List of Publications

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1. Jain M, Trivedi A, Mishra SH. 2010. TLC Determination of Marmesin, a Biologically Active Marker from Feronia Limonia L. American Journal of Plant Sciences 1: 12-16.
2. Jain M, Kapadia R, Jadeja RN, Thounaojam MC, Devkar RV, Mishra SH, 2011. Cytotoxicity evaluation and hepatoprotective potential of bioassay guided fractions from *Feronia limmonia* Linn leaf Asian.J.Trop.Biomed 1: 443-447.
3. Jain M, Kapadia R, Jadeja RN, Thounaojam MC, Devkar RV, Mishra SH, 2011. Effects of standardized Feronia limonia stem bark methanolic extract on in vitro and in vivo CCl_4 induced toxicity and pathological alterations. Experimental and Toxicologic Pathology. (Under communication)
4. Jain M, Kapadia R, Jadeja RN, Thounaojam MC, Devkar RV, Mishra SH, 2011. Hepatoprotective potential of methanolic extract and marmesin isolated from root bark of Feronia limonia: An in vitro and in vivo studies. Food and ChemicalToxicology. (Under communication)
5. Jain M, Kapadia R, Albert S, Mishra SH, 2011. Standardization of *Feronia limonia* (Linn.) leaves by HPLC, HPTLC, physico-chemical and histological parameters. Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas [BLACPMA] (Under communication)

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TLC Determination of Marmesin, a Biologically Active Marker from *Feronia Limonia* L.

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ABSTRACT

Feronia limonia Linn. (Rutaceae) have gained traditional therapeutic importance owing to their high essential oil and coumarins content. Marmesin, a furanocoumarin was identified by TLC and isolated by column chromatography and further purified by Preparative TLC. Presently, there is no appropriate TLC based method available for standardization of F. limonia. A simple, sensitive and accurate high performance thin layer chromatographic (HPTLC) method has been developed for the estimation of marmesin in the methanolic extract of stem bark of Feronia limonia. HPTLC was performed on precoated silica gel $60F_{254}$ aluminium plates (20 cm × 20 cm) with Chloroform: Methanol (9.5:0.5), as mobile phase. Quantitative evaluation of the plate was performed in the absorption-reflection mode at 338 nm. The calibration curve was linear in the concentration range of 20 – 100 ng spot^{-1}. The method was validated for precision, repeatability and accuracy. The technique has been applied, for the first time, for the estimation of marmesin. The proposed method was found to be robust, precise, and accurate, it therefore holds potential for detection, monitoring and quantification of marmesin in Feronia limonia and its related formulation.

Keywords: *Marmesin, Feronia Limonia, HPTLC*

1. Introduction

Standardization and characterization of herbal drugs is a topic of continuous scientific interest in the herbal drug industry [1]. With the advent of modern chromatographic systems there is an ever increasing intent to produce and develop easy, rapid, convenient and cost effective methods for standardization of herbal drugs based on their phytoconstituents. This requirement is fulfilled by thin layer chromatography (TLC) [2,3]. *Feronia limonia* is (family Rutaceae, subfamily Aurantioideae), commonly known as wood-apple , belongs to the tribe Citreae and subtribe Balsamocitrinae[4] which is widely distributed in dry warm regions of India, Bangladesh, Barma, Ceylon, Java & Srilanka [5,6]. This plant recently gained a great therapeutically relevance owing to their high Coumarins and monoterpenoids content, which is explored for treatment of snake bite [7]. Stem bark mainly consists of furan Alkaloid; Coumarins; Flavanones; Lignan; triterpene [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 [8].

Marmesin is one of the most prevalent linear dihydrofuranocoumarin, is abundant in species belonging to the families of Umbelliferae, Apiaceae, Rutaceae, Moraceae, and Leguminoseae [11,12]. It is originally isolated from indigenous indian plants, *Aegle marmelos Correa* [13], and later from the Hawiian shrub *Pelea barbigera* [14] both of these are from rutaceae family. It has an amazing array of scientifically acknowledged benefits for key areas of health, as dermal photosensitizing activity beneficial in the treatment of leucoderma [15], antifungal activity [16], phytoalexin [17], feeding deterrence effects [18] and radical scavenging activity [19]. Currently HPTLC is often used as an alternative to HPLC for the quantification of plant products because of its simplicity, accuracy, cost-effectiveness and rapidity [20]. The present study is based on development of methods for determination of marmesin by HPTLC in *F. limonia* stem bark that may contribute in standardization of raw material of the plant and its formulation.

