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ORIGINAL ARTICLE

Antioxidant property and α -glucosidase, α -amylase and lipase inhibiting activities of *Flacourtia inermis* fruits: characterization of malic acid as an inhibitor of the enzymes

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Abstract *Flacourtia inermis* Roxb. (Flacourtiaceae), is a moderate sized tree cultivated in Sri Lanka for its fruits known as *Lovi*. The current study was undertaken to study the biological activity of extracts of the fruits in an attempt to increase the value of the under exploited fruit crops. Fruits of *F. inermis* were found to be rich in phenolics and anthocyanins. Polyphenol content of the fruits was determined to be 1.28 g gallic acid equivalents per 100 g of fresh fruit and anthocyanin content was estimated as 108 mg cyanidin-3-glucoside equivalents per 100 g of fresh fruits. The EtOAc extract showed moderate antioxidant activity in the DPPH radical scavenging assay with IC_{50} value of 66.2 ppm. The EtOAc and MeOH extracts of the fruits also exhibited inhibitory activities toward α-glucosidase, α-amylase and lipase enzymes with IC_{50}values ranging from 549 to 710 ppm, 1021 to 1949 ppm and 1290 to 2096 ppm, respectively. The active principle for the enzyme inhibition was isolated through activity-guided fractionation and was characterized as (*S*)-malic acid. The results of this study indicate that *F. inermis* fruits have the potential to be used in health foods and in nutritional supplements.

Keywords Antioxidant · *Flacourtia inermis* · Malic acid · α-glucosidase inhibitor · α-amylase inhibitor · Lipase inhibitor

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Introduction

Flacourtia inermis Roxb. (Flacourtiaceae), is a moderate sized tree found in Sri Lanka, and also found in Malaysia, Indonesia, South India, the Philippines and parts of Africa. The fruits, known as *Lovi* in Sri Lanka and Batoko plum in English, are round, cherry-sized, and dark red when ripe. The fruits are usually sour and astringent, though they can be sweet (Morton 1987). Two *Flacourtia* species, *F. inermis* and *F. indica*, both bearing edible fruits, are found in Sri Lanka. *Lovi* fruit juice shows a deep red colour.

In our previous study on the fruits of *F. inermis* (Jayasinghe et al. 2012), five caffeoylquinic acid derivatives, a rare phenolic glucoside (*rel*)-6-α-benzoxy-1α,2α,dihydroxy-5-oxocyclohex-3-enecarboxylic acid 2-(6-*O*-benzoyl-β-D-glucopyranosyloxy)-5-hyrdroxybenzyl ester together with quinic acid and malic acid, were isolated and characterized. Strong antioxidant activity of the chlorogenic acid derivatives was also reported. Antifungal and antibiotic activities of 3,4-dihydroxy benzoic acid isolated from *F. inermis* fruits have also been reported (Benny et al. 2010; George et al. 2011. In these references 3,4-dihydroxybenzoic acid was erroneously reported as 2,3-dihydroxybenzoic acid).

Fruits and vegetables are an important part of the human diet in most parts of the world and provide nutrients that are essential for life. Fruits contain a variety of phytochemicals such as phenolics, anthocyanins and flavonoids that are non-nutrient biologically active compounds, implicated in a wide range of health benefits including lowered rates of cardiovascular disease, decreased risk of cancer, stroke and type 2 diabetes. Antioxidant-rich fruits help to prevent free radical induced oxidative stress (Wang et al. 1996; Eberhardt et al. 2000; Liu et al. 2002).

Plant products may also contain compounds that serve as enzyme inhibitors (Shivaraj and Pattabiraman 1981). These

molecules have the ability to bind to enzymes and reduce their activity. The presence of inhibitors of carbohydrate hydrolyzing enzymes such as α-glucosidase and α-amylase in plant derived foods, is of importance in the control of blood glucose level in patients with type 2 diabetes and contributes to suppression of postprandial hyperglycemia (Sharp et al. 2007).

