JOURNAL OF NATURAL REMEDIES
DOI: 10.18311/jnr/2023/32051

RESEARCH ARTICLE

Phytochemical Profiling and Pancreatic Lipase Inhibitory Activity of *Flacourtia inermis* Roxb. Fruits

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Abstract

Objectives: The present research work was carried out to explore the potential use *Flacourtia inermis* [FI] fruits for the prevention and treatment of obesity through pancreatic lipase inhibition *in vitro.* The study also aimed to investigate the chemical profiling of ethanol extract of FI using High-Performance Thin Layer Chromatography (HPTLC), High-Resolution Liquid Chromatography (HR-LC/MS), and Nuclear Magnetic Resonance Spectroscopy (NMR). **Materials and Methods:** Dried fruits of *Flacourtia inermis* were pulverised and subsequently extracted using various solvents in sequential steps of increasing polarity, such as hexane, ether, chloroform, ethyl acetate, ethanol, and water. After phytochemical analysis by preliminary chemical testing various extracts were evaluated for their ability to inhibit pancreatic lipase, and the ethanol extract was found to have an IC_{50} close to that of reference drug orlistat. The most potent ethanol extract was analysed by HPTLC and separated through column chromatography, and further analysis was performed by HR-LC/MS and 1H-NMR techniques. **Results:** The presence of various phytoconstituents in this plant was detected using different types of analytical techniques. PL lipase inhibitory activity was observed in extracts in a dose dependent manner. Performing PL inhibition assay, it was found that the ethanol fruit extracts have lipase inhibitory activity with an IC_{50} value of 377.15 µg/ml. HPTLC finger printing of the ethanol extract showed the presence of various bioactice compounds. HR-LC/MS study of the most active ethanol extract indicated the presence of different phytochemicals, such as phenolics and flavonoids. Column chromatographic separation of ethanol fruit extract of FI followed by structural elucidation using various spectral studies demonstrated the presence of two compounds namely myricetin and quinic acid. **Conclusion:** The study suggests that the edible fruits of *Flacourtia inermis* have the potential to inhibit pancreatic lipase enzyme and therefore, may be recommended for the management of obesity. Additionally, our research sheds light on the phytochemistry of *flacourtia* species and may lead to the development of novel chemical entities as potential pancreatic lipase inhibitors.

Keywords: Chromatography, *Flacourtia inermis*, Mass Spectrometry, Obesity, Pancreatic Lipase, Phytochemicals, NMR

1. Introduction

The prevalence of obesity is increasing in an alarming rate worldwide, and it is associated with multiple comorbidities like atherosclerosis, diabetes, hypertension, and cancer. Epidemiological studies proved that a high intake of saturated fat in the diet is one of the primary causes of obesity[1]. Pancreatic Lipase (PL), a major lipase in humans, hydrolyzes between 50 and 70 percent of all

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Article Received on: 24.11.2022 Revised on: 19.04.2023 Accepted on: 08.05.2023

ingested lipids, making it a key enzyme in lipid absorption. Inhibiting PL is known to reduce the absorption of fat and is beneficial for the regulation of obesity[1,2].

Orlistat is the only approved PL inhibitor available for the management of obesity[2]. However, the long-term use of orlistat has been associated with significant adverse effects, including gastrointestinal side effects, acute pancreatitis, gallstones, hepatotoxicity, and renal failure. There are published reports describing secondary metabolites derived from plants having lipase inhibitory activity, such as polyphenols, alkaloids, flavonoids, glycosides, saponins, terpenoids, carotenoids, and polysaccharides. As a result, safer and more efficient PL inhibitors are absolutely necessary for the treatment of obesity[4-9].

