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Cytotoxicity evaluation and hepatoprotective potential of bioassay guided fractions from *Feronia limmonia* Linn leaf

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ABSTRACT

Objective: To evaluate the cytotoxicity and hepatoprotective potentials of extracts, fractions or isolated compound from the leaves of *Feronia limonia* (*F. limonia*). **Methods:** Qualitative phytochemical analysis of extracts, fractions or compound was performed by means of thin layer chromatography and spectroscopic assays. The % purity of compound was measured by analytical HPLC. Extracts, fractions or compound have been individually evaluated for their cytotoxicity effects (10, 20, 100, 250, 500, 750 and 1 000 μ g/mL). Based on the inhibitory concentration (IC_{50}) obtained from the cell viability assay, graded concentrations of extracts, fractions or isolated compound were assessed (10, 20, 50, 100, 200 μ g/mL) for its hepatoprotective potential against CCl_4-induced hepatotoxicity by monitoring activity levels of serum glutamatic pyruvatic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT). **Results:** Results indicated that the methanol extract of *F. limonia* was non-toxic and hepatoprotective in nature as compared with the petroleum ether extract. The acetone fraction of methanolic extract also showed similar properties but the subsequent two fractions were cytotoxic. However, the pure compound isolated from the penultimate fraction of methanolic extract was non-toxic and hepatoprotective in nature. Biochemical investigations (SGOT, SGPT) further corroborated these cytological observations. **Conclusions:** It can be concluded from this study that *F. limonia* methanol extract, some fractions and pure isolated compound herein exhibit hepatoprotective activity. However, cytotoxicity recorded in the penultimate fraction and investigation of structural details of pure compound warrants further study.

1. Introduction

Liver is a major organ of human body that plays a crucial role in elimination and biotransformation of toxic substances. During the sojourn of detoxification, reactive oxygen species (ROS) are generated within hepatocytes that result in oxidative damage, gross cellular changes and cell death causing hepatotoxicity or liver damage[1,2]. Since the modern system of medicine is known for inducing liver damage as a part of side effects[3], a hepatoprotectant of herbal origin can be considered as a useful, safe and effective co-supplement to minimize the mentioned manifestations.

Indian subcontinent has historical tradition of using medicine of herbal origin that is often considered to be protective and curative with minimal side effects[4]. It has been reported that 80% of the existing popular drugs in the market have a herbal lineage[5]. There are also reports on "whole plant" studies that have revealed multifaceted therapeutic potential of roots and various aerial parts of medicinal plants[6].

Feronia limonia (*F. limonia*) (family Rutaceae, subfamily Aurantioideae), is commonly known as 'kaitha' or wood apple[7] and widely distributed in deciduous and arid landscapes of several countries in South Asia[8]. *F. limonia* as a whole, or its parts such as unriped fruit, riped fruit, root, bark, trunk gum and leaves have a broad spectrum of traditionally established therapeutic

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properties[9]. Leaf extracts of *F. limonia* has been reported to possess antioxidant[10], larvicidal[11], antidiabetic[12] and hepatoprotective[10] potentials. Decoction of *F. limonia* leaves is consumed by some Indian tribes for treating acidity and related gastrointestinal problems[13].

Phytochemical analysis of *F. limonia* leaves has also extensively been reported[14], but these studies lack scientific investigation pertaining to their therapeutic/ protective role in various facets of human metabolism. In this regard, the present study was aimed to investigate the cytotoxicity and hepatoprotective role of bioassay guided fractions of *F. limonia* in an *in vitro* experimental design.

2. Materials and methods

2.1. Plant material

F. limonia leaves were collected in September to October, 2008 from campus of The M. S. University of Baroda, Vadodara, India. They were authenticated in the Botany Department and a voucher specimen (No.Pharmacy/FL/ 08-09/01/MJ) was deposited in the Pharmacy Department, The M. S. University of Baroda, Vadodara, India.

2.2. Extraction and isolation

The leaves were shade dried, powdered (500 g) and extracted three times with petroleum ether (3×1.5 L) in a soxhlet apparatus. The filtrates were then combined and filtered and concentrated to dryness in a rotary evaporator (Buchi-R-215, Germany) to obtain a crude petroleum ether extract (FL-1). The remaining marc was then dried and again exhaustively extracted at temperature (60-80 ℃) with methanol (3×1.5 L) in a soxhlet apparatus. The pooled extracts obtained were then concentrated under vacuum to give methanolic extract (FL-7). This extract was re-dissolved in water: methanol and partitioned with organic solvents to provide a $CHCl_3$ fraction (FL-9). This fraction was further fractionated by column chromatography using silica gel (60 # 120 mesh) and eluted with chloroform (100%). A total of 22 test tube fractions were collected. Fractions No. 13, 14 were combined (due to their identical TLC characteristics) to obtained a single fraction (FL-10). This fraction was washed with n-hexane FL-11 to obtain its insoluble portion purified with preparative TLC using mobile phase toluene-ethyl acetate (9:1) to yield a pure compound MR-2. The % purity of MR-2 was confirmed by analytical HPLC.

