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Original Article

PHYTOCHEMICAL ANALYSIS, HEPATOPROTECTIVE AND HPTLC ANALYSIS OF FRACTIONS OF *ERYTHROXYLUM MONOGYNUM* METHANOLIC LEAF EXTRACT ON PARACETAMOL INDUCED HEPATIC DAMAGE

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ABSTRACT

Objective: To investigate the hepatoprotective activity of fractions of *E. monogynum* methanolic leaf extract against paracetamol induced hepatic damage and perform HPTLC analysis of active fraction.

Methods: Fractions derived from methanolic extract of leaves of *E. monogynum* (Pet. ether, chloroform and hydroalcoholic) were screened for hepatoprotective activity. Doses of 100mg/kg, 200mg/kg of different fractions administered for seven days and on 8th day toxicity by paracetamol was induced. Levels of biochemical markers along with histopathological changes were monitored to evaluate the extent of hepatoprotection after 24 hrs of toxicity induction.

Results: A significant decrease in the biochemical parameters was evident by the hydroalcoholic fraction as compared to the toxic group which is also confirmed by histopathological changes observed. HPTLC analysis of hydroalcoholic fraction confirmed the presence of rutin, a flavonoidal glycosides besides other phytochemicals.

Conclusion: The results of the present study indicates the significant hepatoprotective activity by the hydroalcoholic fraction of *E. monogynum* against paracetamol induced toxicity.

Keywords: *Erythroxylum monogynum*, Hepatoprotection, Paracetamol, Silymarin, HPTLC.

INTRODUCTION

The liver plays a significant role in regulating various physiological functions of the body, including synthesis, secretion and metabolism of xenobiotics. In the process of clearing and detoxifying chemicals many free radicals are generated which are neutralized by endogenous antioxidants, but once natural protective mechanisms are overpowered it will lead to encountering frequent injuries [1]. From among, drug induced liver injury is one of the most common causative factor that possess a significant clinical and regulatory challenge [2]. Amelioration of liver diseases by synthetic drugs causes unwanted side effects. Hence, herbal drugs have gained importance and popularity in the recent years in view of their cost effectiveness, efficacy and minimal side effects [3]. *Erythroxylum monogynum* (*E. monogynum*) Roxb. Belonging to the family Erythroxylaceae, traditionally reported for its hepatoprotective action [4,5]. In the previous study, we have scientifically reported the hepatoprotective action of methanolic extract of leaves of *E. monogynum* (MEEM) [6]. Hence, the present study was aimed to isolate the active fraction(s) of MEEM by fractionation and screening of the different fractions thereof for hepatoprotective action against Paracetamol induced hepatotoxicity in rats. The active fraction obtained was further analyzed by HPTLC technique.

MATERIALS AND METHODS

Chemical and drugs

Paracetamol (Cipla Ltd., Himachal Pradesh, India), Silymarin (Yucca Enterprises, Mumbai, India), Rutin (Natural Remedies, Bangalore, India). Assay kits for AST, ALT, ALP and TB (Accurex Biomedical Pvt. Ltd., Maharashtra, India), TBA, TCA, Epinephrine, DTNB (Sigma Chemical and Co., St. Louis, MO). All other chemicals and reagents used were of analytical grade and purchased from commercial sources.

Experimental animals

Wistar albino rats weighing 200-250g of either sex were used for these studies. The animals were maintained under standard conditions of temperature (24±2°C) and relative humidity (55±5%) under 12 h light/dark cycles. They were fed with standard pellet diet and water *ad libitum*. All the animals were acclimatized to laboratory conditions before commencement of an experiment. The animal studies were approved Institutional Animal Ethics Committee (CPCSEA/38/2014).

Extraction and fractionation

Plants of *E. monogynum* were obtained from Khammam District, Andhra Pradesh, India in the month of November. A specimen was submitted to Botanical Survey of India (Hyderabad), and authenticated by the same. The dried leaves of the plant were powdered and extracted with methanol by cold maceration technique for 7 days with intermittent shaking. The last trace of methanol was removed by rotaevaporator and finally dried under vacuum to afford methanolic extract of leaves of *E. monogynum*-MEEM (Yield 12.9%).

