

## Hepatoprotective and Antioxidant Activities of *Feronia limonia* (leaves)

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### Summary

Methanolic extract of *Feronia limonia* (ME) was studied against carbon tetrachloride (CCl<sub>4</sub>) induced hepatic injury in albino rats and mice. In addition, antioxidant activity was studied by in vitro models. Pre-treatment with ME reduced biochemical markers of hepatic injury like serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), alkaline phosphatase (ALP), bilirubin, cholesterol, high density lipoprotein (HDL) and tissue glutathione (GSH) levels. Similarly pretreatment with ME reduced the CCl<sub>4</sub> induced elevation in the pentobarbitone sleeping time. Histopathological observations also revealed that pretreatment with ME protected the animals from CCl<sub>4</sub> induced liver damage. ME demonstrated dose dependant reduction in the in vitro and in vivo peroxidation induced by CCl<sub>4</sub>. In addition it showed dose dependant free radical scavenging activity. The amounts of total phenolic compounds were also determined in this study. The results obtained in the present study indicate that the ME can be a potential source of natural antioxidant and hepatoprotective activity.

**Key words:** ME, *Feronia limonia*, Antioxidant activity, hepatoprotective, DPPH assay, CCl<sub>4</sub>

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### Introduction

Liver is the most important organ, which plays a pivotal role in regulating various physiological processes in the body. It is involved in several vital functions, such as metabolism, secretion and storage. It has great capacity to detoxicate toxic substances and synthesize useful principles. Therefore, damage to the liver inflicted by hepatotoxic agents is of grave consequences (1, 2). Liver diseases are mainly caused by toxic chemicals, excess consumption of alcohol, infections and autoimmune disorders. Most of the hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative damages (3-5). In spite of tremendous advances in modern medicine, there are no effective drugs available that stimulate liver function, offer protection to the liver from damage or help to regenerate hepatic cells (6). In absence of reliable liverprotective drugs in modern medicine, there are a number of medicinal preparations in Ayurveda recommended for the treatment of liver disorders and their usage is in vogue since centuries (7).

*Feronia limonia* (Fam: Rutaceae) commonly known as wood apple or elephant apple, is a moderate size tree which is native of India and occurs up to an elevation of 1500 feet in western Himalayas. Ripe fruit of *F.limonia* contain tyramine derivative acicissimol, acidissiminin, sepxide, N.benzoyl tyramine and stigmasterol. [8] It is useful as tonic in diarrhoea, dysentery, stomatitis, tumors, cough, asthma, leucorrhoea, wounds and ulcers. Fruits, leaves and stem bark of *F.limonia* have been studied for anti-tumor [9], larvicidal [10] and antimicrobial activity [11.12].

Furthermore, *F. limonia* literature survey of revealed that no researcher has yet reported hepatoprotective and antioxidant activities of this plant. Therefore, it is worth conducting an investigation on the hepatoprotective and antioxidant methanolic extract of *F. limonia* (ME)

## Materials and Methods

### Plant material

The fresh ripe fruit of *F. limonia* were collected by a local supplier from around the Bangalore in the month of march-April. The Fruit material was Taxonomically identified by the Regional Research Institute, Karnataka, India and Voucher specimen RRI/BNG/DSRU/F54/2006-07. The leaves were dried under shade with and then powdered with a mechanical grinder and stored in an airtight container.

### Preparation of extracts.

The powder obtained was subjected to successive soxhlet extraction with the solvents with increasing order of polarity i.e. Pet. Ether (60-80°), Chloroform (59.5-61.5°), Methanol (64.5-65.5°) and water. Yield 3.29, 6.19, 11.70, and 15.71% respectively.