2. Experimental

2.1. Reagents and Chemicals

All the chemicals, including solvents, were of analytical grade from E. Merck, India. The HPTLC plates Si $60F_{254}$ (20 cm × 20 cm) were purchased from E. Merck (Darmstadt, Germany).

2.2. Plant Materials

The plant material of *Feronia limonia* was collected in the months of September– October 2008 from campus of The M.S. University, Vadodara (Gujarat). They were authenticated in the Botany Department and a voucher specimen (No.Pharmacy/FL/ 08-09/01/MJ) has been deposited in the Pharmacy Department of The M. S. University of Baroda, Vadodara, India.

2.3. Extraction and Isolation of Reference Compound (MR-1) from *Feronia Limonia*

Air-dried and finely powdered stem barks of the plant (500 g) were exhaustively extracted at temperature (60-80°C) with methanol (3 × 1.5 L) in a soxhlet apparatus and the pooled extracts then obtained were concentrated under vacuum to give methanolic extract. Methanolic extract was made hydroalcoholic by addition of hot distilled water in 1:1 ratio partitioned with chloroform (100 mL × 4), and combined chloroform fraction was concentrated in vacuum to afford a brown residue (4.5 g). This residue was chromatographed over a Silica gel (60#120 mesh size) column eluting with toluene followed by increasing concentrations of ethyl acetate and methanol. Fraction 9-10 (toluene: ethyl acetate, 60:40) yielded yellowish crystal resulted in mixture of compounds on TLC. Further purification of MR-1 was achieved by preparative TLC (chloroform: methanol, 9.8:0.2) and confirmed by analytical HPLC. MR-1 obtained as white crystal (118 mg). The structure elucidation of MR-1 was performed with the help of ^{13}CNMR, mass (ESI-MS) spectra and CHN analysis that confirmed as marmesin reported earlier [2,3-dihydro-2-(1-hydroxy-1 methylethyl)- 7H-furo[3, 2-g][1]benzopyran-7-one] (**Figure 1**)[21].

Marmesin (MR-1): $C_{14}H_{14}O_4$ m.p.188-190^0 ($CHCl_3$-petrol); IR spectra: 3479,2977, 2929, 1703, 1630, 1572, 14851444, 1404 and 819 cm^{-1} ;^{1}H NMR: δ1.23 and 1.37(> CMe_2, 1.85(1H, br), 3.23 (2H, br d, *J* 8.8 Hz, H_2-1'), 4.74 (1H, t, *J* 8.8 Hz, H-2'), 6.21(1H, d, *J* 9.5 Hz, H-3), 6.74(1H, s, H-8), 7.22(1H, s, H-5), 7.59(1H, d, *J* 9.5 Hz, H-4) ; m/z (%) 246 (M^+,39), 213(20), 188 (75), 187(100), 175(15), 160(30), 131(19), 59(66), 43(7) ; CHO % elements- (Oxyzen-25.915), (Carbon-67.191) and (Hydrozen-5.480).

2.4. Preparation of Crude Extract

Accurately weighed 5 g of the coarse powder of *F. limonia* stem barks were extracted with methanol (3 × 50 mL) under reflux (30 min each time) on a water bath. The combined extracts were filtered and concentrated, and transferred to a 25 mL volumetric flask and the volume was made up with methanol.

2.5. Preparation of Standard Solution

A stock solution of marmesin (100 μg mL-1) was prepared by dissolving 1 mg of accurately weighed marmesin in methanol and making up the volume of the solution to 10 mL with methanol.