The methanol extract (50g) was suspended in 1:1 ratio of methanol and water (300 ml) and fractionated successively with pet ether (4x 300 ml), ethyl acetate (4x 300 ml) and the left over extract acts as hydroalcoholic extract to afford pet ether fraction PE-MEEM (10.4g), chloroform fraction CF-MEEM (9.2g) and hydroalcoholic fraction HA-MEEM (27.6g) respectively.

Preliminary phytochemical screening

Preliminary phytochemical analysis was performed in different fractions to identify the nature of phytoconstituents present [7].

Acute toxicity study

The acute oral toxicity was performed according to OECD- 425 guidelines. Male wistar albino rats were divided into three groups with six animals in each group. Fractions of MEEM were administered orally as a single dose to rats at different dose levels upto 2000mg/kg b. w. Animals were observed periodically for toxicity symptoms and mortality within 24 hrs and then daily for l4 days.

Experimental protocol

The screening of fractions of MEEM for hepatoprotective action was performed according to the literature [8]. The rats were divided into nine groups of six animals each. Group I (Normal Control) rats received vehicle p. o (1 ml/kg b. w of 5%gum acacia) for 8 days. Group II received 5% gum acacia p. o for seven days and the single dose of paracetamol (2mg/kg) p. o on 8th day. Group III, IV, V, VI, VII, VIII and IX were treated with silymarin (50mg/kg), PE-MEEM (100mg/kg), PE-MEEM (200mg/kg), CF-MEEM (100mg/kg), CF-MEEM (200mg/kg), HA-MEEM (100 mg/kg), HA-MEEM (200mg/kg) daily for seven days. On the 8th day all the groups (III-IX) received paracetamol (2mg/kg) except Group-I. After 24 hrs of toxicity induction i. e., on ninth day blood samples were collected from the retrorbital plexsus. The collected blood is centrifuged at 2500 rpm for 15 min to separate serum which is used for analysis of various biochemical parameters, including AST, ALT, ALP and TB. All rats were sacrificed by cervical dislocation and livers were isolated. Livers isolated were quickly dissected in two halves, one for histopathological studies and the other for biochemical analysis.

Histopathological studies

The liver specimen isolated from treated and control groups was fixed in 10% buffered neutral formalin for 24 hrs and then stained with haematoxylin-eosin for photomicroscopic observation of histopathological changes occurred in the livers.

Measurement of GSH, SOD and MDA in liver homogenate

Livers were washed with ice cold saline to get a 50% homogenate prepared in 0.05M sodium phosphate buffer (pH 7.0). The obtained homogenate was centrifuged at 4000rpm for 10 min at 4°C and the supernatant was used for the estimation of oxidative stress markers like MDA [9], GSH [10] and SOD [11].

Statistical analysis

All the results were expressed as Mean ± SEM. The statistical analysis was carried out by one way analysis of variance (ANOVA) followed by Bonferroni post hoc test using Graph pad Prism-5 software. P<0.05 was considered as significant.

Chromatographic studies

HA-MEEM extract of 10 mg was dissolved in 10 ml of methanol and sample of 5, 10, and 20 µl were applied as 8-mm wide bands, under a continuous flow of nitrogen, using CAMAG LINOMAT V automatic sample applicator [12]. Sample was applied with a 100-µl syringe (Hamilton Bonaduz, Switzerland) at a constant application rate of 0.1 µl/s and the distance between adjacent bands was 15 mm. The plate was developed using the solvent system n-butanol: glacial acetic acid: water: formic acid [4: 4.5: 2: 0.5(v/v)]. Standard rutin (10mg) was dissolved in methanol (10 ml) and scanned by a densitometer (CAMAG) at 540 nm. Vanillin- Sulphuric reagent was used as detecting agent.

RESULTS

Preliminary phytochemical screening

In the preliminary phytochemical analysis different fractions of methanolic extract of *E. monogynum* showed the presence of triterpenoids, saponins, flavonoids, phenols, alkaloids and carbohydrates.

Table 1: Preliminary phytochemical screening of various fractions of MEEM leaves

Name of the phytoconstituent	Chemical test	PE-MEEM	CF-MEEM	HA-MEEM
Flavanoids	Shinoda test	-	+	++
Phenols	5% Alc. $FeCl_3$	-	-	++
Steroidal/triterpenoidal	Libermann-Burchard test	+	++	-
Saponins	Foam test	-	+	+
Alkaloids	Dragendroff's test	+	-	-
Carbohydrates	Molisch and Fehling's test	-	+	++

-, Absent; +, Moderate; ++, Abundance;

PE-MEEM, petroleum ether extract; CF-MEEM, chloroform extract; HA-MEEM, hydroalcoholic extract

Acute oral toxicity

Fractions of MEEM produce no mortality at 2000mg/kg. Therefore, one tenth of the maximum no mortality dose were selected i. e., 200mg/kg as maximum dose along with lowest dose 100mg/kg for this study.