Lovi Lovi (*Flacourtia inermis* Roxb. of flacourtiaceae family) is a small evergreen tree native to Moluccas and also found in India, Malaysia, Sri Lanka, and New Britain in Papua New Guinea (Figure 1). Tropical forests are the natural habitat of this plant. The fruits are grown in the Indian state of Kerala and are known locally as loobikka or lavulolika.The edible fruits are red, pulpy berries with an acidic taste containing 4 to 8 seeds[10-12]. Processed fruits are used in confectioneries and pickles. The fruit juice of *F. inermis* has been reported to contain caffeoylquinic acid derivatives, phenolic glucosides, quinic acid, and malic acid and has been tested for their antioxidant activities. The polyphenols from the fruits possess α-glucosidase and α-amylase inhibitory activities. Several other studies reported on the antibacterial, antifungal, and anti-protozoal activities of the fruit[12-15].

Figure 1. *Flacourtia inermis* tree and fruits.

To uncover its potential biological application, the extracts were subjected to pancreatic lipase inhibitory activity. To the best of the knowledge, limited studies have been reported regarding the chemical profiling of *Flacourtia inermis*. The chemical composition of the fruit extract of FI was studied by HPTLC, column chromatography, and HR-LCMS. Further, the purified fractions were subjected to NMR analysis. Here, we report the identification as well as structural characterisation of the bioactive compounds which may be responsible for PL inhibitory activity.

2. Materials and Methods

2.1 Chemicals and Reagents

Orlistat and pancreatic lipase (Porcine) (Type II, EC 3.1.1.3) for PL inhibition assay were procured from Sigma-Aldrich. 4-nitrophenyl butyrate was obtained from TCI Chemicals (India) Pvt. Ltd. Tris buffer, acetonitrile, and DMSO (dimethyl sulphoxide) were purchased from Hi-media (Mumbai, India.). All other solvents and reagents (analytical grade) were procured from SD Fine Cemicals (Mumbai, Maharashtra, India).

2.2 Collection and Authentication of Plant Materials

Ripe fruits of *Flacourtia inermis* were collected from the Puliyanam region of the Ernakulam district, Kerala, India, following the rainy season. The plant material was authenticated by the Pharmacognosy Division of the Centre for Medicinal Plants Research (CMPR) at Arya Vaidya Sala, Kottakkal, Kerala. The authentication reference number for the plant material is CMPR/AIF/PHG/317.

2.3 Extraction and Phytochemical Screening

Collected fresh fruits were sliced into small pieces, dried in a hot air oven at 60°C and crushed using grinder and extracted sequentially with hexane, chloroform, ethyl acetate, ethanol, and water , using Soxhlet apparatus for 24 hrs. Extraction was repeated in triplicate, followed by filtration and the filtrate was combined and dried in a rotatory evaporator (Roteva, India). The extractive

yield was calculated at percentage scale on dry weight basis. A preliminary phytochemical screening of the extracts was performed to reveal the presence of various phytoconstituents using standard procedure[16]. TLC analysis was further performed to confirm the presence of active phyto constituents in the extract.

2.4 *In vitro* Pancreatic Lipase Inhibition[17]

Ethanol fruit extract of FI was also subjected to analysis to check their inhibitory potential against pancreatic lipase. Lipase inhibition assay performed using an earlier reported method by Zhang *et al*., 4-nitrophenyl butyrate (p-NPB) was taken as the substrate to determine inhibitory activity against pancreatic lipase. The enzyme solutions were made right before usage. Crude porcine pancreatic lipase enzyme was suspended in tris-HCl buffer (2.5 mmol, pH 7.4 with 2.5 mmol NaCl) to make a concentration of 200 units/ml for the *in vitro* assay. A 96-well microtitre plate was used for the assay, which was performed in triplicate. 10 mL of crude extracts were made at five different concentrations viz., 100, 200, 300, 400, and 500 µg/mL, using DMSO was used to dissolve the extracts and Orlistat was taken as positive control. The plates were incubated at 37 °C for 15 mins prior to the experiment. Thereafter, 170 mL of the substrate solution was added to the wells. The plate was incubated at 37 °C for 25 mins and the absorbance was measured at 405 nm using a Multiskan FC Microplate Spectrophotometer. All experiments were performed in triplicate for each extract sample and results are expressed in terms of IC_{50} (µg/mL). The IC_{50} value is the concentration of the extraact to inhibit 50% of its activity under the assay conditions.

