

HPTLC Analysis, Antioxidant and Antidiabetic activities of Ethanol Extract of Moss *Fissidens grandiflora*

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Killari *et al.*: Chemical and Biological Profile of *F. grandiflora*

The phytochemical composition, antioxidant and antidiabetic profile of ethanol extract of *Fissidens grandiflora* was investigated for the first time. The phytochemical analysis of *Fissidens grandiflora* confirmed the presence of flavonoids, phenolics, steroids and tannins. Additionally, *Fissidens grandiflora* contained a high amount of total flavonoids of 101.40±0.38 mg Q/g. The high-performance thin-layer chromatography analysis of *Fissidens grandiflora* identified 3 unknown components with different R_f values and area percentage. *Fissidens grandiflora* showed better inhibition of superoxide free radicals, 2,2-diphenyl-1-picryl-hydrazyl-hydrate free radicals and ferric ions with IC₅₀ values of 81.0, 85.0 and 92.6 µg/ml, respectively. Additionally, *Fissidens grandiflora* depicted a prominent inhibitory profile against α-glucosidase and pancreatic α-amylase with IC₅₀ values of 150.0 and 200.0 µg/ml, respectively. To conclude, the proposed mechanism of action of moss *Fissidens grandiflora* could be by inhibiting free radicals and particularly digestive enzymes.

Key words: Antidiabetic, antioxidant, *Fissidens grandiflora*, moss, pancreatic α-amylase, α-glucosidase

Morphologically, bryophytes are a symbiosis of algae and pteridophytes with around 25 000 species across the globe. Taxonomically, they are classified into liverworts, hornworts and mosses^[1]. Mosses are the simplest-level plants that belong to the second-largest taxonomic group among bryophytes with 14 000 species around the world^[2]. Mosses survive on wet and humid places, and mostly grow on rocks, soil, woods and walls of a building^[3]. Mosses are widely present in forest ecosystems and in the Northern Hemisphere^[4].

Natural sources play a prominent role in the origin of new biological agents that include antioxidants and antidiabetics, which examined to be effectual as well as safe alternative method in the therapy for diabetes mellitus, instead of synthetic molecules^[5]. As a result, search to identify new bioactive molecules and their derivatives for treating diabetes has become an aspect of interest. According to recent research studies, several aquatic organisms including mosses^[6] were found to be a source of these bioactive molecules. Traditionally, tribes of North America utilized mosses for the

management of convulsions, neurasthenia, pneumonia, scald, burns, tuberculosis, and others. In the folklore of China and India, extracts of mosses are well-known for antimicrobial activity and to treat anxiety, snake-bites, heart problems, tuberculosis, cancer, and diabetes^[5,6].

A long time ago, mosses were less considered for the identification of bioactive substances due to problems in identification^[7]. However, in recent times, research attention to mosses chemical profile is increasing, as several biologically active components are identified from these due to their unique adaptations^[8]. However, from a large number of mosses only a very few species have been studied. Additionally, the study of the chemical composition of mosses assists in knowing their metabolism^[9].

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The major chemical constituents of mosses are carbohydrates, essential oils, fatty acids, lipids, flavonoids, cinnamic and benzoic acid derivatives, bibenzyl and benzyl derivatives, volatile oils, polyphenols, terpenoids, steroids and some nitrogen-containing aromatic compounds^[10-12]. There are also reports that extracts of mosses help against a wide range of bacteria and fungi and also act as 15-lipoxygenase inhibitor, wound healing agents, sedatives, cytotoxic, antiHIV, antioxidant, antifeedant, antitumor and proapoptotic and nematocidal due to the presence of terpenoids and aromatic compounds^[5,8,11-17]. *Polytrichum* moss species also present diuretic, antipyretic and antitodal activities and can be used to promote hair growth^[9]. Moss *Taxithelium nepalense* has antioxidant and antidiabetic activities^[18].

Fissidens genus belonging to family Fissidentaceae with about 400 species worldwide, distributed mainly in humid, tropical and warm temperate regions. Particularly, moss *Fissidens grandifolia* reported in the flora of India, China, North and Central America, Africa, West Indies, Mexico and Europe^[19]. Literature survey did not reveal any chemical and biological profiles of the genus *Fissidens*. Hence, to know the chemical profile of *F. grandifolia*, a phytochemical and chromatographic analysis of the ethanol extract (*F. grandifolia*) was carried out to correlate these to its biological profile. The purpose of the present research study was to analyse the chemical composition of *F. grandifolia* by means of phytochemical, high-performance thin-layer chromatography (HPTLC), total flavonoid and phenolic content and to monitor antioxidant and antidiabetic activities.