Hepatoprotective evaluation

The effect of various fractions of MEEM on serum biochemical parameters AST, ALT, ALP and TB are summarized in table 2. A significant increase in the serum AST, ALT, ALP and TB levels were observed in paracetamol (2mg/kg) treated rats as compared to normal control rats. Pretreatment with test fractions (Group IV- IX) and silymarin (Group III) produced a decrease in the above said parameters, but significant decrease was obtained by hydroalcoholic fraction of MEEM and silymarin treated group.

The concentration of Malonaldehyde (MDA) significantly increased in the paracetamol treated group along with reduced levels of antioxidants (GSH and SOD) compared to normal group, whereas there is significant decrease in MDA and increase in antioxidants (GSH and SOD) in hydroalcoholic fraction of MEEM treated group (Table 3).

Table 2: Estimation of rat's biochemical profile of fractions of MEEM on paracetamol intoxicated rats

Groups	AST (U/L)	ALT(U/L)	ALP(U/L)	TB(mg/dl)
Control	195.39 ± 9.59	155.39 ± 9.59	69.95 ± 5.12	0.31 ± 0.06
Toxic group	319.56 ± 7.94	309.69 ±10.48	138.34 ±6.30	2.28 ± 0.05
Silymarin (50 mg/kg)	252.07 ± 9.85[a]	240.78 ± 9.57[a]	83.13 ± 6.32[a]	0.68 ± 0.08[a]
PE-MEEM (100 mg/kg)	274.56 ± 10.48[ns]	279.69 ±11.89[ns]	119.35 ± 4.23[ns]	1.97 ± 0.09[c]
PE-MEEM (200 mg/kg)	268.70 ± 8.48[c]	270.08 ± 9.89[ns]	109.56 ±6.34[c]	1.92 ± 0.07[c]
CF-MEEM (100 mg/kg)	265.34 ± 7.12[b]	258.40 ± 8.87[c]	102.34 ± 6.30[b]	1.58 ± 0.06[b]
CF-MEEM (200 mg/kg)	258.50 ± 8.48[a]	252.50 ± 10.87[b]	97.45 ± 5.23[b]	1.43 ±0.09[b]
HA-MEEM (100 mg/kg)	250.45 ± 7.36[a]	248.58 ±9.80[a]	92.40 ± 5.23[a]	1.28 ± 0.05[a]
HA-MEEM (200 mg/kg)	243.60 ± 9.49[a]	234.09 ± 9.98[a]	88.39 ± 6.30[a]	0.98 ± 0.06[a]

Values are expressed as mean ± SEM (n=6) [a]p<0.001 compared to paracetamol intoxicated group, [b]p< 0.01 compared to paracetamol intoxicated group, [c]p< 0.05 and ns > using one way ANOVA followed by Bonferroni's multiple comparison test.

Table 3: Effect of fractions of MEEM on MDA (nmole/min/mg of protein), GSH (nmole/mg of protein), SOD (unit/mg of protein)

Groups	MDA	GSH	SOD
Control	10.86 ± 0.64	29.58 ± 0.94	15.94 ± 0.52
Toxic group	23.36 ± 0.83	12.64 ± 0.42	4.97 ± 0.94
Silymarin (50 mg/kg)	12.34 ± 0.53[a]	24.59 ± 0.84[a]	14.54 ± 0.73[a]
PE-MEEM (100 mg/kg)	21.93 ± 0.34[ns]	14.24 ± 0.65[ns]	7.58 ± 0.49[ns]
PE-MEEM (200 mg/kg)	19.45 ± 0.45[c]	15.56 ± 0.74[ns]	8.48 ± 0.74[c]
CF-MEEM (100 mg/kg)	18.34 ± 0.63[b]	17.40 ±0.93[b]	9.56 ± 0.47[b]
CF-MEEM (200 mg/kg)	16.15 ± 0.52[a]	19.34 ± 0.62[a]	10.84 ± 0.63[a]
HA-MEEM (100 mg/kg)	15.59 ± 0.83[a]	21.40 ± 0.92[a]	11.34 ± 0.83[a]
HA-MEEM (200 mg/kg)	13.10 ±0.73[a]	23.23 ± 0.83[a]	13.58 ± 0.73[a]

Values are expressed as mean ± SEM (n=6) [a]p<0.001 compared to paracetamol intoxicated group, [b]p< 0.01 compared to paracetamol intoxicated group, [c]p< 0.05 and ns > using one way ANOVA followed by Bonferroni's multiple comparison test.