Fat and obesity are related to type 2 diabetes and heart diseases. Fat absorption may be controlled by pancreatic lipase, which hydrolyses fat in the human body. Lipases are involved in the hydrolysis of glycerides to glycerol and free fatty acids. Powerful synthetic inhibitors of pancreatic lipase are available but the use of naturally occurring inhibitors is considered to be safer (Lee et al. 2010).

The aim of this study is to assess the value of the fruits of *F. inermis* for a possible use as a nutritional supplement. First, total phenolics and the anthocyanin contents of the fruits were determined. Second, antioxidant activity and enzyme inhibitory activities toward α-glucosidase, α-amylase and lipase of organic extracts of the fruits were evaluated. Finally, an active principle of the enzyme inhibitors was isolated through activity guided fractionation and characterized as malic acid.

Material and methods

General

Solvents used for extractions and assays were distilled and all chemicals used were purchased from Sigma Aldrich, unless otherwise stated. Extraction of plant materials was carried out using an ultrasound sonicator (VWR Ultrasonic cleaner, model – USC 1700 D). UV absorptions were measured on a UV-1601, Shimadzu spectrophotometer using 10 mm quartz cell. ^{1}H and ^{13}C NMR spectra of pure compounds were recorded on a Bruker DRX 500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) spectrometer in CD_3OD. The ^{1}H and ^{13}C shifts are expressed in reference to the solvent signal (δ_H 3.30 and δ_C 49.0). Optical rotation was measured on a Jasco P-2200 polarimeter Column chromatographic separations were performed using silica gel (70–230 mesh size, Fluka 60741) and Sephadex LH-20. HPLC separations were carried out on a Shimadzu LC-6A apparatus equipped with a UV detector, reverse phase C_{18} column and gradient solvent elution.

Collection and extraction of fruits

Fresh fruits of *F. inermis* (1.5 kg), collected from the Central Province of Sri Lanka. A voucher specimen of the plant and photographic evidence of the fruits are deposited at the Natural Products laboratory of the Institute of Fundamental Studies. Fruits were blended and the fruit pulp was separated from the wine red fruit juice by filtering. The fruit juice was then extracted with EtOAc, while the fruit pulp residue was sequentially extracted with EtOAc and MeOH using a sonicator. Thin layer chromatography (TLC) indicated that the EtOAc extracts from the fruit pulp and fruit juice were identical and the two extracts were combined. Solvents were evaporated by using a rotary evaporator at 40 °C to give the EtOAc extract (10.5 g) and MeOH extract (4.5 g).

A 70 % aqueous MeOH extract of the fruits was used for the determination of the total phenol content. Fresh fruits were blended and used to determine anthocyanin content. EtOAc and MeOH extracts from fruits were screened for antioxidant activity, α-amylase inhibition, α-glucosidase and lipase inhibition activities and bioactivity guided fractionation was carried out to isolate pure active compounds.

Total polyphenol content (TPC)

TPC was determined by the Folin-Ciocalteu method (Turkmen et al. 2006; ISO 2005). Gallic acid which was dried at 102 °C to a constant weight (GA) (0.5–50 mg) was used to prepare a standard curve by mixing a 500 μL aliquot of each GA solution with 500 μL of distilled water and 2.5 mL of 10–fold diluted Folin-Ciocalteu reagent. After 5 min, 4.0 mL of 7.5 % (*w*/*v*) Na_2CO_3 solution was added, allowed to stand at room temperature for 60 min, and absorbance at 765 nm was measured against distilled water. A 500 μL aliquot of a 1000 ppm solution of test solution was prepared from a 70 % aqueous MeOH extract of fresh fruits and treated as described above. The phenolic content (mass of polyphenols) was read from the standard curve as GA equivalents (GAE) per 100 g of fruit.

Determination of anthocyanin content

Total anthocyanins were determined by a pH differential method (Sutharut and Sudarat. 2012). Briefly, 20 g of fruits and 40 mL of extracting solvent (95 % ethanol:1.5 M HCl, 85:15) were blended, filtered, and the residue was washed with 30 mL of the same solvent. The residue was extracted once more using 70 mL extracting solvent. The combined extracts were made up to 200 mL with the same solvent (pH was adjusted to 1.0 by the addition of 1.5 N HCl). A 1.0 mL of the extract was diluted up to 10 mL with pH 1.0 KCl buffer (prepared by mixing 50 mL of 0.2 M KCl and 134 mL of 0.2 M HCl). Another 1 mL portion of the extract was added with two drops of conc. NaOH, made up to 10 mL with pH 4.5 NaOAc buffer and the two solutions allowed to equilibrate (15 min).

