the hepatoprotective potential of coded plant (222) leaf extract against paracetamol induced hepatotoxicity and oxidative stress in Wistar rats.

Keywords: Hepatotoxicity, Paracetamol, Oxidatives stress, Antioxidant.

1. Introduction

Liver is the vital organ of our body regulating homeostasis by various functions such as drug elimination, detoxification and regulation of physiological processes involving storage, secretion and metabolism (Pal & Manoj, 2011). Liver injury may be caused by toxic chemicals, certain drugs, chronic alcoholism, malnutrition, infections and oxidative stress (Wolf, 1999). Hepatotoxicity by various toxins results in serious debilities ranging from severe metabolic disorders to even mortality. Paracetamol (Acetaminophen or *N*-acetyl-*p*-aminophenol) is a commonly used analgesic and antipyretic drug that belongs to the para-aminophenol class of the non-steroidal anti-inflammatory drug (Aghababian, 2010).

Liver diseases remain a serious health

* Corresponding author

* Name of the medicinal plant species will be disclosed only after obtaining the Patent

problem and the management of liver disorders is still a challenge to modern medicine. Hepatic complications are the major cause for liver transplantation and mortality recorded worldwide. The available pharmacotherapeutic options for liver disorders are limited and there is a great demand for the development of new alternative effective drugs (Akindele *et al*., 2010). The modern conventional synthetic drugs used for the treatment of liver diseases are inadequate and can have serious side effects. So there is a worldwide trend to go back to traditional medicinal plants. Many herbal products from medicinal plants are in use for the treatment of liver ailments and studies are being conducted for finding therapeutically more efficient, safer and less expensive drugs from medicinal plants (Mitra *et al*., 2000).

The present scientific study was carried out based on traditional knowledge related to a coded medicinal plant (Code No. 222) disclosed by a traditional healer and the claim of the traditional healer was that he was using the particular medicinal plant species for treating diabetes and related complications. According to him, the coded plant also provides protection to the liver. On verification, no pharmacological studies related to the hepatoprotective potential of the coded plant 222 had been so far conducted and its therapeutic usage was kept as trade secret by the healer. The present ethnopharmacological study revealed the hepatoprotective potential of the coded plant 222 leaf ethanolic extract on paracetamol-induced hepatotoxicity in Wistar rats.

2. Materials and Methods

2.1. Preparation of the coded plant extract

The coded plant 222 leaves were collected from the Traditional Healer at Karunagappally, Kerala. The plant washed thoroughly under tap water, shade dried, powdered and 100 g of the leaf powder was extracted with 1000 mL of ethanol overnight, at room temperature with constant stirring (200 rpm). The extract was filtered, concentrated and the solvent evaporated completely on a rotary evaporator at 40^0C temperature under reduced pressure, dried in a desiccator and it was reconstituted in 0.5 % Tween-80 to required concentrations and used for the experiments.

2.2. Animals

Wistar albino rats, males (200–250 g) obtained from the Institute's Animal House were used for the present study. They were housed in poly-acrylic cages with three animals per cage and maintained under standard laboratory conditions (temperature 24-28 ± 1°C, relative humidity 60 ± 5% and 12 h light/dark cycles), fed commercial rat feed (Lipton India Ltd., Mumbai, India) and boiled water *ad libitum*. All experiments involving animals were carried out according to National Institute of Health (NIH) guidelines, after getting the approval of the Institute's Animal Ethics Committee (B-form No. B-01/12/2011/EM & EP - 07).