2.6. Chromatography

A Camag TLC system equipped with Camag Linomat V an automatic TLC sample spotter, Camag glass twin trough chamber (20 × 10 cm), Camag scanner 3 and integrated win CATS 4 Software were used for the analysis. TLC was performed on a pre-coated TLC plate silica gel$60F_{254}$

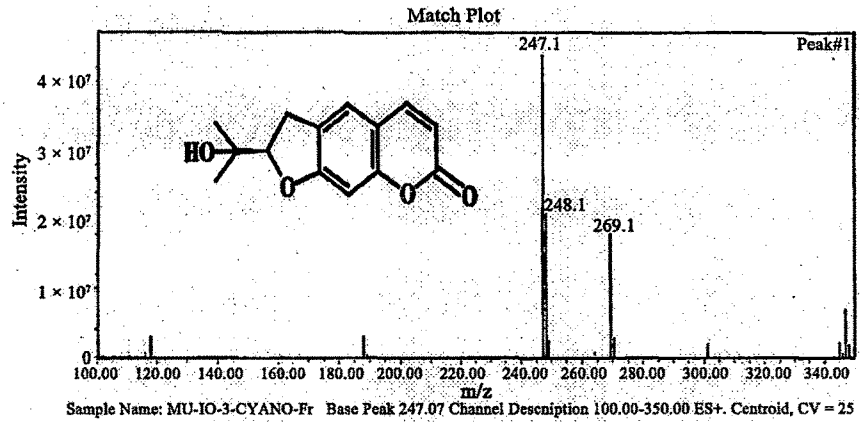


Figure 1. Mass spectroscopy and chemical structure of marmesin.

(20 cm × 10 cm). Samples and standards were applied on the plate as 8 mm wide bands with an automatic TLC sampler (Linomat V) under a flow of N_2 gas, 10 mm from the bottom and 10 mm from the side and the space between two spots were 15 mm of the plate. The linear ascending development was carried out in a Camag twin trough chamber (20 cm × 10 cm) which was presa- turated with 20 mL mobile phase chloroform: methanol (9.5:0.5 v/v) for 20 min at room temperature (25 ± 2°C and 40% relative humidity). The length of the chromatogram run was 9 cm. Subsequent to the development, TLC plates were dried under stream of hot air and then subjected to densitometric scanning using a Camag TLC scanner III (Camag, Switzerland) with win CATS software (version 1.4.1) in the absorbance- reflectance scan mode. Quantitative evaluation of the plate was performed in absorption-reflection mode at 338 nm. Quantification of marmesin in the extract of *F. Limonia* stem barks was performed by external standard method, using pure marmesin as standard.

2.7. Calibration Curve for Marmesin

Stock solution of marmesin (100 μg mL-1) was prepared in methanol and different amounts (20–100 ng spot^{-1}) were applied on a TLC plate, using Linomat V for preparing five point calibration graphs of peak area versus concentration. The regression equation for marmesin was 1089.554 + 230.603x and co-relation coefficient (r^2) was 0.999.

2.8. Quantification of Marmesin in Test Sample

Ten microlitres of sample solution were applied in triplicate on a TLC plate and developed, scanned as above. Peak areas were recorded and the amount of marmesin was calculated using the calibration plot.

2.9. Specificity

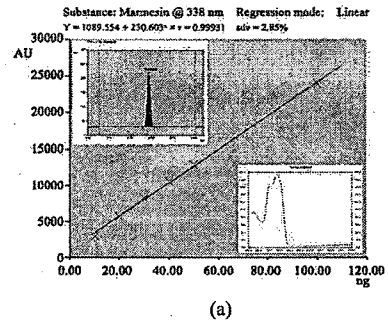
The specificity of the method was ascertained by co-analyzing standard and sample. The band for marmesin in sample was confirmed by comparing the *Rf* (0.49) and absorption spectra of the spot to that of reference compound. The peak purity of marmesin peak in sample track was assessed by comparing the spectra at peak start, peak apex and peak end positions of the band. Good correlation was also obtained between standards and sample overlay spectra ($r^2 > 0.99$).