The absorbance of each dilution at 510 and 700 nm against distilled water was measured. The anthocyanin

content of *F. inermis* fruits (as cyanidin-3-glucoside equivalents) was calculated using the equation,

$$\text{Monomeric anthocyanin pigment}\left(\text{mg}/\text{L}\right) = \frac{A \times MW \times DF \times 1000}{\varepsilon}$$

where

$$A = (A_{510} - A_{700})pH1 - (A_{510} - A_{700})pH4.5, MW = 449.2, \varepsilon = 26,900 \, and DF(Dilution factor) = 10.$$

Antioxidant activity (DPPH radical scavenging activity)

The crude extracts (25–1000 ppm) and pure compound (1–100 ppm) were tested using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (Silva et al. 2005). Each solution (0.75 mL) was mixed with 0.3 mL of a 0.3 mM DPPH MeOH solution and kept in the dark at room temperature (30 min). The absorbance was measured at 518 nm against a reaction blank (0.3 mL of MeOH and 0.75 mL of the plant extract). A mixture of 0.3 mL of 0.3 mM DPPH solution and 0.75 mL of MeOH was used as a reaction control against MeOH. Percent inhibition was calculated for each concentration and IC_{50} was calculated for each extract. Ascorbic acid and BHA were used as positive controls (1–100-ppm).

The following formula was used to calculate the percent inhibition.

$$\text{Percent inhibition} = \frac{Abs_{Reaction\ control} - Abs_{sample}}{A_{Reaction\ control}} \times 100$$

IC_{50} values (concentration at 50 % inhibition) were calculated by plotting dose response curves of % inhibition vs. concentration.

α-glucosidase inhibition assay

A 2 units/mL solution of α-glucosidase (Sigma-G5003, from *Saccharomyces cerevisiae*, 100 U/mg of protein) and 5 mM *p*-nitrophenyl-α-D-glucopyranoside solution were prepared in 20 mM phosphate buffer (pH 6.0) (Sharp et al. 2007). Plant extracts and pure compound were dissolved in distilled water with 1 % DMSO (500–5000 ppm extracts/ 1–250 ppm for pure compounds). The reaction was carried out at 25 °C using 10 μL enzyme, 50 μL substrate and 10 μL plant extract for 7 min. Absorption was measured at 415 nm after the addition of 80 μL 0.4 M glycine, pH 10.4. A control reaction was performed using 10 μL enzyme, 50 μL substrate and 10 μL of aqueous 1 % DMSO of acarbose tablets (1–100 ppm) (ACARB 25 – Orchid Healthcare, Mumbai, India) and absorption was recorded in the same manner as described above. The IC_{50} value was calculated by plotting % inhibition of enzyme activity against sample extract concentration.

α-amylase inhibition assay

The dinitrosalicylic acid (DNSA) color reagent was prepared using 96 mM 3,5-dinitrosalicylic acid in deionized water (20 mL), 5.31 M sodium potassium tartrate in 2 M NaOH (8 mL) and deionized water (12 mL) (Nickavar et al. 2008). α-Amylase (Type VI-B, porcine pancreas, 23 U /mg of protein) was dissolved in 20 mM phosphate buffer (pH 6.9, containing 6.7 mM NaCl). Plant extracts were dissolved in deionized water with 1 % DMSO (500–5000 ppm extracts/ 1–250 ppm for pure compounds). A 100 μL of α-amylase (8U /mL) was mixed with plant extract and incubated at 25 °C for 30 min. A 100 μL of this mixture was mixed with starch (0.5 % *w/v*) solution (100 μL), incubated at 25 °C for 3 min, DNSA reagent (100 μL) was added, incubated at 85 °C in a water bath for 15 min, allowed to cool and then diluted with distilled water (900 μL). Negative controls were conducted in the same manner replacing plant extracts with 1 % DMSO (100 μL) in deionized water. Blanks were prepared by adding DNSA reagent prior to the addition of starch solution to denature the enzyme, kept in 85 °C water bath for 15 min and then diluted with distilled water (900 μL) as before. Absorbance was measured at 540 nm and percent inhibition was plotted against concentration to calculate IC_{50}. Acarbose (1–100 ppm) was used as the positive control.