$$\% \text{ Lipase inhibition} = [1- (A/B)] \times 100$$

2.5 High-Performance Thin-Layer Chromatography

Ethanol fruit extract of FI was used for HPTLC analysis. The sample was dissolved in methanol and 5 µl of the test solution was impregnated on a precoated silica gel 60 F254 aluminium plates (5 x 10 cm) to a band length of 8 mm using CAMAG Automatic TLC Sampler 4 (ATS4). Plate imprignated with sample was placed in a twin trough TLC developing chamber (20 x 10 cm) for 15 mins to attain complete saturation of the chamber. The mixture of Tolune:ethylacetate:methanol (7:3:10) was used as a mobile phase, and the solvent was allowed to move 90 mm from the bottom of TLC plate. The developed plate was then dried using a heat gun and the plates were visualized under UV light at 254 nm and 366 nm, and after derivatization the plate was viewed under white light using WinCATS.

2.6 High Resolution-Liquid Chromatography-Mass Spectroscopy (HR-LCMS) Analysis

The HR-LC/MS analysis was performed to identify the phytoconstituents present in the ethanol extract of FI. The Sophisticated Analytical Instrument Facility (SAIF) of the Indian Institute of Technology (IIT), Bombay, was availed to perform the HR-LC/MS analysis. Chromatographic separation was performed using UHPLC System, Agilent Technologies, USA, attached with an electrospray ionization source, a binary pump, an autosampler, and a PDA detector. The column employed was Hypersil GOLD C18 100 x 2.1 to 3 mm. The composition of mobile phase was water (A) and acetonitrile (B), both containing formic acid (0.1%). An injection volume of 5.00 ml was employed. Acquisition time was kept 30 mins with an initial 2 mins for the flow of solvent. The identification of compounds were performed by comparing the mass fragmentation, molecular mass, abundance, and library database similarity.

2.7 Chromatographic Separation

The ethanol extract (1 g) was subjected to column chromatography on silica gel (60-120 mesh) (Merck). The Column chromatographic separation of ethanol fruit extract has been carried out using chloroform:methanol (95:5 to 85:15) as the mobile phase. Two compounds were isolated and the characterization was performed.

2.8 Structure Elucidation

Structure elucidation of the isolated Phytoconstituent was performed using IR, NMR, and mass spectra. NMR spectra was recored using Bruker Avance Neo instrument. DMSO-d6 was used as solvent and TMS was kept as internal standard. MRMS spectra was done on a Maldi-TOF Synapt XS HD Mass Spectrometer (Waters) and Infrared spectra were recorded on Bruker ATR.

2.9 Statistical Analysis

The PL lipase inhibitory assay was performed in triplicate and the datas are reported as mean ± S.D. The IC_{50} values were calculated using graphpad prism 6.0 software.

3. Results

3.1 Extraction and Phytochemical Screening

The yield of various extracts was found to be 1.56% w/w, 0.92% w/w, 17.48% w/w, 46.8% w/w, and 32% w/w for hexane, chloform, ethyl acetate, ethanol, and water respectively. The preliminary phytochemical identification using chemical test revealed the presence of phyto constituents such as carbohydrates, alkaloids, glycosides, phenolics, saponins, flavonoids, and steroids in various extracts. Based on TLC analysis and phytochemical screening, EtOAc, ethanol, and water extracts of *F. inermis* were tested for PL inhibitory activity.

3.2 *In vitro* PL Inhibition

The inhibitory potential of the FI fruit extracts against PL was tested and the findings are shown in Table 1. The ethyl acetate (FF1), ethanol (FF2), and aqueous extracts (FF3) showed inhibitory activities in dose-dependent manner with IC_{50} (μg/mL) values 397.80, 377.15, and 390.69 respectively. Orlistat had IC_{50} value of 323.17 μg/ml. However, all extracts were less potent than orlistat (control) in inhibiting pancreatic lipase (Figure 2). As the results indicate, the ethanol extract of FI fruits (FF2) was the most potent among the other two fractions.