MATERIALS AND METHODS

HPTLC plates silica gel 60 F 254 was purchased from Merck Ltd. (Mumbai, India). 1,1-diphenyl-2-picrylhydrazyl (DPPH) and intestinal acetone powders from rats were purchased from Sigma Aldrich (Mumbai, India). Amylase HR reagent was obtained from Pro Lab Marketing Pvt. Ltd. (New Delhi, India). All other chemicals used in this study are of analytical grade.

Plant collection:

Specimens of moss *F. grandifolia* were collected from tree barks near Coonoor, Nilagiri hills, Tamil Nadu, India (11° 21' 0.7488" N 76° 47' 45.9744" E, 6070 feet above sea level) in August 2019. Authenticated specimens of *F. grandifolia* was deposited with an accession number LWG-36/VB-Orissa-2019 at Bryophyte Herbarium,

CSIR-National Botanical Research Institute (NBRI), Lucknow, India.

Extraction and phytochemical analysis:

The dried specimens of moss *F. grandifolia* (about 50 g) was extracted thrice with ethanol and concentrated under reduced pressure to obtain ethanol extract of *F. grandifolia* as a dark greenish residue (2 g, 4 % w/w), preserved at 4° for further use. Phytochemical analysis^[20] was carried out on the extract of *F. grandifolia* using standard stratagems for the identification of chemical constituents.

HPTLC analysis:

HPTLC analysis^[21] of *F. grandifolia* was performed on Camag Linomat 5 instrument. Initially, *F. grandifolia* was dissolved in ethyl acetate (10 mg/0.5 ml, HPLC grade), which was centrifuged at 3000 rpm for 5 min and used for analysis. Sample (0.2 µl) was loaded as 6 mm band at length on 10×10 cm HPTLC silica gel plate 60 F 254 using Hamilton syringe (100 µl size). The loaded plate was placed in a thin-layer chromatography twin trough developing chamber and the plate was developed up to 70 mm in hexane:ethyl acetate (1:4) solvent system. The developed plate was dried and sprayed with stannic chloride reagent and placed in a Camag TLC Scanner to capture images at 254 nm using a UV lamp (D2 and W). Finally, the peak display along with peak tables, was identified and measured.

Total flavonoid content:

The total flavonoid content^[22] of the extracts was determined using aluminium chloride spectrophotometric method, in which AlCl₃ forms a complex with hydroxyl groups of flavonoids in the test sample. To the extract (1 mg/ml) or standard quercetin solution (3.125, 6.25, 12.5, 25, 50, 100 µg/ml) 3 ml of methanol, 1 ml of 2 % AlCl₃ solution, 200 µl of 1 M potassium acetate was added and the volume was made up to 10 ml with distilled water and incubated for 60 min at room temperature. The blank contained only reagents, and the absorbance was read at 415 nm. Based on the measured absorbance of the test sample, the total flavonoid content was determined from the calibration graph and the total flavonoid content was expressed in terms of quercetin equivalents (mg quercetin/g of extract).

Total phenolic content:

The total phenolic content^[23-25] was estimated by

Folin-Ciocalteu's method. To the extract (1 mg/ml) or standard gallic acid solution (3.125, 6.25, 12.5, 25, 50, 100 µg/ml) 0.5 ml of Folin-Ciocalteu reagent, 1.5 ml of 20 % sodium carbonate was added, the volume was made up to 10 ml with distilled water and incubated at room temperature for 120 min and the absorbance was measured at 750 nm on a spectrophotometer against blank. Based on the measured absorbance of the test sample, the total phenolic content was read on the calibration graph and the total phenolic content was expressed in terms of gallic acid equivalent (mg of gallic acid/g of extract).

In vitro antioxidant activity, DPPH assay:

F. grandifolia extract was subjected to the DPPH assay^[26] in triplicate and results were reported as % inhibition of DPPH free radical. Initially to known concentrations of the sample, 0.004 % DPPH dissolved in methanol was added and incubated for 30 min at 37°. Sample absorbance was read 517 nm against a suitable blank on a UV/Vis spectrophotometer (Spectra MAX plus 384, USA) Plotting concentrations against the % inhibition determined IC₅₀ values of *F. grandifolia* extract.

