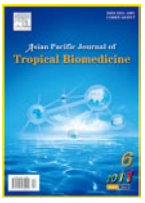
Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage:www.elsevier.com/locate/apjtb



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

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ARTICLE INFO

Article history:
Received 25 March 2011
Received in revised form 17 April 2011
Accepted 3 May 2011
Available online 20 May 2011

Keywords:
Feronia limonia leaf
HepG2
Cytotoxicity
Hepatoprotective
Phytochemical analysis
TLC
HPLC
Hepatoprotective activity
SGPT
SGOT
Isolated compound
Spectroscopic assay

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