2.3. HPTLC fingerprinting of the extract, fractions and isolated compound

Qualitative fingerprinting of FL-1, FL-7, FL-9, FL-10 and isolated compound MR-2 was performed by thin layer chromatography (TLC). TLC analysis were carried out on A Camag TLC system equipped with Camag Linomat V an automatic TLC sample spotter, Camag glass twin trough chamber (20 cm × 10 cm), Camag scanner 3 and integrated win CATS 4 Software. TLC was performed on a pre-coated TLC plate silica gel 60 F254 plates (Kieselgel 60 F254, Merck, Germany)[15], using the mobile phases of toluene-ethyl acetate (85:15). Detection of chemical constituent was done under UV at 365 nm as reported by Wagner *et al*[16].

2.4. Maintenance of HepG2 cells

Human liver hepatoma cells (HepG2) (obtained from National Centre for Cell Sciences, Pune, India) were seeded (1×10^5 cells/25 mm T Flask) and cultured in DMEM containing 10% FBS and 1% for 24 h at 37 ℃ with 5% CO_2 (Thermo scientific, forma II water jacketed CO_2 incubator). Cells were sub-cultured every third day by trypsinization with 0.25% Trypsin-EDTA solution. All the reagents were sterile filtered through 0.22 μ filter (Laxbro Bio-Medical aids Pvt. Ltd, Mumbai, India) prior to use for the experiment.

2.5. In vitro cytotoxicity assay

HepG2 cells (5.0×10^3 cells /well) were maintained in 96 well culture plate (Tarson India Pvt Ltd) for 72 h in presence of FL-1, FL-7, FL-9, FL-10, FL-11 or MR-2 at the concentrations of 10, 20, 100, 250, 500, 750 and 1 000 μg/mL. At the end of incubation period, 10 μL of MTT (5 mg/mL in PBS) was added to wells and the plate was incubated at 37 ℃ for 4 h. At the end of incubation, culture media was discarded and the wells were washed with PBS (Himedia Pvt Ltd, Mumbai, India). Later, 150 μL of DMSO was added to all the wells and, were incubated for 30 min at room temperature with constant shaking. Absorbance was read at 540 nm using ELX800 Universal Microplate Reader (Bio-Tek instruments, Inc, Winooski, VT) and subsequently % cell viability was calculated[17].

2.6. In vitro CCL_4 induced hepatotoxicity in HepG2 cells

HepG2 cells (5.0×10^3 cells /well) were maintained in culture media containing 1% CCL_4 in presence or absence of FL-1, FL-7, FL-9, MR-2 or sylimarin at the concentrations of 10, 20, 50, 100, 200 μg/mL for 24 h. Later, supernatants from each well were removed and activity levels of serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT) were determined using commercially available enzymatic kits Merck microlab300 semi-autoanalyzer as per the instruction of manufacturer.

2.7. Morphological analysis of HepG2

HepG2 cells (1.0×10^5 cells /well) were maintained in culture media containing 1% CCL_4 in presence or absence of FL-1, FL-7, FL-9, MR-2 or sylimarin at the concentrations of 10, 20, 50, 100, 200 μg/mL for 24 h. At the end of experimental period, cells were fixed in 4% paraformaldehyde for 10 min, mounted in glycerin and examined under Leica DMIL inverted microscope (40×) and photographed.

2.8. Statistical analysis

Data were analysed for statistical significance using one way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test and results were expressed as mean±SEM using Graph Pad Prism version 3.0 for Windows, Graph Pad Software, San Diego California USA.