Superoxide radical scavenging assay:

F. grandifolia extract was screened in the superoxide radical scavenging assay^[27] in triplicate and results were expressed as % inhibition of superoxide free radicals. NADH (73 µM), 15 µM of PMS and NBT (50 µM) in 20 mM phosphate buffer (pH 7.4) was mixed and standardized. Then 1 ml of known concentrations of test sample was added to the above-standardized mixture and incubated for 30 min. After incubation, absorbance of all the samples were read at 562 nm against blank. IC₅₀ values of *F. grandifolia* extract were determined by plotting concentrations against % inhibition.

Ferric ion (Fe³⁺) reducing power assay:

F. grandifolia extract was screened in the ferric ion (Fe³⁺) reducing power assay^[28] in triplicate and results were expressed as % of inhibition of Fe³⁺ ions. To 2.5 ml of phosphate buffer (pH 6.6, 0.2 M), 2.5 ml potassium ferricyanide (1 %) and known concentrations of sample were added and incubated for 20 min. Later, 2.5 ml of 10 % trichloroacetic acid and 0.5 ml of 0.1 % ferric chloride was added to each sample and the absorbance was read at 700 nm against a blank. IC₅₀ values of *F. grandifolia* were determined by plotting concentrations against % inhibition. Percent inhibition was calculated using the formula, $(C-S)/C \times 100$, where

C is the absorbance of the control, S is the absorbance of sample.

In vitro antidiabetic activity:

The assay of α -glucosidase inhibitory activity^[22] was performed in a triplicate. A stock solution of α -glucosidase acetone powder from rat intestine acetone powder was made prepared (1.0 mg/ml in 10 mM phosphate buffer, pH 6.8, diluted 40-fold with the same buffer) and 2.0 µl of this solution was mixed with 20 µl of the samples of different concentrations (25, 50, 75 and 100 µg/ml dissolved in DMSO) and 100 µl of 50 mM phosphate buffer (pH 6.8) in 96 well microplates and incubated for 5 min at 37°. After incubation, 50 µl of the substrate (5 mM of *p*-nitrophenyl- α -D-glucopyranoside prepared in 50 mM of phosphate buffer, pH 6.8) was added and the entire reaction mixture was again incubated for 20 min at 37°. After that, the reaction was terminated by adding 50 µl of Na₂CO₃ (1 M) and the final volume was made to 150 µl. The amount of *p*-nitrophenol released from the substrate was noted at 405 nm spectrophotometrically. DMSO and glibenclamide were used as control and standard, respectively. Percent inhibition of α -glucosidase enzyme was calculated using the formula, $\% \text{ inhibition} = (C-S)/C \times 100$, where C is the absorbance of the control, S is the absorbance of sample

The porcine pancreatic α -amylase inhibitory assay^[26] was determined in a triplet (n=3). Amylase HR reagent (100 µl) and 40 µl of sample of various concentrations (50, 100, 150 and 200 µg/ml of extract) were mixed and incubated for 10 min at 37°. Then 60 µl of 0.1 mg/ml BPNPG7 (blocked *p*-nitrophenylmaltoheptaoside) in 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer pH 6.9 was added and further incubated for 10 min at 37°. The quantity of *p*-nitrophenol released from *p*-nitrophenyl- α -D-glucopyranoside (substrate) was measured at 405 nm on a UV/Vis spectrophotometer (Electron 420 series spectrophotometer). DMSO was used as control and glibenclamide as a standard. IC₅₀ values of test samples were determined by plotting concentrations of the sample against % inhibition. The % inhibition of α -amylase activity was calculated using the formula, $\% \text{ inhibition} = (C-S)/C \times 100$, where C is the absorbance of the control, S is the absorbance of sample.

RESULTS AND DISCUSSION

Phytochemical analysis^[20] of *F. grandifolia* showed

presence of different phytochemical constituents (Table 1), such as carbohydrates, flavonoids, phenolics, steroids, and tannins, but it gave negative results for alkaloids, carbohydrates, saponins, and glycosides. The HPTLC analysis^[21] gave the fingerprinting profile of *F. grandifolia* extract, which was illustrated in fig. 1. From the HPTLC chromatogram, 3 unknown compounds represented by peak 1, 2 and 3 with R_f values and percent areas of -0.04, -0.01 and 0.37, and 16.67, 54.43 and 28.82 %, respectively (fig. 1).