2.3. Paracetamol induced hepatotoxicity study

The hepatoprotective potential of the ethanolic leaf extract of coded drug 222 was evaluated on paracetamol (APAP)-induced hepatotoxicity in Wistar rats. Rats were divided into different groups, each group containing six rats. Group 1 and 2 were the normal control and toxin control groups respectively and both received 0.5 % Tween-80 (1 mL, p. o) for six days. Group 3 was given silymarin (100 mg/kg) and group 4 was given the coded drug extract 222 at 125

mg/kg dose for six days (1 mL, p. o). The coded drug extract 222 (125 mg/kg) dose was found effective in the primary *in vivo* studies, so that the single dose of the coded drug 222 was fixed. Paracetamol (2.5 g/kg in 0.5 % Tween-80, 1 mL, p. o) was administered to all groups except the normal control group on the fifth day, 30 min after drug administration. 48 h after paracetamol administration, the animals were sacrificed by carbondioxide inhalation. Blood samples were collected for evaluating the biochemical parameters and liver tissue slices were collected in ice cold phosphate buffered saline to estimate the antioxidant enzymes. The liver tissues were also preserved in formalin (10%) for histopathological studies (Suja *et al*., 2003).

2.3.1. Biochemical Estimation

The collected blood was allowed to coagulate for 1h at room temperature and centrifuged at 1500 rpm for 15 min at 37ºC to separate the serum. The serum was then used for the assay of marker enzymes, namely Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Serum Alkaline phosphatase (ALP) and Serum Bilirubin (SB) according to standard methods. Other serum parameters such as serum cholesterol, triglyceride and total protein levels were estimated and compared to the standard modern drug, silymarin.

2.3.2. Estimation of antioxidant enzymes in liver tissue homogenate

0.5 g of the rat liver tissues of all the groups were sliced and homogenized with 10 mL of 150 mM KCl-Tris-HCl buffer (pH 7.2) to prepare the tissue homogenate. Superoxide dismutase (SOD) was estimated by using the method of Misra and Fridovich (1972), catalase by Aebi (1974) and reduced glutathione (GSH) was estimated by using the method of Ellman (1959).

2.3.3. Lipid peroxidation study (*in vivo*)

Lipid peroxidation was estimated by the method of Ohkawa *et al.,* (1979). Liver homogenate was mixed with 100 µL of 8.1% sodium dodecyl sulfate and 600 µL of 20% acetic acid solution, kept for 2 min at room temperature, then 600 µL of 0.8% solution of TBA was added, heated at 95^0 C for 60 min in water bath and cooled with ice cold water at 4^0 C. Then n-butanol was added, shaken vigorously and centrifuged at 10,000 rpm for 5 min. The absorbance of the organic layer was measured at 532 nm. Lipid peroxidation was expressed as n moles of MDA/mg of protein.

2.3.4. Histopathological studies

Liver specimens obtained from the control and the treated groups of the experiment were trimmed to small pieces and preserved in formalin (10% solution) for 24 h. They were subjected to dehydration with acetone of strength 70, 80 and 100% respectively, each for 1 h. The infiltration and impregnation was done by treatment with paraffin wax, twice each time for 1 h. Specimens were cut into sections of 3-7 µm thickness, stained with haematoxylin and eosinand mounted the specimens was done using Distrene Phthalate Xylene (DPX). The specimens were observed under high resolution microscope (magnification × 400) with camera and attachment (Carl Zeiss, Germany).

2.4. Statistical analysis

All the analyses were carried out in triplicate. The statistical significance between the samples were determined by the analysis of variance (ANOVA) and the data were recorded as mean ± Standard Deviation (SD). $P \leq 0.05$ was considered to be statistically significant. Significant differences between means were determined by Duncan's Multiple Range tests

(Raghava, 1987). The computer software employed for the statistical analysis was IBM SPSS Statistics, version 20 (USA).

3. Results and Discussion

Several investigators have used paracetamol (APAP) induced hepatotoxicity as an experimental model to evaluate the potential of hepatoprotective agents (Dwivedi *et al*., 1991). The hepatotoxicity of APAP has been attributed to the formation of highly reactive toxic metabolite *N*-acetyl-*p*-benzo-quinone imine (NAPQI) that causes oxidative stress and glutathione depletion (Shah and Deval, 2011). The covalent binding of *N*-acetyl-*p*-benzoquinone imine to sulphydryl groups of protein resulting in cell necrosis and lipid peroxidation induced by decrease in glutathione in the liver as the cause of hepatotoxicity has been reported (Handa and Sharma, 1990). NAPQI can also induce DNA strand breakage which promotes apoptosis and hepatic necrosis (Hinson *et al*., 2004).