3. Results

3.1. Phytochemical analysis

Phytochemical analysis using HPTLC assays provided qualitative insights into the bioactive constituents of the FL-1, FL-7, FL-9, and FL-10 and isolated compound MR-2. TLC characterizations of all the extracts, fractions and isolated compound was done at UV 360 nm because some secondary metabolites like flavanoids, coumarins, *etc* showed fluorescence at 360 nm. The chromatogram of extracts and fractions showed many spots with different R_f values such as FL-1, 0.04, 0.16, 0.19, 0.22, 0.27, 0.36, 0.40, 0.48, 0.57, 0.65, 0.67, 0.79, 0.86, 0.89, 0.94; FL-7, 0.03, 0.16, 0.36, 0.48, 0.60, 0.67, 0.78, 0.84; FL-9, 0.03, 0.15, 0.24, 0.44, 0.56, 0.60, 0.66, 0.72, 0.77, 0.84, 0.92; Fl-10, 0.33, 0.60 and chromatogram of isolated compound showed single spot at R_f 0.60. Among the several spots present in FL-7, FL-9, FL-10 one spot exactly matched with the isolated compounds R_f value, and it was found to be more intense compared with the other spots. Hence, it indicated the extract or fractions contain isolated compound. TLC chromatogram of extracts, fractions and isolated compound were shown in Figure 1 and 2.

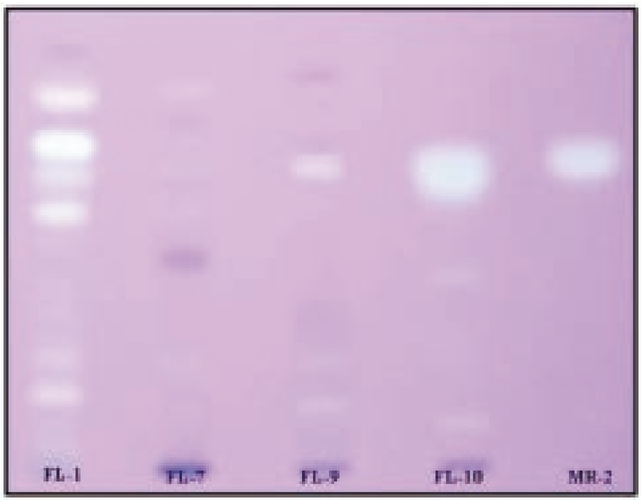

Figure 1. TLC fingerprinting of extracts, fractions and isolated compound from *F. limonia* leaf at UV 365 nm.

3.2. Characteristic and analytical HPLC of isolated compound MR-2

The isolated compound has white needle shaped crystals having melting point at 188 ℃. UV λ max was 265 nm. The percentage purity of isolated compound was found to be 96%. HPLC chromatogram was shown in Figure 3.

Table 1
Effect of *F. limonia* extracts, fractions, isolated compound (MR-2) and sylimarin on CCL_4 induced hepatotoxicity (mean±SEM) (n=3).

Treatments		SGOT (IU/L)	SGPT (IU/L)	Cell viability (%)
Control		5.00±0.57	4.00±0.88	100.00±0.00
1% CCL_4		15.00±3.46$^{###}$	10.00±1.78$^{###}$	20.81±1.21$^{###}$
1% CCL_4 + Sylimarin (μg/mL)	10	6.33±0.33**	5.33±0.88**	74.28±1.70***
	20	6.33±0.66**	3.33±0.66***	81.17±1.99***
	50	4.00±0.57***	2.66±0.88***	84.41±1.87***
	100	4.00±1.00***	2.66±0.33***	94.54±4.10***
	200	3.33±0.88***	2.33±0.88***	96.85±3.45***
1% CCL_4 + FL-1 (μg/mL)	10	7.33±0.88*	6.66±1.00**	84.06±2.00***
	20	7.00±0.57**	3.00±0.57***	84.43±4.99***
	50	5.00±0.58***	2.33±0.66***	78.48±5.49***
	100	3.66±0.88***	2.00±0.57***	76.79±6.29***
	200	2.33±0.88***	1.33±0.33***	79.60±8.00***
1% CCL_4 + FL-7 (μg/mL)	10	7.00±0.57*	4.66±1.10***	87.85±4.32***
	20	6.33±0.88**	4.33±0.89***	85.18±1.93***
	50	3.33±0.88***	3.33±0.66***	85.56±2.29***
	100	2.00±0.58***	3.00±0.57***	78.01±4.12***
	200	1.66±0.33***	1.66±0.33***	77.40±3.46***
1% CCL_4 + FL-9 (μg/mL)	10	7.66±0.67*	2.66±0.66***	90.13±2.08***
	20	5.00±0.58**	2.00±0.57***	98.82±2.78***
	50	4.00±0.56***	2.33±0.42***	85.23±3.23***
	100	3.00±0.44***	1.33±0.33***	87.57±1.44***
	200	2.33±0.34***	1.33±0.33***	88.65±2.81***
1% CCL_4 + MR-2 (μg/mL)	10	7.00±0.65*	7.6±0.88*	54.21±2.00**
	20	6.00±1.00**	6.00±0.57**	65.13±1.16***
	50	6.33±0.88**	6.66±0.88**	83.02±2.11***
	100	4.12±0.65***	4.22±0.32***	83.40±2.79***
	200	3.11±0.23***	2.00±0.21***	85.48±2.99***

$^{###}P$<0.001 compared with control; *P<0.05, **P<0.01, ***P<0.001 compared with 1% CCL_4.