Total flavonoid content^[22] and phenolic content^[23]

TABLE 1: PHYTOCHEMICALS IN ETHANOL EXTRACT OF *FISSIDENS GRANDIFOLIA*

Phytochemical	Ethanol extract <i>F. grandifolia</i>
Steroids	+
Saponins	-
Alkaloids	-
Carbohydrates	+
Flavanoids	+
Tannins	+
Glycosides	-
Phenolics	+

‘+’ indicates presence; ‘-’ indicates absence

expressed as quercetin equivalent and gallic acid equivalent, respectively, arrived at from the standard calibration line Eqns. $y = 0.0096x + 0.0052$; $R^2 = 0.999$ and $y = 0.0038x + 0.003$; $R^2 = 0.9997$, respectively (fig. 2). *F. grandifolia* extract contained higher total flavonoid content (101.40 ± 0.38 mg Q/g) compared to total phenolic content (65.71 ± 0.95 mg GA/g) as shown in Table 2.

Among the phytochemicals from natural sources, polyphenols and flavonoids are usually regarded as the substances with high antioxidant capability. These compounds also reported to be effective against Alzheimer's, diabetes, eye disorders, cancer, and heart problems. Additionally, the most vital feature of phenolic and flavonoids is their ability to be effective against oxidative diseases like diabetes by reducing LDL oxidation^[24,25].

In general, natural antioxidants have pronounced safety to mankind and are without any significant side effects. Based on the aforementioned points, *F. grandifolia* extract was screened initially against DPPH^[26],

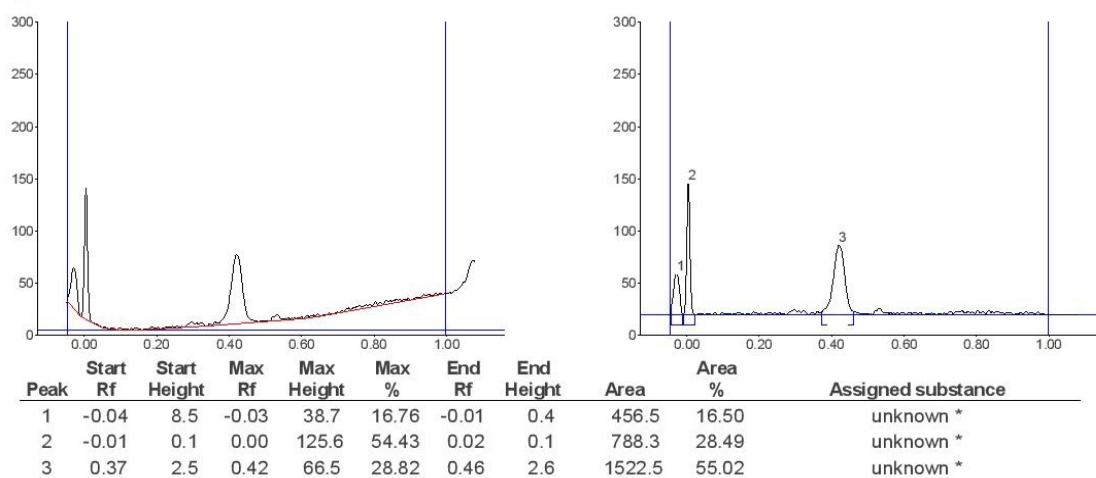


Fig. 1: HPTLC chromatogram of ethanol extract of *F. grandifolia*

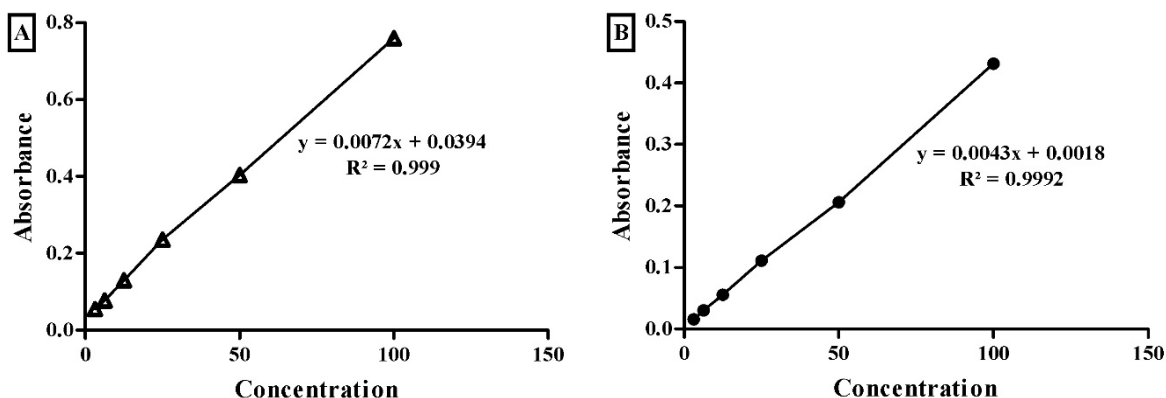


Fig. 2: Calibration graphs of ethanol extract of *F. grandifolia*
Calibration graph for A. total flavonoid and B. phenolic contents of ethanol extract of *F. grandifolia*