The results show that paracetamol effectively induced hepatotoxicity in APAP control group as reflected by the increased serum enzyme levels, decreased antioxidant enzyme status of the liver and histopathological architecture of toxin control animals. In the assessment of liver damage by paracetamol, the determination of enzyme levels such as ALT, AST and ALP are largely used (Howell *et al*., 2014). In toxin control paracetamol treated group, elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in liver. But the elevated levels of the serum enzymes such as ALT, AST and ALP were reduced to near normal levels in the 222 (125 mg/kg) extract treated group compared to the standard control silymarin (100 mg/kg) (Table 1). Silymarin is a known hepatoprotective compound isolated from *Silybum marianum* & it is reported to exhibit protective effect on plasma membrane of hepatocytes (Ramellini and Meldolesi, 1978). Silymarin exhibited liver regenerating effect induced by the stimulation of RNA polymerase enzyme in the nucleus of liver cells and this result in an increase in the synthesis of ribosomal proteins which helps to regenerate hepatocytes (Gruenwald, 2004).

Serum bilirubin, triglycerides and cholesterol levels are significantly ($P \leq 0.05$) decreased in 222 (125 mg/kg) to normal level, compared to the standard control. Paracetamol seems to cause impairment in lipoprotein metabolism and also alterations in cholesterol mechanism (Kobashigania & Kasiska, 1997). The levels of cholesterol and triglyceride were significantly ($P \leq 0.05$) increased in paracetamol treated rats. Elevation of triglyceride level during paracetamol intoxication could be due to increased availability of free fatty acids, decreased hepatic release of lipoprotein and increased esterification of free fatty acids. Administration of 222 extract at 125 mg/kg reduces the cholesterol and triglyceride levels significantly ($P \leq 0.05$) (Table 2). The decrease in total protein level is one of the diagnostic symptoms of liver diseases (Mossa *et al*., 2012) and there was a significant ($P \leq 0.05$) decrease in total protein level in APAP control group compared to the normal control group. But 222 leaf extract and silymarin treated groups showed a significant ($P \leq 0.05$) increase in total protein level after APAP intoxication.

As a result of APAP toxicity, reactive oxygen species (ROS) are generated causing damage to various membrane components of hepatocytes. The necrotic changes lead to elevated levels of

biomarker enzymes of the liver of APAP induced hepatotoxic animals and this can be correlated to the increased level of lipid peroxidation (Mossa *et al*., 2012). The antioxidant enzymes such as SOD, catalase and GSH are responsible for the detoxification of deleterious free radicals. SOD is one of the most important enzymes in the antioxidant enzymatic system which catalyses the disputation of superoxide radicals to produce H_2O_2 and molecular oxygen, hence diminishing the toxic effects caused by the radicals. The enzyme catalase catalyses the reduction of H_2O_2 and protects the tissues from highly reactive hydroxyl radicals (Baynes, 1995). During metabolism, APAP is converted to NAPQI by cytochrome P-450 system (Dahlin *et al*., 1984) and NAPQI is normally detoxified by conjugation with GSH (Potter & Hinson, 1986). Table 3 shows the estimated values of catalase, SOD and GSH in the liver of normal and hepatotoxic rats treated with the coded plant 222 leaf ethanolic extract and standard drug silymarin. The levels of these antioxidant enzymes were found to be lowered in APAP-induced hepatotoxic rats. However, treatment with the coded plant 222 leaf ethanolic extract (125 mg/kg) and silymarin (100 mg/kg) resulted in the elevation of these enzymes compared to toxin control group. Lipid peroxidation is the major reason for the hepatocyte membrane damage causing changes in membrane permeability and fluidity (El-Megharbel *et al*., 2014). The coded drug 222 ethanolic extract at 125 mg/kg exhibited 43.82 % of MDA inhibition compared to standard drug silymarin (55.57%) in *in vivo* lipid peroxidation. The present study revealed an increased activity of antioxidant enzyme system of the liver indicating an adaptive mechanism in response to oxidative stress generated by APAP.