2.10. Method Validation

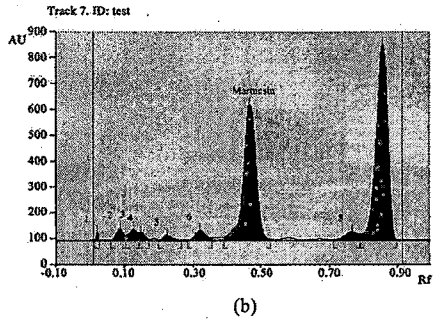
The method was validated for precision, accuracy and repeatability [22]. Instrumental precision was checked by repeated scanning of the same spot 20 and 100 ng five times and was expressed as coefficient of variance (% RSD). Method precision was studied by analyzing the standards 20 and 100 ng per spot under the same analytical procedure and lab conditions on the same day and on different days (inter-day precision) and the results were expressed as % RSD. Accuracy of the method was tested by performing the recovery studies of the pre-analysed sample with standard at three levels (55.02, 68.78 and 82.53 μg mL^{-1}), % recovery and average % recovery were calculated.

3. Result and Discussion

Chloroform: methanol (9.5:0.5 v/v) gave the best resolution and satisfactory separation of the components in the extracts with well resolved peaks. A total of nine peaks were observed methanol extracts of samples. A comparative chromatographic display is shown in **Figures 2(a) & 2(b).** The densitometric scanning was therefore performed at a wavelength of 338 nm. The identities of the bands of marmesin (*Rf* = 0.49), in the sample extract were confirmed by overlaying their absorption spectra with those of the standard compounds using the TLC Scanner 3. The peak purity of the separated marmesin was confirmed by recording the absorption spectra at start to middle and middle to the end of the peak.



(a)



(b)

Figure 2. (a) TLC chromatogram, for a standard marmesin in methanol, Calibration curve and Three-dimensional overlaid chromatogram of standard track and sample track for marmesin; (b) TLC chromatogram, for stem barks methanolic extract of *Feronia Limonia*.

3.1. System Suitability Test

3.1.1. Linearity and Detection Limit

Linearity was checked by applying standard solutions of marmesin at five different concentration levels. The calibration curve was drawn in the concentration range of 20–100 ng spot^{-1}(**Figures 2(a)** and **2(b)**). The equation for the calibration curve of marmesin is Y = 1089.554 + 230.603x and the correlation coefficient of the calibration plot was 0.999 indicating good linearity. Results of regression analysis on the calibration curve and detection limits are presented in **Table 1(a)**.

3.1.2. Precision Studies

Instrumental precision was checked by repeated scanning of the same spots (20 and 100 ng spot^{-1}) of standard marmesin five times and the RSD values were 1.56 and 1.82 for 20 and 100 ng spot^{-1}, respectively. To determine the precision of the developed assay method 20 and 100 ng spot^{-1} of the marmesin standard was analysed five times within the same day to determine the intra-day variability. The RSD values were 3.41 and 6.29 for 20 and 100 ng spot^{-1}, respectively. Similarly the inter-day precision was tested on the same concentration levels on 2 days and the RSD values were 2.68 and 2.83, respectively (**Table 1(b)**).

Table 1. Method validation parameters for quantification of marmesin using proposed TLC densitometric method.

(a) Linearity regression data

Sl no.	Parameter	Results
1	RF	0.49
2	Dynamic range (ng spot^{-1})	20–100
3	Equation	Y = 1089.554 + 230.603x
4	Slope	230.603
5	Intercept	1089.554
6	Limit of detection	5 ng
7	Limit of quantification	15.15 ng
8	Linearity (correlation coefficient)	0.999
9	Specificity	Specific

(b) Precision studies data

Concentration (ng spot^{-1})	Instrumental precision (% RSD)	Method precision (% RSD)	
		Intra-day	Inter-day
20	1.56	3.41	2.68
100	1.82	6.29	2.83

(c) Recovery studies of marmesin

Sl no.	Amount of marmesin present in the sample (µg)	Amount added (µg)	Amount found (µg)	Avg. Recovery (%)
1	68.78	55.02	122.11	98.83
2	68.78	68.78	136.92	
3	68.78	82.53	148.81	

3.1.3. Sample Analysis and Recovery Studies

This developed TLC method was subsequently applied for the analysis of marmesin in the methanolic extract of *Feronia limonia* stem barks. The marmesin content of the stem barks by this proposed method was found to be 0.03412%. For the examination of recovery rates, 80, 100 and 120% of pure marmesin were added to preanalyzed sample and quantitative analysis was performed. The average recovery was 98.83 (**Table 1(c)**).