superoxide^[27], and ferric ion assays^[28], the results of which were illustrated in fig. 3. The lower IC₅₀ values indicate higher inhibition of free radicals. The principal of DPPH radical assay in which decoy of the DPPH radical to a non-radical form (DPPH-H) takes place. The IC₅₀ values of *F. grandifolia* extract against DPPH was found to be 85.0 µg/ml, whereas ascorbic acid's value was 29.5 µg/ml.

On the other hand, the superoxide radicals arise from metabolic process interact with other substrates in presence of enzyme or metal catalysed routes to engender hydroxyl radical, peroxides and oxygen free radicals. These free radicals induce oxidative damage to lipids, DNA and proteins^[25]. The concentration of *F. grandifolia* extract needed to cause 50 % inhibition of superoxide radical was 81.0 µg/ml, while ascorbic acid was 26.5 µg/ml. The IC₅₀ value of *F. grandifolia*

extract was 92.6 µg/ml, whereas ascorbic acid value was 34.5 µg/ml (fig. 3). From all the antiradical assays, it can be concluded that *F. grandifolia* is preferably acting by exhibiting radical scavenging properties rather than reducing antioxidant power.

Natural sources with a high amount of phenolics and flavonoids, which display effective antioxidant profile have been recommended for use in the treatment of diabetes^[26]. Generally, the main biological target in diabetes of phenolics and flavonoids are α-glucosidase and DPP-4, by acting as radical scavengers^[29]. Therefore, as *F. grandifolia* revealed good phenolic and flavonoid content with prominent antioxidant activity, the study was extended to evaluate its ability to manage diabetes.

The α-glucosidase inhibitory assay^[22] was performed using *p*-nitrophenyl-α-D-glucopyranoside as a substrate and glibenclamide as a standard inhibitor. From the assay, it was estimated that the IC₅₀ concentration needed for *F. grandifolia* to inhibit α-glucosidase enzyme was 150.0 µg/ml, while glibenclamide's was 29.3 µg/ml (fig. 4). The porcine pancreatic α-amylase inhibition assay^[26] was performed using BPNPG7 and

TABLE 2: TOTAL FLAVONOID AND PHENOLIC CONTENT OF *FISSIDENS GRANDIFOLIA*

Sample	Total flavonoid content*	Total phenolic content*
<i>Fissidens grandifolia</i>	101.40±0.38 mg Q/g	65.71±0.95 mg GA/g

*n=3; mean±SD

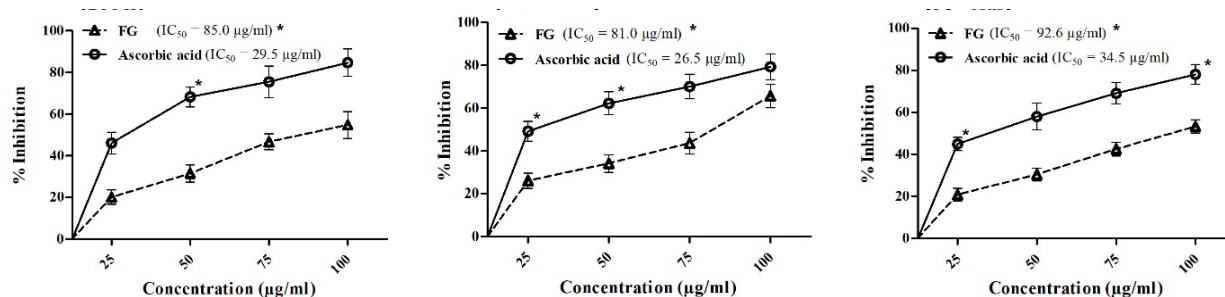


Fig. 3: Antioxidant activity profiles of ethanol extract of *F. grandifolia*

IC₅₀ value determination of ethanol extract of *F. grandifolia* (FG) against A. DPPH, B. superoxide and C. ferric ions, n=3; Mean±SD; *statistically significant at p<0.05. (—○—) ascorbic acid