Lipase inhibition bioassay

The substrate *p*-nitrophenyl butyrate (PNPB) was dissolved in a 50 mM Tris–HCl buffer containing 6 % *v/v* Triton X-100 (pH 7.2) (Choi et al. 2003). *Candida rugosa* lipase (Sigma L1754, 724 U/mg of protein) was dissolved in 10 mM Tris–HCl buffer (pH 7.5, containing 10 mM KCl). A 20 μL aliquot of 10 mM PNPB, 4 μL of 0.25 M EDTA, 5 μL of 10 % Triton X-100, and 50 μL of 1 M Tris–HCl (pH 7.5) were mixed in a micro centrifuge tube and 721 μL of deionized water was added to make a final volume of 800 μL, so that the working substrate contained 0.2 mM PNPB. Test samples were dissolved in 1 % DMSO in deionized water (500–5000 ppm extracts/ 1–250 ppm for pure compounds and a 100 μL of test sample was mixed with 100 μL of 2 U/mL enzyme and incubated (30 min, 37 °C). The working substrate (800 μL) was mixed, incubated (20 min, 37 °C) and absorbance at 405 nm was measured against a blank that contains 100 μL of enzyme buffer instead of enzyme. Absorbance of the reaction control (100 μL of optimum enzyme concentration and 100 μL of 1 % DMSO in deionized water), incubated under the same conditions, was obtained as before, after the addition of working substrate, against a blank consisting of 100 μL enzyme buffer, 100 μl of 1 % DMSO, and 800 μL of working

substrate. Percent inhibition was plotted vs concentration to calculate IC_{50}. Orlistat (1–100 ppm) was used as the positive control.

Activity guided fractionation of the EtOAc extract of *F. inermis* fruits

The EtOAc extract (2.5 g) which exhibited high inhibitory activity against α-amylase enzyme, was passed through a silica gel column with n-hexane: EtOAc: MeOH system and yielded five fractions F1 (124 mg), F2 (317 mg), F3 (1160 mg), F4 (256 mg) and F5 (355 mg). These fractions were separated comparing TLC patterns of eluted fractions; F1- n-hexane:EtOAc 100:0 to 70:30; F2 n-hexane:EtOAc 60:40 to 30:70; F3 n-hexane:EtOAc 20:80 to EtOAc:MeOH 96:04; F4 EtOAc:MeOH 94:06 to 90: 10, F5 EtOAc:MeOH 80:20 to 100 % MeOH). Fraction F3 (IC_{50} value of 544 ppm) was further fractionated using a silica gel column with an n-hexane: CH_2Cl_2: MeOH system to afford SF3; eluted fractions from n-hexane:CH_2Cl_2 05:95 to CH_2Cl_2: MeOH 98:02 (388 mg, IC_{50} 205 ppm) as the most active fraction. SF3 (350 mg) was passed through a Sephadex LH-20 column with MeOH and further purified by HPLC (1 mL/min, UV monitor at 224 nm) using stepwise gradient solvent systems of 25, 50, 60 and 70 % MeOH in water to isolate AAL1 (40 mg as a white solid). $[\alpha]_D^{25}$ –1.8 (*c* 0.38, H_2O) (lit. –2.3 (The Merck Index 2006); FABMS *m/z* 135 $[M+H]^+$, 117 $[M+H\text{-}H_2O]^+$; ^{1}HNMR (500 MHz in CD_3OD) δ: 2.63 (1H, *dd*, *J*=16.0, 7.7 Hz), 2.78 (1H, *dd*, *J*=16.0, 4.4 Hz), 4.47 (1H, *dd*, *J*= 7.7, 4.4 Hz); ^{13}C NMR (125 MHz in CD_3OD) δ176.6 (C-1), 174.2 (C-4), 68.5 (C-2), 40.1 (C-3).