4. Conclusions

Thin layer chromatography is a globally accepted, rational and practical solution to characterize the crude plant drug along with pharmacologically active constituent enriched standardized extracts and their formulations. TLC method on silica gel $60F_{254}$ with chloroform–methanol (9.5:0.5, v/v) was developed and densitometric evaluation was performed at 338 nm. This method is simple, specific, precise, accurate and robust for the determination of marmesin [2,3-dihydro-2-(1-hydroxy-1 methylethyl)-7H-furo[3,2-g][1]benzopyran-7-one]. This standardized TLC procedure may be used effectively for the screening analysis as well as quality evaluation of the plant or its derived herbal products.

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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 μg/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 μg/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.

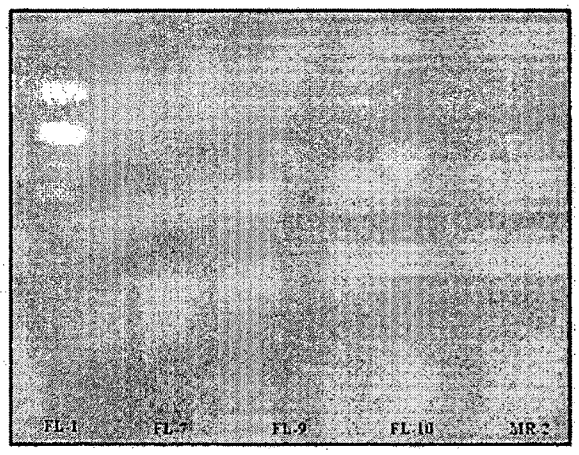

Figure 1. TLC fingerprinting of extracts, fractions and isolated compound from *F. limonia* leaf at UV 365 nm.

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±0.80***	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).

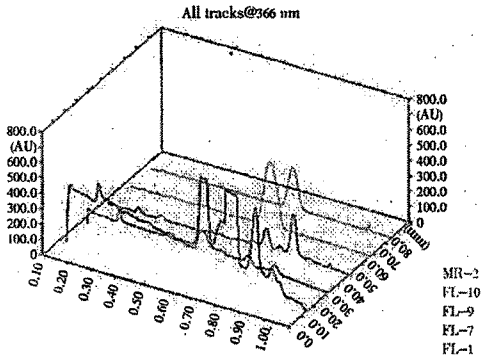

Figure 2. Three-dimensional overlaid chromatogram of extracts, fractions and isolated compound from *F. limonia* leaf.

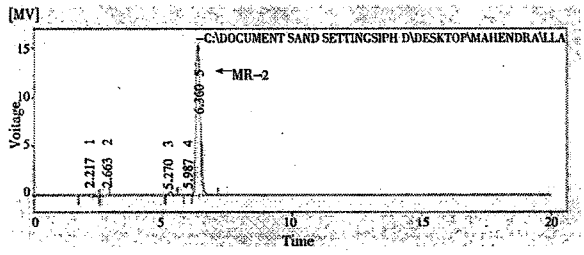

Figure 3. Parameters and chromatogram of isolated compound MR-2 by HPLC process.

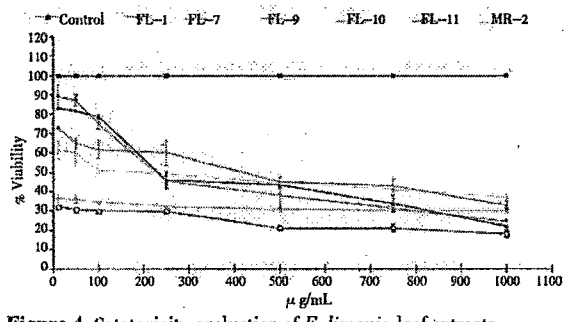

Figure 4. Cytotoxicity evaluation of *F. limonia* leaf extracts, fractions, isolated compound (MR-2).