### Phytochemical screening

A preliminary phytochemical screening of all extracts carried out as described by Khandelwal K.L (13)

### Animals

Albino rats (Wistar) weighing 150-200g and albino mice weighing 20-25g of either sex were used in this study. They were procured from Sri Venkateshwara Enterprises, Bangalore. The animals were acclimatized for one week under laboratory conditions. They were housed in polypropylene cages and maintained at 27°C ± 2°C under 12 hrs dark / light cycle. They were fed with standard rat feed (Gold Mohr Lipton India Ltd.) and water *ad libitum* was provided. The litter in the cages was renewed thrice a week to ensure hygeinicity and maximum comfort for animals. Ethical clearance for handling the animals was obtained from the Institutional Animals Ethical Committee (MMU/IAEC/O8/2007) prior to the beginning of the project work. (131/1999/CPCSEA)

## **ANTIOXIDANT ACTIVITY**

### **Reducing power**

The reducing power of ME was determined according to the method of Oyaizu(33) . 10 mg of ME in 1ml of distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K<sub>3</sub>Fe(CN)<sub>6</sub>] (2.5 ml, 1%).The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 RPM for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl<sub>3</sub> (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Sodium metabisulphite was used as the reference material. . All the tests were performed in triplicate and the results averaged. The percentage decrease in absorbance was calculated. (14)

### **Inhibition of Hydroxyl radical**

Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the test compounds (ME) for hydroxyl radical generated by Fe<sup>3+</sup>-Ascorbate-EDTA-H<sub>2</sub>O<sub>2</sub> system (Fenton reaction) according to the method of Kunchandy and Rao . The reaction mixture contained in a final volume of 1.0 ml, 100 µl of 2-deoxy-2-ribose (28 mM in KH<sub>2</sub>PO<sub>4</sub>-KOH buffer, 20 mM, pH 7.4), 500µl of the various concentrations of ME and the reference compound sodium metabisulphate (25 µg) in KH<sub>2</sub>PO<sub>4</sub>-KOH buffer (20 mM, pH 7.4), 200 µl of 1.04 Mm EDTA and 200 µM FeCl<sub>3</sub> (1:1 v/v), 100 µl of 1.0 mM H<sub>2</sub>O<sub>2</sub> and 100 µl of 1.0 mM ascorbic acid was incubated at 37°C for 1h. 1.0 ml of thiobarbituric acid (1%) and 1.0 ml of trichloroacetic acid (2.8%) were added to the test tubes and incubated at 100°C for 20 min. After cooling, absorbance was measured at 532 nm against a control sample containing deoxyribose and buffer. Reactions were carried out in triplicate. Sodium metabisulphite used as the reference compound. All the tests were performed in triplicate and the results averaged. The percentage decrease in absorbance was calculated. (14)

#### **Inhibition of DPPH radical**

The free radical scavenging activity of ME was measured by 1, 1-diphenyl-2-picrylhydrazil (DPPH) using the method of Blois (31). 0.1 mM solution of DPPH in methanol was prepared and 1ml of this solution was added to 3 ml of various concentrations of ME and the reference compound (5, 10, 25, 50 and 100 µg). After 30 min, absorbance was measured at 517 nm. BHT was used as the reference material. All the tests were performed in triplicate and the graph was plotted with the mean values. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples (14).

#### **Inhibition of Nitric oxide radical**

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction (15,16). The reaction mixture (3 ml) containing sodium nitroprusside (10 mM) in phosphate buffered saline (PBS) and ME and the reference compound in different concentrations (5, 10, 25, 50 and 100 µg) were incubated at 25°C for 150 min. Each 30 min, 0.5 ml of the incubated sample was removed and 0.5 ml of the Griess reagent (1% sulphaniamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H<sub>3</sub>PO<sub>4</sub>) was added. The absorbance of the chromophore formed was measured at 546 nm. All the tests were performed in triplicate and the results averaged. BHT used as the reference compound. All the tests were performed in triplicate and the results averaged. The percentage decrease in absorbance was calculated. (14).

#### **Inhibition of Superoxide anion radical**

Measurement of superoxide anion scavenging activity of ME was performed based on the method described by Nishimiki (17) and slightly modified. About 1 ml of nitroblue tetrazolium (NBT) solution containing 156 µM NBT which is dissolved in 1.0 ml of phosphate buffer (100 mM, pH 7.4), 1 ml of NADH solution containing 468 µM of NADH which is dissolved in 1 ml of phosphate buffer (100 mM, pH 7.4) and 0.1 ml of various concentration of ME and the reference compounds (5, 10, 25, 50 and 100 µg) were mixed and the reaction started by adding 100 µl of phenazine methosulphate (PMS) solution containing 60 µM of PMS 100 µl of phosphate buffer (100 mM, pH

7.4). The reaction mixture was incubated at 250C for 5 min and the absorbance at 560 nm was measured against the control samples. Vitamin C used as the reference compound. All the tests were performed in triplicate and the results averaged. The percentage decrease in absorbance was calculated. (14).