Statistical analysis

All samples were assayed six times, unless stated otherwise. Data were descriptively analyzed using ORIGIN Pro 8.0.

Results and discussion

The wine red EtOAc extract and dull red MeOH extract from the *F. inermis* fruit extract showed positive results for antioxidant activity, α-amylase, α-glucosidase and lipase enzyme inhibitory activities, and were also rich in phenolics and anthocyanins. The total phenol content (TPC) of fresh fruits of *F. inermis* was found to be 1.28 g of GA equivalents/100 g fresh fruits. Literature reports indicate, of the eleven common fruits analysed (Sun et al. 2002), cranberry had the highest phenolic content of 507 mg GA equivalents/100 g of fresh fruits (Sun et al. 2002). The highest polyphenol content was measured (Gąstol et al. 2013) for the Cornelian cherry (*Cornus mas* L.) while values ranging from 657 to 2611 mg GA equivalents/ =100 g of dry weight were reported in Greek Cornelian cherries (*Cornus mas* cv. Vermio) (Pantelidis et al. 2007). Therefore the polyphenol content of *F. inermis* fruits is relatively high.

The anthocyanin content wasfound to be 108 mg of cyanidin-3-glucoside equivalents/100 g of fresh fruits of *F. inermis*. According to these results, anthocyanins contribute approximately 10 % of the total phenol content of *F. inermis* fruits. The anthocyanin content of *F. inermis* fruits is comparable or higher than those reported for blueberries and blackberries (84.12–116.59 mg/100 g fresh weight) (Sellappan et al. 2002). In another study the anthocyanin content of red fruits such as blackberry, chokeberry, red raspberry and strawberry, were reported to vary from 16.92 mg (strawberry) to 357.19 mg (chokeberry) per 100 g fruit (Jakobek et al. 2007). Therefore, fruits of *F. inermis* provide a dietary source that is rich in these phenolic phytochemicals.

The EtOAc and MeOH extracts showed significant radical scavenging antioxidant activity against the DPPH free radical (Table 1). Higher activity was observed in the EtOAc extract (IC_{50} 66.2 ppm) and compared well with the antioxidant activity determined by the DPPH method, reported for some red fruits (Jakobek et al. 2007). These red fruits are well known to be dietary sources of antioxidants. The higher antioxidant activity of the EtOAc extract in the current study could be due to the presence of anthocyanins, phenolics, including the phenolic acids reported in our previous paper (Jayasinghe et al. 2012).

The EtOAc extracts showed high inhibition activities against α-amylase, α-glucosidase and lipase enzymes under in vitro conditions (Tables 2 and 3). IC_{50}values of the EtOAc extract against α-amylase, α-glucosidase and lipase were 1020.91, 549.13 and 1290.92 ppm, respectively. These values are significant for a crude extract when compared with the IC_{50} values of clinical drugs such as Acarbose, the inhibitor of α-amylase and α-glucosidase (19.85 and 13.83 ppm respectively) and lipase inhibitor Orlistat (55.13 ppm) determined under identical conditions.

The pure compound AAL1, separated after bioactivity guided fractionation, was identified as malic acid by direct comparison of TLC behavior and spectral properties (^{1}H and ^{13}C NMR and FABMS data) with those of authentic material.

Table 1 IC_{50} values for [b]DPPH radical scavenging activity of the EtOAc and MeOH extracts of *F. inermis* fruits

Extract/Compound	[c]IC_{50} (ppm)
EtOAc	66.20
MeOH	212.95
[a]BHA	26.57
Ascorbic acid	22.27

[a] BHA butylated hydroxyanisole; [b] DPPH 1,1-diphenyl-2- picrylhydrazyl; [c] IC_{50} Concentration of extracts in ppm required to cause 50 % scavenging of DPPH radical

Table 2 [c]IC_{50} values for α-glucosidase and α-amylase inhibition of the EtOAc and MeOH extracts of *F. inermis* fruits