Histopathological studies of the liver samples confirmed the hepatoprotective property of the coded drug 222. The APAP control group showed fatty changes, swelling and necrosis with loss of hepatocytes in the liver (Fig. 1). It was seen that histopathological damage induced by paracetamol were improved in rat liver, treated with the extract which is evident from the normalization of fatty changes and necrosis of the liver. These findings are in agreement with the commonly accepted view that serum levels of transaminase return to normal with healing of hepatic parenchyma and regeneration of normal hepatocytes. This implies that concomitant administration of the extract prevented hepato-necrotic changes, induced by the toxic dose of paracetamol, indicating the ability of the extract to maintain the normal functional status of the liver.

Table 1: The effect of the coded plant 222 leaf ethanolic extract on rat serum enzymes and serum bilirubin after paracetamol (APAP) intoxication

Groups	**ALT (IU/L)**	**AST (IU/L)**	**ALP (KA units/100 mL)**	**Serum Bilirubin (mg/dL)**
Normal Control	36.13±3.25	85.57±0.70	113.56±0.70	0.28±0.06
APAP Control (2.5 g/kg)	98.64±1.52	406.66±3.38	265.61±1.39	0.93±0.02
222 ethanolic extract (125 mg/kg)	18.98±3.73*	296.55±0.01*	171.21±0.01*	0.37±0.01*
Silymarin (100 mg/kg)	16.72±1.57*	303.70±2.96*	142.90±1.98*	0.30±0.02*

Table 2: The effect of the coded plant 222 leaf ethanolic extract on rat serum parameters after paracetamol (APAP) intoxication

Groups	Triglycerides (mg/dL)	Cholesterol (mg/dL)	Total protein (g/dL)
Normal Control	94.58 ± 4.52	32.76 ± 2.93	7.28 ± 0.41
APAP Control (2.5 g/kg)	326.13 ± 5.87	53.28 ± 0.80	1.23 ± 0.01
222 ethanolic extract (125 mg/kg)	109.38 ± 0.01*	34.31 ± 4.40*	3.50 ± 0.01*
Silymarin (100 mg/kg)	98.96 ± 2.16*	25.30 ± 1.64*	5.39 ± 0.47*

Table 3: Effect of coded plant 222 leaf ethanolic extract on oxidative stress of paracetamol induced hepatotoxicity in Wistar rats

Groups	Catalase (U/mg protein)	SOD (U/mg protein)	GSH (nmoles/mg protein)
Normal Control	94.21± 0.24	15.31 ± 0.94	47.25 ± 0.15
APAP Control (2.5 g/kg)	35.62±0.37	3.77 ± 0.32	18.24 ± 0.31
222 ethanolic extract (125 mg/kg)	69.27± 0.13*	9.44 ± 0.27*	37.52 ± 0.24*
Silymarin (100 mg/kg)	82.71± 0.47*	10.72 ± 0.66*	40.72 ± 0.28*

Table 4: The effect of the coded plant 222 ethanolic leaf extract on lipid peroxidation *in vivo* after paracetamol intoxication

Groups	MDA (n mol/g wet liver)	MDA inhibition (%)
Normal control	± 0.01	----------
APAP control (2.5 g/kg)	14.72 ± 0.03	----------
222 ethanolic extract (125 mg/kg)	8.27 ± 0.11*	43.82 %
Silymarin (100 mg/kg)	6.54 ± 0.12*	55.57 %

4. Conclusion

In conclusion, the coded plant 222 leaf ethanolic extract exhibited hepatoprotective activity by significantly reducing the elevated serum enzymes levels in paracetamol (APAP)-induced hepatotoxic rats. The extract also showed protection against APAP-induced oxidative stress by significantly reducing the formation of reactive oxygen species (ROS) or by scavenging the free radicals by antioxidant system. The histopathological studies along with antioxidant and biochemical evaluation suggests the protective effect of coded plant 222 leaf ethanolic extract against paracetamol induced hepatotoxicity.