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

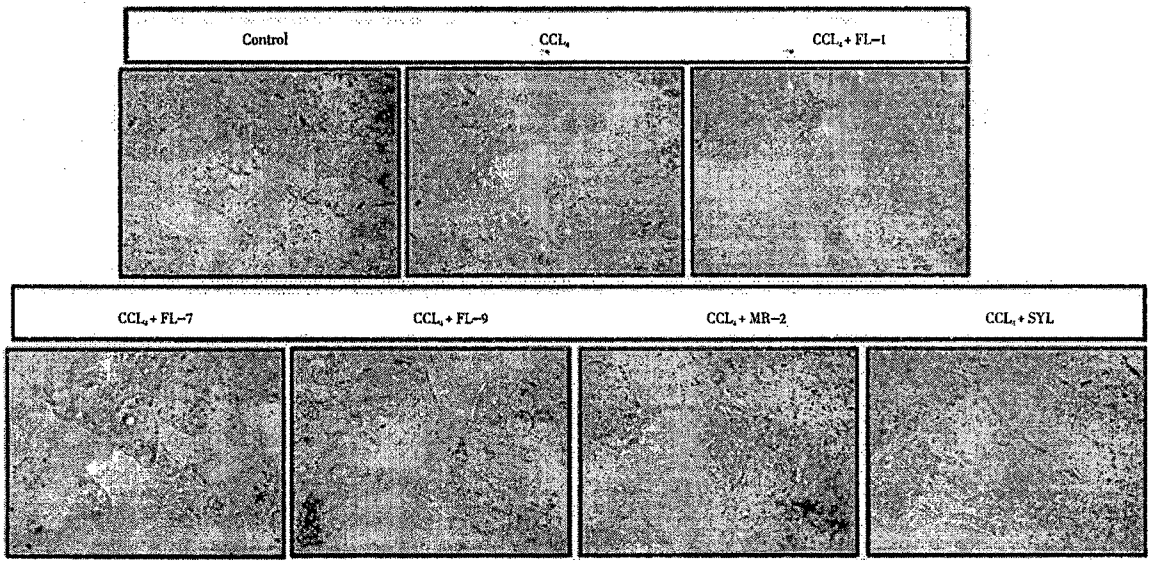

Figure 5. Effect of *F. limonia* extracts, fractions, isolated compound (MR-2) and sylimarin on CCl_4 induced hepatotoxicity. SYL: Sylimarin.

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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Effects of standardized *Feronia limonia* stem bark methanolic extract on *in vitro* and *in vivo* CCl_4 induced toxicity and pathological alterations

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Abstract: Present study was aimed at evaluating the hepatoprotective potential of a methanolic extract and marmesin (MR) isolated from root bark of Feronia limonia (FL) using in vitro and in vivo experimental models. In the in vitro study, activity levels of AST and ALT, cell viability and cell death were evaluated in HepG2 cells treated with CCl4 in presence or absence of FL extract or MR. Also, plasma activity levels of AST, ALT, billirubin, ALP, protein, hepatic antioxidants, LPO and histopathological evaluations were carried out in rats treated with CCl4 alone or co-supplemented with FL extract or MR in a dose dependent manner. In vitro co-supplementation of FL methanolic extract or MR significantly minimized alteration in the AST and ALT and improved cell viability. Also, oral administration of FL methanolic extract or MR significantly prevented CCL4 induced elevation in the plasma markers of hepatic damage and hepatic lipid peroxidation and, decrement in hepatic antioxidants. Also, in vivo hepatoprotective potential of FL methanolic extract and MR was evident in the form minimal alterations in the histoarchitecture of liver. Present study is the first scientific report on hepatoprotective potential of FL root bark methanolic extract and MR.

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