Extract	IC_{50} (ppm) (α -glucosidase inhibition)	IC_{50} (ppm) α -amylase inhibition)
EtOAc	549.13	1020.91
MeOH	710.69	1948.39
[a]AAL1	58.15	96.40
[b]Acarbose	13.83	19.85

[a] AAL1 *S*-(−) malic acid isolated from *F. inermis* fruits; [b] Acarbose – clinically used inhibitor of α-amylase and α-glucosidase; [c] IC_{50} Concentration (ppm) of extract required for 50 % inhibition of enzyme activity

The ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data recorded in CD_3OD were listed in Materials and Method Section, since the data has not been reported thus far. It is known that natural malic acid present in fruits including green apples and other fruits is a (*S*)-(−) form (Coppola and Schuster 2002), and thus AAL1 can be reasonably assumed to be (*S*)–malic acid. This was confirmed by the negative sign of the specific optical rotation.

AAL1 (malic acid) showed high activity for inhibition of α-glucosidase, α-amylase enzymes (Table 2) and lipase enzymes (Table 3). Malic acid was also found to be the principal organic acid detected in potent anti-obesity lipase inhibitor containing extracts of a mushroom, *Phellinus linteus* (Lee et al. 2010). These findings suggest that malic acid is responsible for the inhibitory activities of *F. inermis* fruit extracts against α-glucosidase, α-amylase and lipase enzymes. Malic acid is also reported to be one of the most abundant organic acid in different varieties of blackberries (Kafkas et al. 2006) and in several varieties of other fruits (Ryan and Dupont 1973).

Malic acid is a permitted food additive and is often added to food products (to add a sour/tart taste), to obtain value added products, as food additives, and as a preservative in animal feed (European Food Safety Authority 2014). Malic acid has also been used for relief of pain, fibromyalgia and the chronic fatigue syndrome (Russell et al. 1995). Therefore consuming *F. inermis* fruits or incorporating extracts of these fruits which contain a valuable combination of antioxidants such as anthocyanins and phenolic compounds as well as malic acid in a food supplement, will provide a safe and valuable nutritional supplement from a natural source. The inclusion of *Flacourtia inermis* in formulating a product to provide a lipase inhibitor with safe and excellent lipase inhibitory activity has been patented (Chonan et al. 2005). It has been suggested that cranberry (*Vaccinium macrocarpon*) fruit powders, containing organic acids, as well as polyphenolic compounds including phenolic acids and displaying inhibition of both α-glucosidase and α-amylase, could be developed for the management of postprandial hyperglycemia (Pinto et al. 2010). In a study of organic acids in Chinese herbs, it was found that organic acids such as citric acid and L-malic acid may be the active ingredients responsible for the cardioprotective effects *Fructus choerospondiatis* (Tang et al. 2013).

Table 3 IC_{50} values for lipase inhibition of EtOAc and MeOH extracts of *F. inermis* fruits

Extract	[b]IC_{50} value (ppm) (Lipase inhibition)
EtOAc extract	1290.92
MeOH extract	2095.46
[a]AAL1	69.03
[c]Orlistat	55.13

[a] AAL1 *S* (−) malic acid;; [b] IC_{50} Concentration (ppm) of extract required for 50 % inhibition of enzyme activity;[c] Orlistat clinically used inhibitor of lipase

Conclusion

The present study demonstrated that *F. inermis* fruits have antioxidant property as well as enzyme inhibitory activities toward α-glucosidase, α-amylase and lipase. Also, (*S*)-malic acid was isolated and characterized as an active principle that inhibits the three enzymes. These beneficial properties increase the value of the *F. inermis* fruits and suggest that the fruits and fruit extracts can be used as health foods and nutritional supplements.

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Author's contribution A.G.A.W. Alakolanga (25 %). Laboratory bench work and analysis

N. Savitri Kumar (25 %). Research supervision, interpretation of results, manuscript writing

Lalith Jayasinghe (25 %). Research supervision, interpretation of results, manuscript writing

Yoshinori Fujimoto (25 %). NMR spectral data,, interpretation of results, manuscript writing

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