3.3. Cytotoxicity assessment of F. limonia extracts/fractions and MR-2

Cytotoxicity assessment of *F. limonia* extracts (Petroleum ether and methanol, respectively) revealed an identical pattern of cytotoxicity with both showing less that 50% cell viability at 250 μg /mL dose. However, methanolic fractions (FL-9, FL-10 or FL-11) showed a different pattern of cytotoxicity of HepG2 cells. FL-9 exhibited the highest percentage of cell viability (65%) at 200 μg/mL. However, FL-10 and FL-11 recorded significant cytotoxicity, which was characterized by less than 50% cell viability at all the doses. MR-2 recorded much improved cell viability as compared with its preceding fractions (Figure 4).

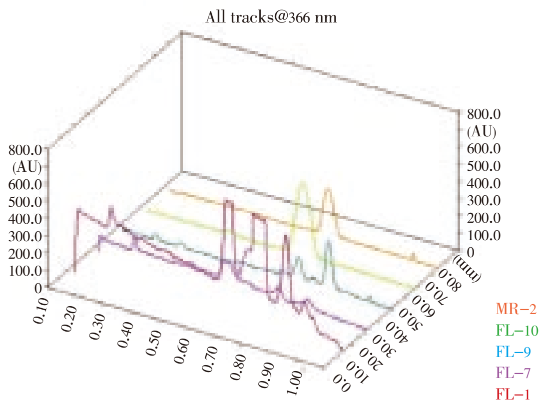

Figure 2. Three-dimensional overlaid chromatogram of extracts, fractions and isolated compound from *F. limonia* leaf.

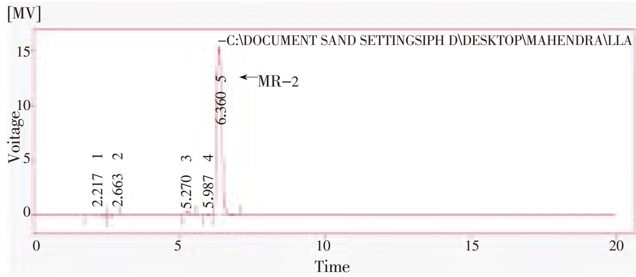

Figure 3. Parameters and chromatogram of isolated compound MR-2 by HPLC process.

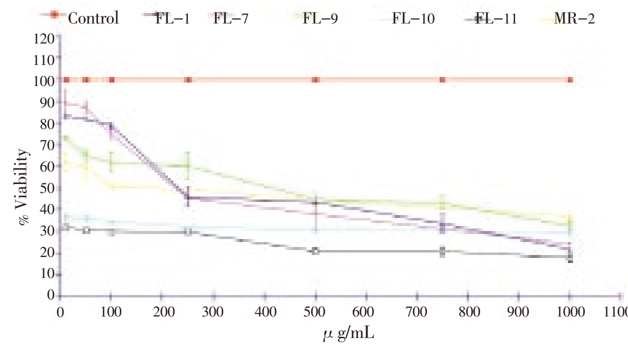

Figure 4. Cytotoxicity evaluation of *F. limonia* leaf extracts, fractions, isolated compound (MR-2).