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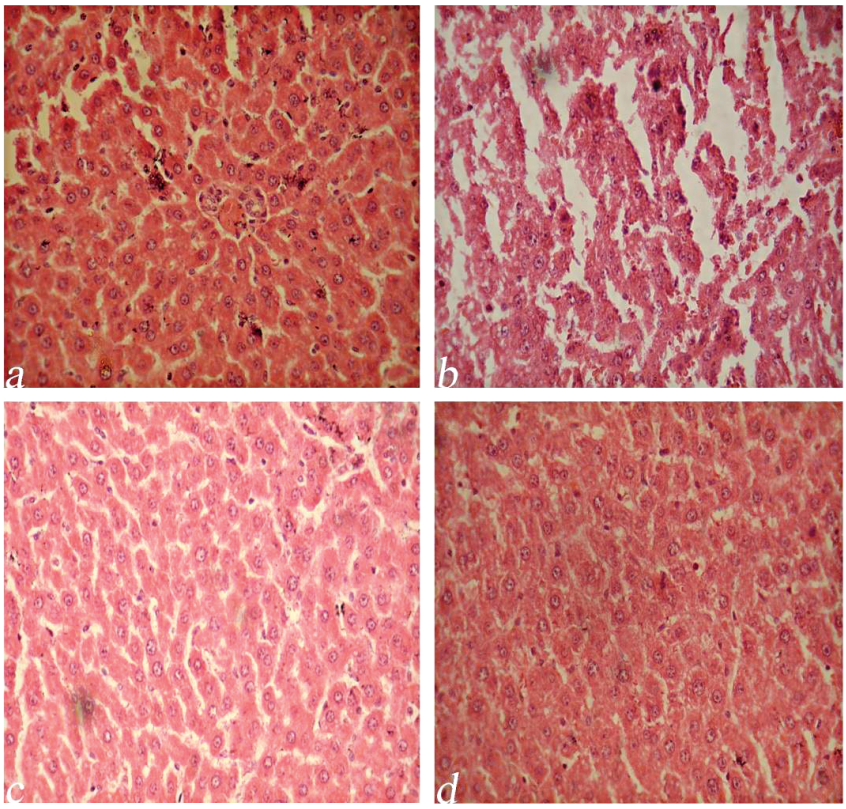
Fig. 1



Effect of coded drug 222 ethanolic extract on paracetamol –induced liver damage in Wistar rats. a) Normal liver with well defined nuclei and cytoplasm (x 400). b) Paracetamol-treated liver with hepatic cell necrosis (x 400). c) Liver treated with 222 (125 mg/kg) extract showing normalcy of hepatocytes (x 400). d) Silymarin-treated group with almost normal hepatocytes (x 400).

References

1. Aebi H 1974. Catalase, In: Methods on enzymatic analyses edited by Bergmeyar, Academic Press New York. pp. 674-684.
2. Aghababian R V 2010. Essentials of Emergency Medicine. Jones & Bartlett Publishers, Burlington. pp. 814.
3. Akindele A J, Ezenwanebe K O, Anunobi C C, Adeyemi O O 2010. Hepatoprotective and *in vivo* antioxidant effects of *Byrsocarpus coccineus* Schum. and Thonn. (Connaraceae). J. Ethnopharmacol. 129: 46–52.
4. Dahlin D C, Miwa G T, Lu A Y and Nelson S D 1984. *N*-Acetyl-*p*-benzoquinone imine: a cytochrome P-450-mediated oxidation product of acetaminophen. Proc. Natl. Acad. Sci. USA. 81 (5): 1327-1331.
5. Dwivedi Y, Rastogi R, Garg N K and Dhawan B N 1991. Prevention of paracetamol-induced hepatic damage in rats by picroliv, the standardized active fraction from *Picrorrhiza kurroa*. Phytother. Res. 5: 115.
6. El-Megharbel S M, Hamza R Z and Refat M S 2014. Preparation, spectroscopic, thermal,

antihepatotoxicity, hematological parameters and liver antioxidant capacity characterizations of Cd(II), Hg(II), and Pb(II) mononuclear complexes of paracetamol anti-inflammatory drug. Spectrochim. Acta. Mol. Biomol. Spectrosc. 131: 534-544.