3.3 HR-LC/MS Analysis

HR-LC/MS chromatograms identified a total of seven compounds and were represented by a compound name, mass abundance, and chemical nature (Table 2). The identified compounds were Myricetin, Gibberellin A3, Histidinyl-Glutamine, N-(1-Deoxy-1-fructosyl)leucine and 6,7,3',4'-Tetrahydroxyflavanone (Fustin), and Quinic acid. HR-LC/MS chromatogram and the representative MS/MS spectra of the selected compounds are dipicted in Figures 3 and 4.

3.4 HPTLC

The ethanol fruit extract of FI was spotted on an HPTLC plate and developed using Tolune:ethylacetate:methanol (7:3:10) as a mobile phase. The fingerprint of ethanol extract demonstrated unique chromatographic of bands which was in accordance to the phenolics and flavonoids, and it can be considered unique because they are visually different from each other. Figure 5 dipicts the HPTLC fingerprints of ethanol fruit extract under 254 nm before derivatization, under 366 nm before derivatization and under white light after derivatization (FF1-FF3). Under 366 nm major bands were observed at R_f of 0.02 (blue), 0.07 (blue), 0.12 (blue), 0.16 (blue), 0.23 (blue), 0.34 (blue), and 0.36 (blue) . Under 254 nm, the most prominent bands were seen at R_f of 0.02 (black), 0.04 (black), 0.10 (black), 0.27 (black), 0.32 (black), 0.44 (black), 0.60 (black), and 0.88 (black) (Figure 5). After derivatization, new bands at 0.09 (light blue), 0.14 (purple), 0.26 (light blue), 0.37 (light blue), 0.67, 0.82 (blue) and 0.87 (light blue), and more intense bands at 0.44 can be observed.

Table 1. Pancreatic lipase inhibitory activity of different extracts

Concentration (μg/ml)	PLinhibitory activity of ethyl acetate extract FF1	PL inhibitory activity of ethanol extract FF2	PL inhibitory activity of aqueous extract FF3	PL inhibitory activity of Orlistat (Standard)
100	11.20 ± 0.10	12.55 ± 0.30	11.25 ± 0.40	15.83 ± 0.40
200	24.50 ± 0.50	26.89 ± 0.45	24.50 ± 0.40	33.40 ± 0.55
300	37.20 ± 0.40	38.10 ± 0.10	35.20 ± 0.60	46.30 ± 0.15
400	50.30 ± 0.10	53.62 ± 0.50	51.52 ± 0.50	63.20 ± 0.10
500	64.50 ± 0.50	67.28 ± 0.40	65.30 ± 0.55	75.50 ± 0.30
IC_{50}	**397.80**	**377.15**	**390.69**	**323.17**

Values are mean ± SD in triplicae

3.5 Structural Elucidation

Structure elucidation of the constituent which was isolated from ethanol extract (Compound 1), appeared as white crystals and identified as quinic acid (Figure 6 (a)). In IR spectra, the OH stretching of carboxylic group was characterized by the presence of a strong band appearing approximately at 3,511-3,340 cm^{-1}. The aliphatic C–H stretching was present at 2,976 cm^{-1}. The C = O stretching was observed at 1,682 cm^{-1}. The ^{1}H NMR and ^{13}C NMR spectra of the isolated compounds were recorded using BRUKER Avance Neo 500 spectrometer on (δ) scale, using DMSO-d6 as a solvent and the spectra are shown

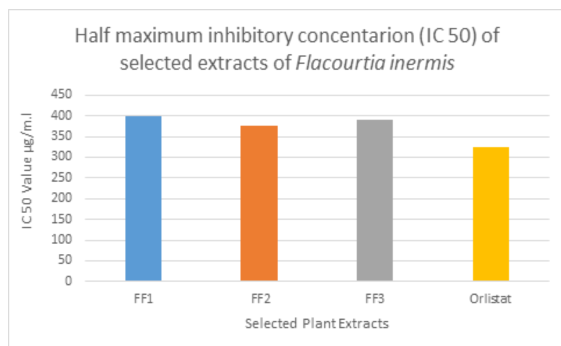

Figure 2. Comparison of IC 50 values of extracts FF1, FF2, and FF3 with orlistat.