3.4. Hepatoprotective potential of F. limonia extracts/ fractions and MR-2

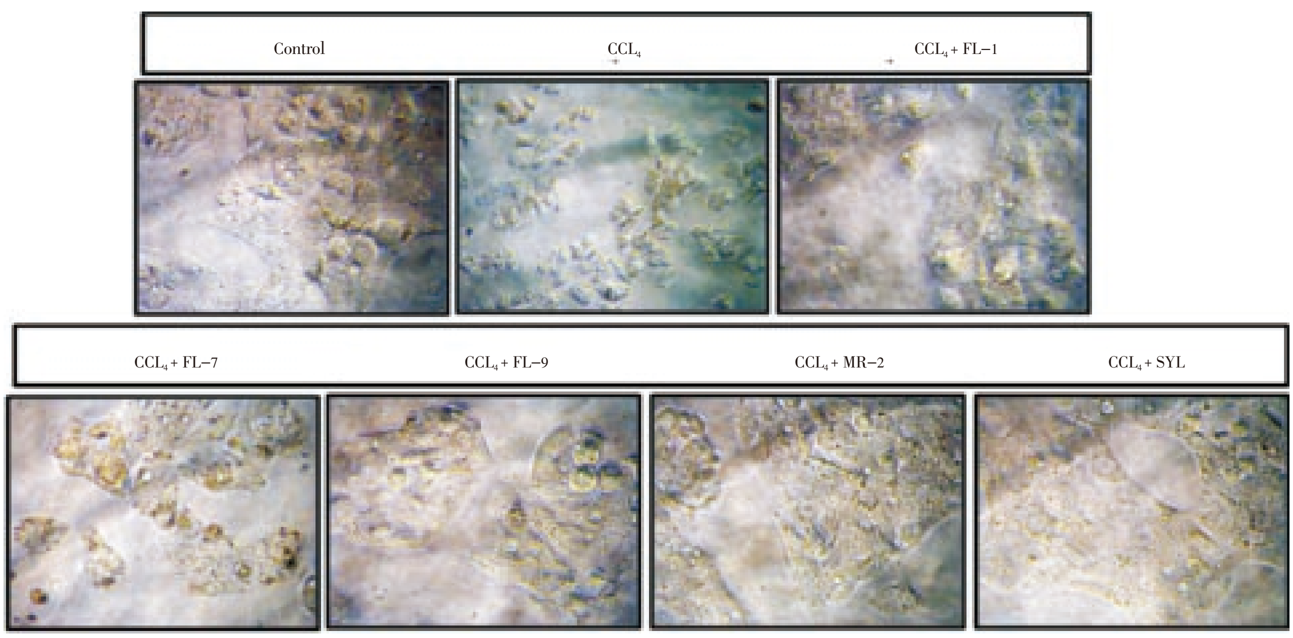

Figure 5. Effect of *F. limonia* extracts, fractions, isolated compound (MR-2) and sylimarin on CCL_4 induced hepatotoxicity.
SYL: Sylimarin.

Activity levels of SGOT and SGPT in cell supernatants revealed that there was a significant increment in their activity levels in cells treated with 1% CCl_4. However, co-supplementation of FL-1, FL-7, FL-9 or MR-2 recorded a non-linear dose dependent decrease in activity levels of SGOT and SGPT. These activity levels were comparable to that of dose dependent decrease in CCl_4 and sylimarin treated groups (Table 1 and Figure 5).

4. Discussion

Extracts of *F. limonia* leaves has been extensively studied for its therapeutic potential and its chemical analysis has been reported extensively. These reports have demonstrated the presence of flavonoids, steroids, volatile oils, fatty acid and coumarins[18-20]. In the present study petroleum ether and methanolic extract, and subsequent bio-assay guided fractions of methanolic extract have been obtained and assessed. Also a pure compound MR-2 has been isolated from one of the methanolic fractions. They were thought to be cytotoxic. Purity of MR-2 was validated (96%) and its structural analysis using 13C NMR, Mass (ESI-MS) spectra and CHN analysis is currently in progress. This study investigates the cytotoxicity and hepatoprotective potential of bioassay guided fractions of *F. limonia* leaf.

In recent times *in vitro* cytotoxicity of plant extracts and bioassay guided fractions has gained importance for primary level screening. Also HepG2 is a popular and an effective *in vitro* model for assessing hepatoprotective potential of phyto compounds or bioassay guided fractions due to its functional similarity with an intact liver[21].

Results of the study clearly indicate that FL-7 extract of *F. limonia* provides superior hepatoprotection to FL-1 because methanolic extract has been extensively reported for the presence of copious amounts of coumarins and flavanoids[19]. Positive results from FL-9 are also attributed to the same reason. However, cytotoxicity in FL-10 and FL- 11 is inexplicable and warrants further study. FL-9 fraction imparts superior hepatoprotection to FL-7 possibly because of the flavanoids and coumarins undergoing concentration due to fractionation. It can also be assumed that reappearance of hepatoprotection in MR-2 and its non-toxic nature can be attributed to the possible removal of the unknown toxic substance in the insoluble fraction.

It can be concluded from the present study that the hepatoprotective potential of methanolic extract and some of its fractions is attributed to flavanoids and coumarins content rich in its leaf extract. Structural details of MR-2 shall provide further insight into its hepatoprotective potential. Ongoing studies for assessment of *in vivo* hepatoprotection using these bioassay guided fraction is under progress in our laboratory. This study provides the first scientific evidence about the hepatoprotective nature of bioassay guided fractions of *F. limonia*.

Conflict of interest statement

We declare that we have no conflict of interest.

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