#### **Amount of total phenolic compounds**

Total phenolic content of ME was assessed approximately by using the Folin–Ciocalteu phenol reagent method. To 200 ml of the, sample extracts were added 1.0 ml of Folin–Ciocalteu, reagent and 0.8 ml of sodium carbonate (7.5% w/v), and the contents were mixed and allowed to stand for 30 min. Absorption at 765 nm was measured in a, UV–Vis Spectrophotometer. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams, per gram of sample, using a standard curve generated with gallic acid (19).

### **HEPATOPROTECTIVE ACTIVITY**

#### **Acute oral toxicity studies**

The acute oral toxicity was performed according to OPPTS following up and down procedure. Colony bred female albino rats Wistar strain (150-200gm) were maintained under controlled animal house condition with access to food and water *ad libitum*. The limit test carried out first at 5000mg/kg. b.w. All animals were observed for toxic symptoms and mortality for 72 h. (20).

#### **CCl<sub>4</sub>-induced hepatotoxicity**

In this experiment 24 hr fasted animals were randomly divided in to 6 groups 6 animals each. Animals of group I and II received 1 ml of distilled water, whereas animals of group III, IV, V and VI received silymarin (100mg/kg), ME (200, 400, and 600mg/kg) respectively for 5 days orally. Animals of group I received 1ml/kg of liquidparaffin on 2<sup>nd</sup> and 3<sup>rd</sup> day, where as animals of group II,III,IV,V and VI received 2 ml CCl<sub>4</sub>: paraffin (1:1) simultaneously on 2<sup>nd</sup> and 3<sup>rd</sup> day 30 min after vehicle, silymarin or test extract. Animals were sacrificed under mild ether anesthesia. Blood samples and liver tissue were collected. (21) Blood samples collected from the above mentioned group of biochemical markers of liver damage like serum glutamate pyruvate transaminase

(SGPT) (22), serum glutamate oxaloacetate transaminase (SGOT) (23), alkaline phosphatase (ALP) (24), serum bilirubin (25), cholesterol (26) and HDL (27). Liver slices were collected from the above groups of animals were subjected to the determination of tissue glutathione (GSH) level and lipid peroxidation. Tissue GSH levels were estimated by using Ellman and modified by Aykac et al (28,29) similarly inhibition of CCl<sub>4</sub> induced tissue lipid peroxidation was done using the method of Buege and Steven (30).

#### **Effect of pentobarbitone sleeping time**

This test was performed to find out the influence on the CCl<sub>4</sub> altered functional performance of the liver. Albino mice of either sex were randomly divided in to 6 groups of 6 animals each. The animals of group I were administered pentobarbitone (40mg/kg) and noted the duration of sleep (duration of loss of righting reflex) was determined. The animals of group II, III, IV, V and VI received ip, CCl<sub>4</sub> (CCl<sub>4</sub>:paraffin, 1:1, 4ml/kg), silymarin (100mg/kg), ME 200, 400 and 600mg/kg respectively. Half an hour after the respective treatments, animals of group II, III, IV, V and VI were administered pentobarbitone (40mg/kg, ip) and sleeping times were determined. (21).

#### **Statistical analysis**

Results were expressed as mean±SE. statistical analysis was performed with one way analysis of variance (ANOVA) followed by Student's 't' test. P value less than 0.05 was considered to be statistically significant when compared to control.

#### **Histopathological observation**

Liver tissue collected were used for the preparation of histopathological slides by using microtome and were suitably stained and observed under microscope for the architectural changes seen during CCl<sub>4</sub> challenge in ME treated and control groups.