7. Gruenwald J 2004. PDR for herbal medicines. 3rd Ed. Thomson PDR, Montvale.
8. Handa S S and Sharma A 1990. Hepatoprotective activity of andrographolide from *Andrographis paniculata* against CCl_4. Indian J. Med. Res. 92: 276-283.
9. Hinson J A, Reid A B, McCullough S S and James L P 2004. Acetaminophen induced hepatotoxicity: Role of metabolic activation, reactive oxygen/ nitrogen species and mitochondrial permeability transition. Drug Metab. Rev. 36: 805-822.
10. Howell B A, Siler S Q, Shoda L K M, Yang Y, Woodhead J L and Watkins P B 2014. A mechanistic model of drug-induced liver injury aids the interpretation of elevated liver transaminase levels in a phase I clinical trial. CPT Pharmacometrics Syst. Pharmacol. http://dx.doi.org/10.1038/psp.2013.74.
11. Kobashigania J A and Kasiska B L 1997. Hyperlipidemia in solid organ transplantation. Trasplantation. 63: 333-338.
12. Misra H P and Fridovich I 1972. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J. Biol. Chem. 247: 3170-3175.
13. Mitra S K, Seshadri S J, Venkataranganna M V, Gopumadhavan S, Udupa V and Sarma D N K 2000. Effect of HD-03-a herbal formulation in galactosamine-induced hepatopathy in rats. Indian J. Physiol. Pharmacol. 44: 82–86.
14. Mossa A T H, Heikal T M and Omara E A A 2012. Physiological and histopathological changes in the liver of male rats exposed to paracetamol and diazinon. Asian Pac. J. Trop. Biomed. 2: S1683-1690.
15. Ohkawa H, Oshishi N and Yagi K 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acids. Anal. Biochem. 95: 351-358.
16. Pal R K and Manoj J 2011. Hepatoprotective activity of alcoholic and aqueous extracts of fruits of *Luffa cylindrica* Linn. in rats. Ann. Biol. Res. 2 (1): 132-141.
17. Potter D W and Hinson J A 1986. Reactions of *N*-acetyl-*p*-benzoquinone imine with reduced glutathione, acetaminophen, and NADPH. Mol. Pharmacol. 30 (1): 33-41.
18. Raghava R D 1987. Statistical Techniques in Agricultural and Biological Research, Oxford & IBH Publishing Co., New Delhi.
19. Ramellini G and Meldolesi J 1976. Liver protection by Silymarin. *In vitro* effect on dissociated rat hepatocytes. Arzneim Forsch (Drug Research). 26: 69–73.
20. Rotruck J T, Pope A L, Ganther H E, Swanson A B, Hafeman D G and Hoekstra W G 1973. Selenium: biochemical role as a component of glutathione peroxidase. Science, 179: 588-590.
21. Shah V N and Deval K 2011. Hepatoprotective activity of leaves of *Parkinsonia aculeata* Linn. against paracetamol induced hepatotoxicity in rats. Int. J. Pharm. 1 (2): 59-66.
22. Suja S R, Latha P G, Rajasekharan S and Pushpangadan P 2003. Antihepatotoxic activity of *Spilanthes ciliata* on paracetamol-induced liver damage in rats. Pharmaceut. Biol. 41: 30-34.
23. Wolf P L 1999. Biochemical diagnosis of liver diseases. Indian J. Clin. Biochem. 14 (1): 59-90.