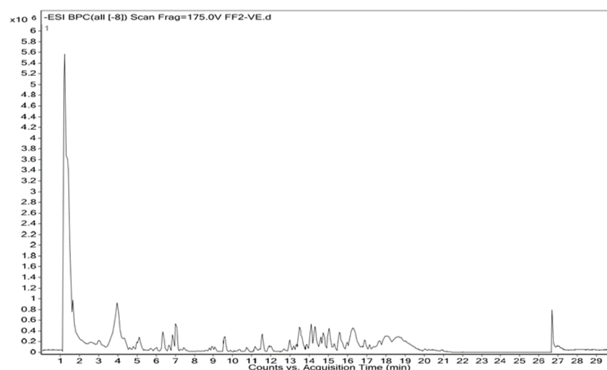

Figure 4. HR-LC/MS chromatogram (Negative ESI) of ethanol fruit extract of *Flacourtia inermis.*

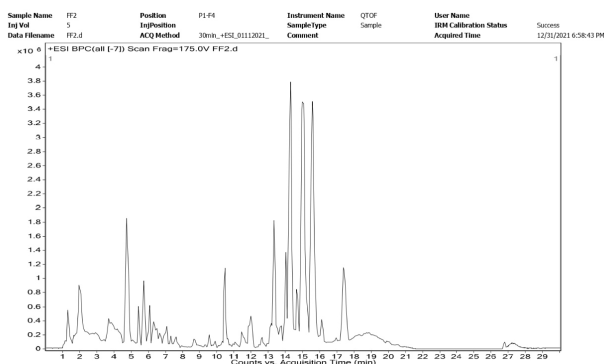

Figure 3. HR-LC/MS chromatogram (Positive ESI) of ethanol fruit extract of *Flacourtia inermis.*

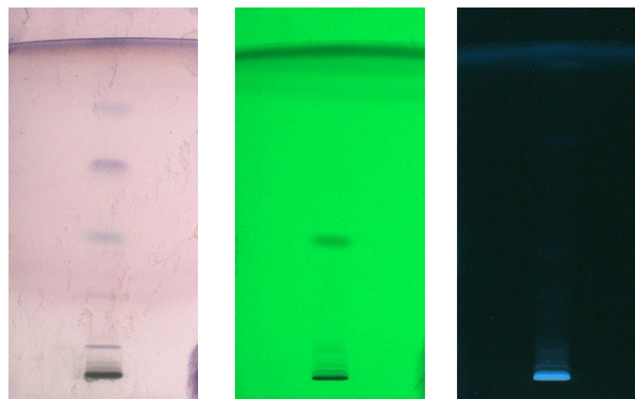

Figure 5. HPTLC Chromatogram at visible UV 254 nm and 366 nm.

Table 2. HRLC/MS analysis of ethanol extract of *Flacourtia inermis*

Compound	Mass	Abundance	Chemical nature
Myricetin	318.09	310934	Hexahydroxyflavone
Gibberellin A3	346.127	477896	hormone
Histidinyl-Glutamine	283.1277	73676	Aminoacid
N-(1-Deoxy-1-fructosyl)leucine	293.1484	218524	Aminoacid
6,7,3',4'-Tetrahydroxyflavanone (Fustin)	288.10	118531	Flavanonol
Demethoxycentaureidin 7-O-rutinoside	638.33	313102	Flavanonol
Quinic acid	192.06	650345	cyclic polyol

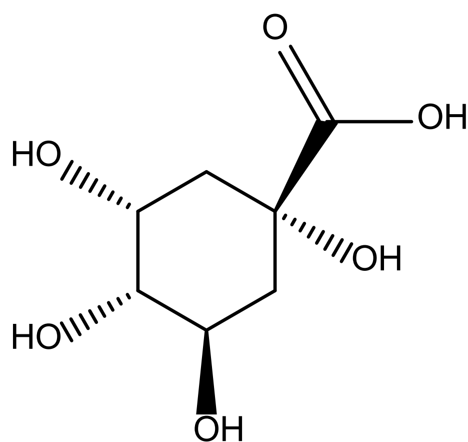

Figure 6. **(a)** Quinic acid.