### **Results**

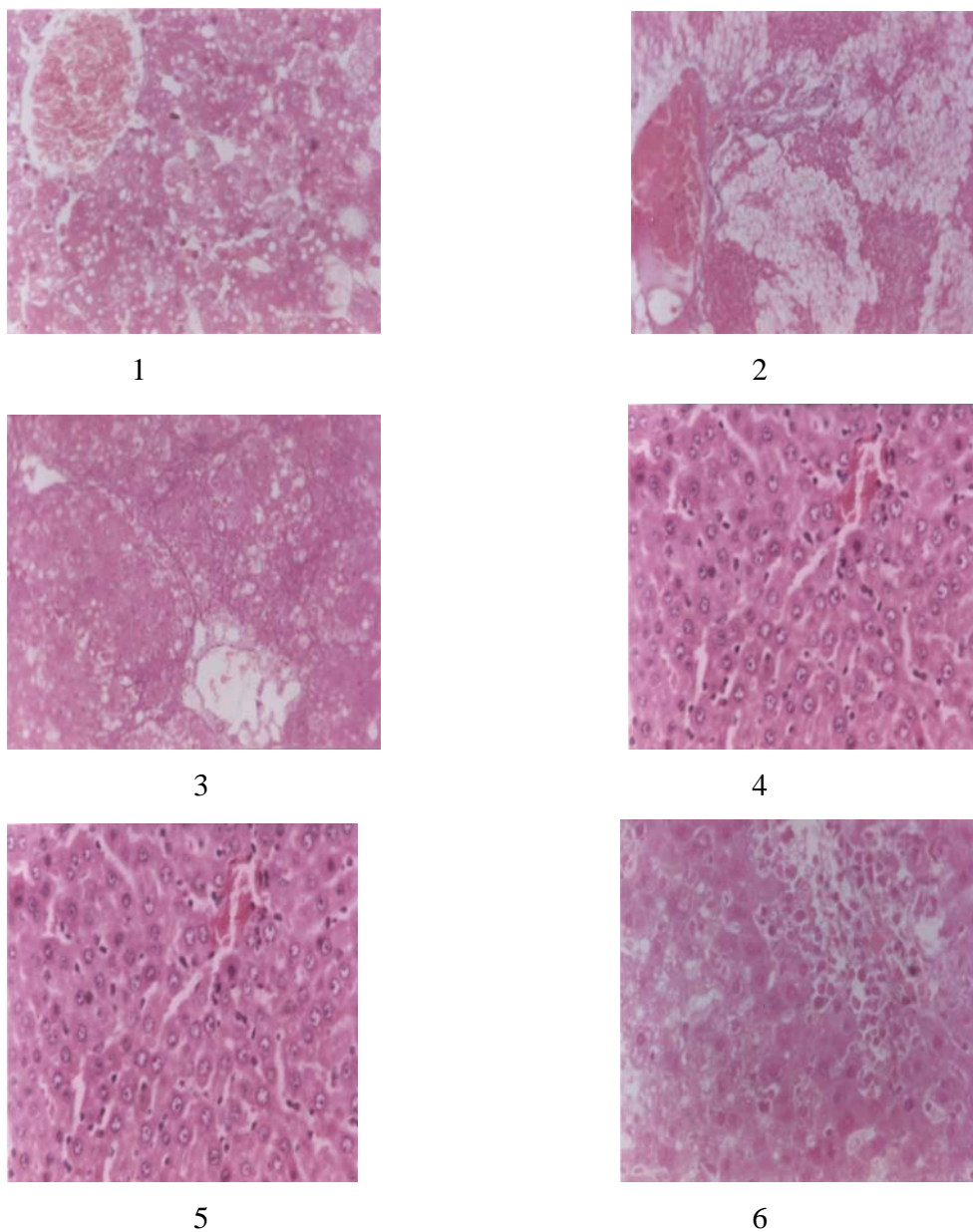
Preliminary phytochemical studies revealed the presence of tannins, phenols, proteins, aminoacids, flavanoids, triterpenoids, β-sitosterol, Vitamins, glycosides. The ME found to be non toxic up to 5000 mg/kg.