The biochemical analysis results were supported by histopathological examination of the liver tissue of control and treated groups. The histological changes in the liver architecture like piecemeal necrosis, ballooning degeneration and sinusoidal congestion due to intoxication by paracetamol was attenuated by the fractions of MEEM, particularly by hydroalcoholic fraction (fig. 1).

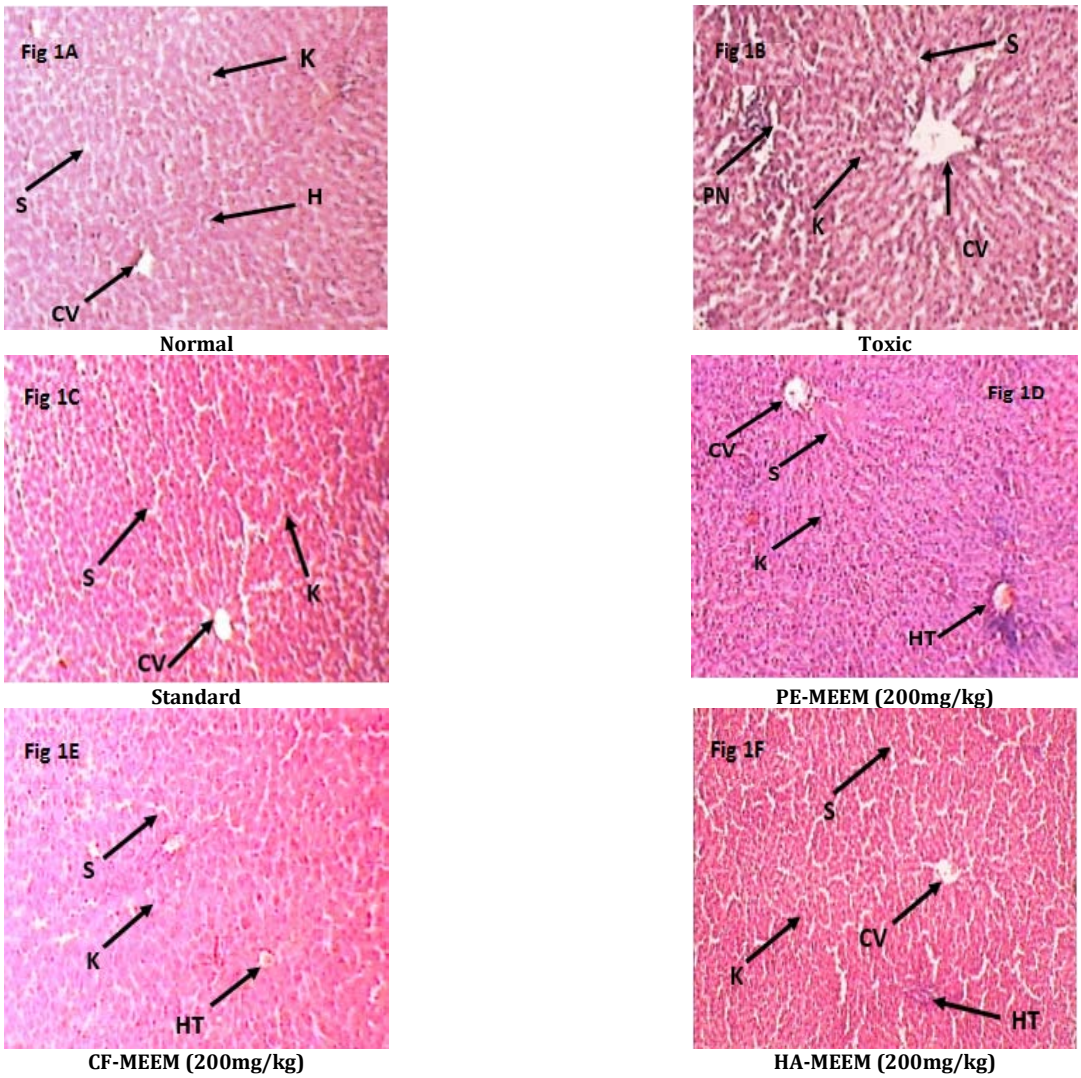

Fig. 1: Histopathological changes occurred in rats during paracetamol intoxication and prevention by the treatment with fractions of methanolic extract of leaves of *E. monogynum* (CV- Central Vein, H- Hepatocytes, HT- Hepatic Triad, K- Kupffer Cells, PN- Piecemeal necrosis S-Sinusoids)

Chromatographic evaluation

Chromatographic profile of HA-MEEM in n-butanol: glacial acetic acid: water: formic acid [4: 4.5: 2: 0.5(v/v)] showed the presence of six spots. One of the spot showed an identical R_f value with that of standard rutin (R_f-0.47) when visualized under UV-250 nm fig. 2(a-b).

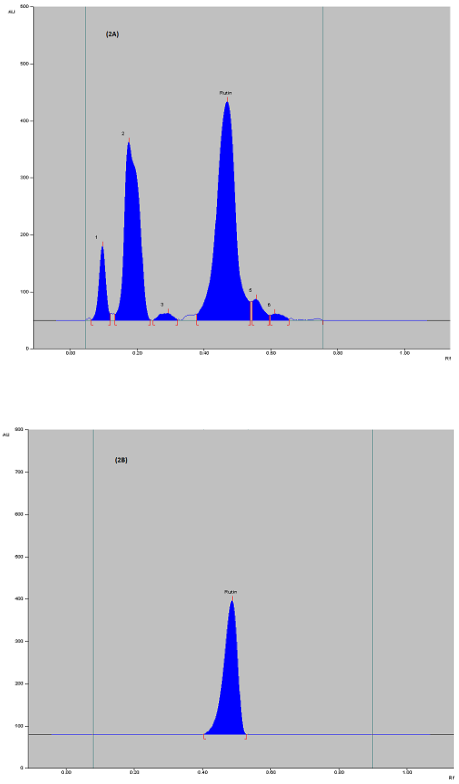

Fig. 2: HPTLC fingerprinting analysis of hydroalcoholic fraction of *E. monogynum* revealing the presence of rutin (Rutin λmax-250 nm, R_f-0.47). Fig. 2A: Rutin in HA-MEEM, fig. 2B: Standard of Rutin

DISCUSSION

The protective effect of methanolic extract of leaves of *E. monogynum* against is already known [6]. Here, the fractions of methnolic extract of leaves of *E. monogynum* have been revealed to have an important hepatoprotective effect against paracetaomol induced liver toxicity.