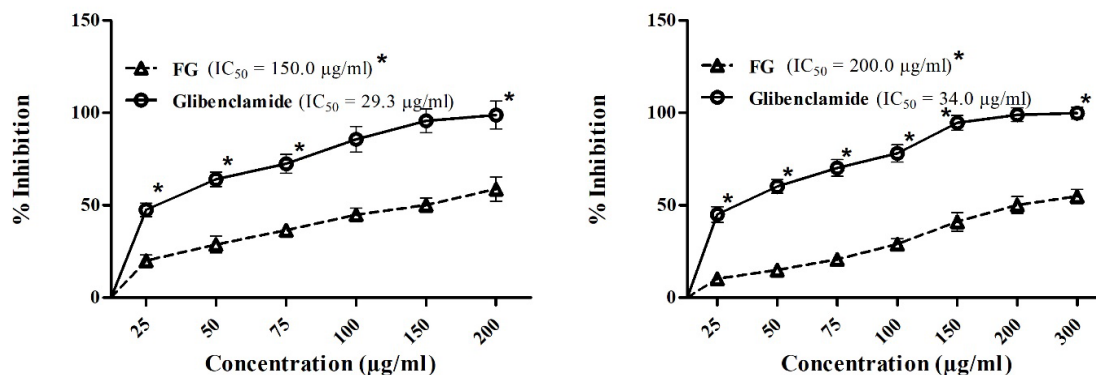


Fig. 4: α-Glucosidase and α-amylase inhibitory activity of ethanol extract of *F. grandifolia*

IC₅₀ value determination of ethanol extract of *F. grandifolia* against A. α-glucosidase and B. α-amylase n=3; mean±SD; **statistically significant at p<0.05. (—○—) glibenclamide

glibenclamide as substrate and standard, respectively. The concentration of *F. grandifolia* extract needed to cause 50 % inhibition of porcine pancreatic α -amylase was found to be 200.0 μ g/ml, while glibenclamide was 34.0 μ g/ml (fig. 4). From the outcomes of *in vitro* assays, it could be confirmed that the *F. grandifolia* extract could be antidiabetic by virtue of inhibiting digestive enzymes.

Diabetes mellitus is a metabolic disorder well-defined by hyperglycaemia resulting due to alteration in the metabolic rate of carbohydrate, lipid and proteins. Additionally, oxidative stress conditions assumed to augment free radical production. According to recent studies, the oxidative stress-induced free radicals have been associated in the pathology of diabetes mellitus^[25-27].

In the current study, phytochemical analysis of *F. grandifolia* revealed different phytochemical constituents (Table 1). The *F. grandifolia* was subjected to HPTLC analysis in order to develop the finger-print profile (fig. 1). Based on the phytochemical analysis data, the total flavonoid and phenolic contents of *F. grandifolia* were determined which revealed their presence in significant amounts (Table 2). Further, the antioxidant (DPPH and superoxide assays) activity, *in vitro* antidiabetic activity of *F. grandifolia* extract was evaluated and quantified to identify the extracts higher scavenging capacity towards DPPH and superoxide free radicals (fig. 3), which revealed that the antioxidant capability of *F. grandifolia* is due to free-radical quenching mechanism.

In addition, the outcomes of *in vitro* bioassays on digestive enzymes revealed that the *F. grandifolia* was specific towards α -glucosidase enzyme and α -amylase, which indicated the proposed mechanism of action of *F. grandifolia* might be acting by inhibiting particularly digestive enzymes like α -glucosidase enzyme and α -amylase (fig. 4).

In conclusion, present study provided primary information on the chemical composition, antioxidant, α -glucosidase and α -amylase inhibitory properties of *F. grandiflora*. The examination of the *F. grandifolia* revealed the existence high flavonoid content. The main substances identified from phytochemical analysis of *F. grandifolia* were carbohydrates, steroids, polyhydroxy compounds and aliphatic fatty acids. Additionally, evaluation of antioxidant and antidiabetic activities suggested that *F. grandifolia* possessed DPPH and superoxide free radical suppressing property and α -glucosidase and porcine pancreatic α -amylase

inhibitory activity. This information provided some evidence to the traditional medical application of *F. grandiflora* to manage diabetes. These active constituents needs to be isolated and further investigated to find out how *F. grandiflora* reduced free radicals in diabetes.

Conflict of interest:

No conflict of interest between any of the authors.

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