The antioxidant activity of ME in six in vitro models was studied. ME demonstrated antioxidant property in all the models. However, ME showed better reducing power

than the standard i.e. sodium metabisulphite (25 µg). In all other models(i.e DPPH, superoxide, nitric oxide, hydroxyl radical scavenging and inhibition of in vitrolipid peroxidation) antioxidant activity of even 100 µg of ME was less than of standards (Table 1) In the control group (+ve control) CCl<sub>4</sub> significantly enhanced the biochemical markers like SGPT, SGOT, ALP, cholesterol, and bilirubin and reduced the levels of HDL (Table 2). Pretreatment with ME (200, 400 and 600 mg/kg) reduced the elevated levels of all the above mentioned biochemical indicators and reduced the liver tissue GSH levels and increased the tissue peroxidation. Pretreatment with ME enhanced the tissue GSH levels and reduced lipid peroxidation (Table 3) Histopathological observations reveal that CCl<sub>4</sub> treatment has damaged the liver architecture (i.e. fatty degeneration of hepatic cells as indicated by ballooning of hepatocytes, fatty cyst, infiltration of lymphocytes and proliferation of Kupffer cells. Liver sinusoids were congested) and pretreatment with ME prevented/reversed the CCl<sub>4</sub> induced liver damage in a dose dependant manner. Fig 1 CCl<sub>4</sub> treatment enhanced the pentobarbitone sleeping time and pretreatment with ME reversed it (Fig 2).The total phenolic content of the ME was estimated to be 272.0 ± 2.2 mg Gallic acid equivalents/g of plant extract from triplicate measurements.



**FIG 1. Effect of ME of *F.limonia* on Liver architecture in normal and carbon tetrachloride treated rats**



- 1. Negative control
- 2. Carbon tetrachloride treated
- 3. Silymarin (100mg/kg)

- 4. ME (200mg/kg) +Carbon tetrachloride
- 5. ME (400mg/kg)+ Carbon tetrachloride
- 6. ME (600mg/kg) + carbon tetrachloride

Table 1: *In vitro* antioxidant effect of ME of *F.limonia*

[Values are mean SE from triplicate readings. Figures in parentheses are % increase (+) and decrease (-) in absorbance]

Treatment	Reducing power	Hydroxyl radical scavenging	DPPH scavenging	Nitric oxide scavenging	Superoxide anion scavenging	lipid peroxidation
Control	0.1270±0.015	0.310±0.014	0.855±0.009	0.292±0.003	0.592±0.004	0.2321±0.016
CCl4 (10µl)	----	-----	-----	-----	-----	0.3617±0.015
Sodium metabisulphite (25µg)	0.2382±0.014* (+87.56)	0.071±0.003* (-77.66)	-----	-----	-----	0.0583±0.012* (-83.88)
BHT (25µg)	-----	-----	0.123±0.008* (-85.56)	0.074±0.003* (-74.66)	-----	-----
Vitamin C (25µg)	----	-----	-----	-----	0.0162±0.008 (-72.64)	-----
ME (5µg)	0.1723±0.033* (+31.61)	0.1723±0.035* (+31.61)	0.701±0.005* (-18.01)	0.233±0.006* (-20.21)	0.469±0.016* (-20.78)	0.2170±0.014* (-40.01)
ME (10µg)	0.1992±0.023* (+56.85)	0.213±0.002* (-31.29)	0.636±0.007* (-25.61)	0.212±0.008* (-27.4)	0.401±0.005* (-32.26)	0.1975±0.016* (-45.39)
ME (25µg)	0.2181±0.018* (+71.73)	0.161±0.004* (-41.40)	0.584±0.03* (-31.7)	0.165±0.004* (-43.49)	0.325±0.015* (-45.1)	0.1830±0.022* (-49.40)
ME (50µg)	0.2341±0.034* (+84.33)	0.131±0.008* (-57.74)	0.409±0.008* (-52.16)	0.120±0.007* (-58.9)	0.272±0.013* (-54.05)	0.1623±0.025* (-55.13)

Significant at P<0.001 compared to control

**Table 2: Effect of ME on biochemical parameters of CCl4 induced hepatic injury.**  
[Values are mean ± SE from 6 animals in each treatment]