Paracetamol is a well known analgesic and antipyretic drug and it is known to cause severe hepatic damage in humans and experimental animals when used at high doses. N-acetyl-*p*-benzoquinonimine, NAPQI a reactive metabolite formed by the action of cytochrome P- 450 enzyme system is responsible for the toxicity [13]. When the structural intergrity of liver cell damaged a variety of cytoplasmic enzymes are released into the blood stream. A significant hepatic damage is evident by the quantitative estimation of these enzymes in serum which reveals the extent and type of hepatocellular damage [14].

However, pretreatment with fractions of MEEM attenuated the increased levels of biochemical parameters like AST, ALT, ALP and TB as compared to paracetamol treated group. Among the fractions, hydroalcoholic fraction (HA-MEEM) significantly reduced the levels of biochemical parameters indicating that it could maintain the functional and structural integrity of the liver cell membrane against paracetamol toxicity, which is also from the histopathological findings.

"Oxidative Stress"- is resulted due to the imbalance between ROS and antioxidant defense system which leads to hepatocellular necrosis. GSH and SOD are the biological antioxidants which scavenges the reactive metabolites of paracetamol [15]. In our study, reduced levels of these antioxidants were observed in paracetamol treated group. However, hydroalcoholic fraction significantly increased the levels of antioxidants against paracetamol treatment suggesting enhanced antioxidant properties. Hydroalcoholic fraction also attenatued the levels of MDA, product of lipid peroxidation when compared to toxic paracetamol treated group. The decrease in levels of MDA indicates the interaction of hydroalcoholic fraction with polyester fattyacids and thereby inhibiting the augumentation of the lipid peroxidation process leading to production of MDA.