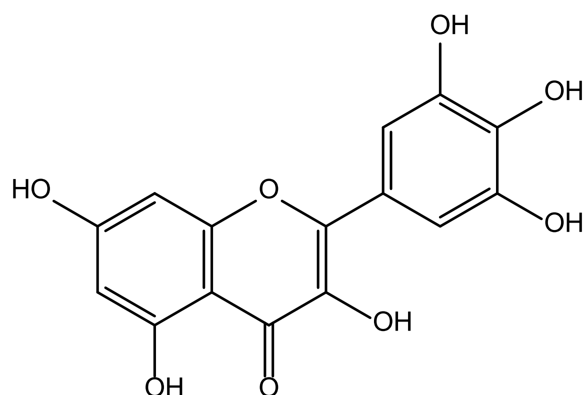

Figure 6. **(b)** Myricetin.

in (Figure 1 in supplementary data). ^{1}H-NMR 1.78-1.68 (dd,2H,CH), 1.89-1.85 (dd,2H,CH), 3.25 (d,1H,CH), 3.75 (q, 1H, CH), 3.88 (dt,1H,CH), 4.55 (br,3H,-OH), 5.37 (br,1H,-OH group), 12.30 (br, 1H, COOH).^{13}C NMR (125 MHz) DMSOd6: (Figure 3 in supplementary data) 37.35, 40.40, 66.63, 68.91, 74.34, 175.52 ESI-MS (m/z): 193.0694 (M + 1).

The second compound isolated from the ethanol fraction as light yellow-coloured crystals was identified as Myricetin. The signals were in accordance with the structure of Myricetin. The 1H-NMR spectra of isolated compound 2 (Figure 6(b)) exhibited characteristic chemical shift values (δ, ppm; d6-DMSO):1H NMR:δ 6.20 (1H, d, J = 1.91 Hz), 6.38 (1H, d, J = 1.9 Hz), 7.26 (2H, d, J = 1.91 Hz), 8.80 br, 4^{1} –OH, 9.22,2H,br, 3^{1} –OH, 5^{1} –OH, 9.33br, 1H, 3 –OH, 10.78,br, 1H,7-OH, 12.50, S, 5-OH. ^{13}C NMR (125 MHz) DMSOd6: 93.12, 98.08, 102.9, 107.9, 120.72, 135.79, 145.64, 146.76, 156, 160.65, 16379, 175.68. ESI-MS (m/z): 319.0463 (M + 1).

4. Discussion

The current study identified and characterized various phytoconstituents found in the ethanol fruit extract of *Flacourtia inermis by* qualitative phytochemical screening, HPTLC, HR-LCMS, and NMR analysis.

PL is lead enzyme in lipid absorption and responsible for the hydrolysis of dietary fats; therefore, reducing fat absorption by inhibiting PL is a useful strategy for managing cobesity. Orlistat is a powerful lipase inhibitor; however, it is associated with severe gastrointestinal side effects[1,18-20]. The study investigated the PL inhibitory activity of FI fruits, which is an edible and underutilized fruit from Kerala.