Treatment	SGPT (U/l)	SGOT (U/l)	ALP (U/l)	Total bilirubin (mg/dl)	Direct bilirubin (mg/dl)	Cholesterol (mg/dl)	HDL (mg/dl)
Control (Saline 0.5ml)	47.51±1.75	0.89±1.16	131.15±1.75	0.92±0.05	0.18±0.05	106.76±2.83	48.41±1.44
Silymarin (100mg/kg+CCl4)	61.32±2.33*	134.72±1.77*	93.12±1.99*	1.26±0.06*	0.37±0.04*	120.16±4.85	46.81±1.25*
ME (200mg/kg+CCl4)	143.71±0.013*	255.32±1.54*	153.32±1.99*	2.12±0.04*	0.83±0.05*	147.12±4.24*	31.38±1.69*
ME (400mg/kg+CCl4)	114.32±0.018*	197.91±2.15*	128.1±2.05*	1.86±0.04*	0.67±0.06*	139.31±3.67*	36.71±1.85*
ME (600mg/kg+CCl4)	87.32±0.07*	163.67±0.97*	93.81±1.196*	1.53±0.04*	0.52±0.04*	126.32±3.83*	41.32±1.703*

\*P<0.01 Vs CCl4 group.

ME- Methanolic Extracts of *F.limonia*

**Table 3: Effect of ME on the liver tissue levels of GSH and lipid peroxidation in the CCl4 treated rats.**

[Values are mean±SE from 6 animals. Figures are % increase (+) and decrease (-) in absorbance]

Group	Tissue levels	Tissue lipid peroxidation
Control Saline (0.5ml)	0.9251±0.05	0.2540±0.06
CCl4 (2ml/kg)	0.4281±0.09	0.3732±0.06
Silymarin (100mg/kg+CCl4)	0.8329±0.05 (+92.46).	0.1332±0.06 (- 64.30)
ME (200mg/kg+CCl4)	0.5994±0.08 (+40.01)	0.2010±0.06 (- 46.14)
ME (400mg/kg +CCl4)	0.6871±0.06 (+60.05)	0.1732±0.05 (- 53.59)
ME (600mg/kg +CCl4)	0.8032±0.05 (+77.80)	0.1320±0.06 (-64.63)

\*P<0.01 Vs CCl4 group

ME- Methanolic Extracts of *F.limonia*

### **Discussion**

CCl<sub>4</sub> induced hepatic damage is due to its cytochrome P-450 enzyme system catalyzed hepatic conversion in to highly reactive trichloromethyl radical (CCl<sub>3</sub>\*), which upon reaction with oxygen radical gives trichloromethyl peroxide radical (OCCl<sub>3</sub>\*). This radical forms covalent bond with sulfhydryl group of several membrane molecules like glutathione, which is considered as the initial step in the chain of events leading to lipid peroxidation and hepatic tissue destruction. Extent of decrease in tissue GSH and tissue lipid peroxidation is a measure of tissue destruction (32) ME demonstrated potent DPPH, superoxide, nitric oxide, hydroxyl radical scavenging property. Therefore, it may be inferred that antioxidant property of the study plant may prevent formation of trichloromethylperoxide radical. Thereby inhibit the lipid peroxidation and offer hepatoprotection against CCl<sub>4</sub> challenge. Prevention of tissue GSH depletion by ME treatment also indicates that the natural inbuilt tissue protective mechanism is kept intact and oxidative degeneration of tissue is prevented. However it is necessary to rule out the inhibitory effect on the cytochrome P-450 enzyme system. The ME has exhibited antioxidant property which may be responsible for hepatoprotective activity. The phytochemical test indicated presence of tannins, flavanoids, phenols, glycosides, vitamins and triterpenes. Some of these constituents have been responsible for observed activity.

### **Conclusion**

In conclusion, the present study demonstrated that the leaves of *Feronia Limonia* possess hepatoprotective property may be attributed to the antioxidant principles of this plant. Further study is warranted to isolate, characterize and screen active principles from ME that possess antioxidant and hepatoprotective properties.

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