HPTLC analysis of hydroalcoholic fraction (HA-MEEM) revealed the presence of six peaks of which one is comparable with that of standard rutin. Rutin- a flavanoidal glycoside reported having potent hepatoprotective action [16]. Rutin present in the hydroalcoholic possess maximum area compared to other peaks which reveals it as a major constituent in the extract. Moreover, preliminary phytochemical screening of hydroalcoholic fraction was also found to possess phenolic compounds. Therefore, we can consider that these identified compounds either individually or synergistically might be responsible for its hepatoprotective action against paracetamol induced toxicity.

In conclusion, the hydroalcoholic extract of leaves of methanolic extract of *E. monogynum* showed significant hepatoprotective activity. The extract showed the presence of rutin majorly, which is reported for its hepatoprotective action. Therefore, the hydroalcoholic extract should be considered for possible clinical application for the treatment of hepatic diseases.

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CONFLICT OF INTERESTS

Declared None

REFERENCES

1. Kamisan FH, Yahya F, Ismail NA, Din SS, Mamat SS, Zabidi Z, *et al*. Hepatoprotective activity of methanol extract of *Melastoma malabathricum* leaf in rats. J Acupunct Meridian Stud 2013;6(1):52-5.
2. Russmann S, Gerd A, Grattagliano I. Current concept of mechanisms in drug induced hepatotoxicity. Curr Med Chem 2009;16:3041–53.
3. Sheetal V, Singh SP. Current and future status of Herbal medicine. Veterinary World 2008;11:347-50.
4. Parrotta J. A Healing plants of peninsular India. CAB International; 2001. p. 279-80.
5. Kirtikar KR, Basu BD. Indian medicinal plant. 2nd ed, Vol I. Dehradun: International Book Distributors; 1987. p. 415.
6. Sabeena SH, Namdeo AG. Heptoprotective effects of leaves of *Erythroxylum monogynum* Roxb. on paracetamol induced toxicity. Asian Pac J Trop Biomed 2013;3(11):877-81.
7. Trease GE, Evans WL. *Pharmacognosy*. 16th ed. London: Bailliere Tindall Ltd; 2009. p. 60-75.
8. Jafri MA, Jalis Subhani M, Kalim javed, Surender Singh. Hepatoprotective activity of leaves of *Cassia occidentalis* against Paracetamol and ethylalchohol intoxofication in rats. J Ethnopharmacol 1999;66:355-61.
9. Slater TF, Sawyer BC. The stimulatory effects of carbon tetrachloride and other halogenoalkanes or peroxidative reactions in rat liver fraction *in vitro.* Biochem J 1971;123:805-14.
10. Morgan MS, Depirre JW, Mannervik B. Levels of glutathione, gulathione reductase and glutathione S-transferase activities in rat lung and liver. Biochem Biophy Acta 1979;582:67-78.
11. Misera HP, Fridocich. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for SOD. J Biol Chem 1972;3170-5.
12. Mythili S, Arunachalam S. High performance thin layer chromatography profile of Cassytha *filiformis*. Asian Pac J Trop Med 2012:S1431-5.
13. Yen FL, Wu TH, Lin LT, Lin CC. Hepatoprotective and antioxidant effects of *Cuscuta chinensis* against acetaminophen induced hepatotoxicity in rats. J Ethnopharmacol 2007;111:123-8.
14. Sivakrishnan S, Kottaimuthu A. Hepatoprotective acitivity of ethanolic extract of aerial parts of *Albizia procera* Roxb (Benth) against paracetamol induced liver toxicity. Int J Pharm Pharm Sci 2014;6:233-8.

15. Sathya A, Siddhuraju P. Protective effect of bark and empty pod extracts for *Acacia auriculiformis* against paracetamol intoxicated liver injury and alloxan induced type II diabetes. Food Chem Toxicol 2013;56:162-70.

16. Domitrovic R, Jakovac H, Marchesi VV, Knezevic SV, Cvijanovic O, Tadic Z, *et al*. Differential hepatoprotective mechanism of rutin and quercetin in CCl_4 intoxicated BALB/Cn mice. Acta Pharmacol Sin 2012;33:1260-70.