Multiple solvents were utilized for extraction because the choice of solvent is crucial in the extraction of phytoconstituents from plant sources. Crushed fruits of FI were extracted using successive solvent extraction like hexane, chloroform, ethyl acetate, ethanol, and water. The preliminary phytochemical analysis followed by pancreatic lipase inhibitory assay showed higher activity for ethanol extract in a dose-dependent manner. Phytochemical analysis revealed diverse componds in the extract leading to focus on studying the ethanol extract. Ethanol is a polar solvent that can dissolve a wide range of componds such a alkaloids, flavonoids, terpenoid,s and phenolic componds. Comparing the percentage yield of the extract using solvent ethanol to other organic and aqueous solvents, the ethanol solvent yield appears to be promising. The presence of various phytochemical classes in it supports this selection. As the results indicated the ethanol extract exhibited higher activity than other ethylacetate and water extracts with an IC_{50} close to that of standard drug orlistat. However, the activity was less potent than that of orlistat. Our literature review has shown that polar compounds are crucial for pancreatic lipase activity in selected plants[4,21]. And, ethanol is a polar solvent that can dissolve wide range of bioactive compounds.

HPTLC is an useful tool for fingerprinting, identification of herbal medicines and products. The developed HPTLC method can be utilized for the qualitative analysis of *Flacourtia inermis*. The phytoconstituent of ethanol fraction, as identified by HR-LC/MS, included myricetin, Gibberellin A3, Histidinyl-Glutamine, N-(1-Deoxy-1-fructosyl) leucine and 6,7,3',4'-Tetrahydroxyflavanone (Fustin), and Quinic acid. These substances might act synergistically

or additively to inhibit pancreatic lipase. It should be highlighted that HR-LC/MS-QTOF provided a trustworthy method for speculative identification of the phytoconstituents in flacourtia fruits due to its faster acquisition rate and precise mass acquisition capabilities.

Column chromatographic purification of the ethanol extracts lead to the identification of Myricetin, belonging to flavonoid category which is present abundantly in tea, red wine, vegetables, fruits, and medicinal herbs. Myricetin derivatives are reported to possess antioxidant, antihyperglycemic, anticancer, anti-inflammatory, and antimalarial activities[22]. In the present study, we have successfully isolated another phytoconstituent, quinic acid, a naturally occurring phenolic compound found in plants. The spectral studies further confirmed the presence of myricetin and quinic acid. All spectral data are in accordance with anticipated structure. There are currently a number of food plants with abundant amounts of polyphenolic chemicals that may have pancreatic lipase inhibitory effects, preventing obesity and problems associated with it. The compounds such as phenolic acids, flavones, flavonols, tannins, and chalcones are documented to possess activity against PL. The observed activity could reasonably referred to the presence of flavonoid compounds such as myricetin and phenolic compound quinic acid. Quinic acid was previously reported in coffee fruits which is a potent antiobesity lipase inhibitor[18,19,22]. The combination of the aforesaid constituents in a single plant is expected to have promising PL inhibitory activity. On the basis of this study, we propose that myricetin and quinic acid may be the phytoconstituents responsible for prevention of dietary fat absorption by inhibiting pancreatic lipase.

5. Conclusion

The current study depicts the initial investigation, reporting PL lipase inhibitory activity of *Flacourtia inermis* fruit extract. The HR-LC/MS analysis identified several active compounds in ethanol fruit extract. The pancreatic lipase inhibitory effect of the fruit extract may be due to the presence of potentially bioactive compounds such as myricetin and quinic acid and their synergistic activities along with other phytoconstituents. Findings in this study illustrate the pancreatic lipase inhibitory potential of the flacourtia fruits, making a good choice for future prospects with potential applications in functional food supplements as well as nutraceuticals. Further works are essential to explore the underlying mechanism associated with the antiobesity potential.

6. Acknowledgements

The authors gratefully acknowledge SAIF, IIT Bombay, Mumbai for providing HR-LC/MS facilities. They also extend their gratitude to SAIF, Punjab University, for the instrumental facilities for NMR. The authors would like to thank the Centre for Medicinal Plants Research (CMPR), Arya Vaidya Sala, Kottakkal for plant authentication. Additionally, the authors wish to express their appreciation to Al Shifa College of Pharmacy, Kerala for providing the necessary facilities to conduct the present study.

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