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PHYTOCHEMICAL SCREENING AND IN VITRO EVALUATION OF ANTIDIABETIC ACTIVITY OF *FICUS PALMATA* FRUITS

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ABSTRACT

The present study was aimed to investigate the phytochemical composition and in-vitro substantiation of the α-amylase and α-glucosidase enzymes inhibitory activity of *Ficus palmata* fruits. The fruits were extracted with methanol: water (4:1 v/v) and the extract obtained were further fractionated using n-butanol, ethylacetate, methanol and water. All the four fractions were phytochemically screened for presence of various phytochemical groups and evaluated for α-amylase and α-glucosidase inhibitory activity at different concentrations (50-500 µg/ml). Phytochemical screening revealed highest number of different phytochemicals in the MeOH fraction followed by EtOAc and aqueous fractions. All the fractions exhibited inhibitory action against α-amylase and α-glucosidase enzymes in a concentration dependant manner. The highest inhibitory activities of BuOH, EtOAc, MeOH and aqueous fraction were recorded as 61.77, 85.41, 90.63, and 78.25% against α-amylase and 69.81, 86.39, 93.43, and 79.55 % against α-glucosidase respectively at a concentration of 500 µg/ml. Among all, methanol fraction recorded the best α-amylase and α-glucosidase inhibitory activity with an IC_{50} value of 166.91±2.73 and 118.73±0.67 µg/ml respectively comparable to that of acarbose (154.87±2.33 and105.63±1.71 µg/ml). The present study amply established the potent α-amylase and α-glucosidase inhibitory activity of *F. palmata* fruits. The highest inhibitory efficacy of methanol fraction of *F. palmata* fruit which may be due the presence of bioactive phytochemicals in higher concentration could be beneficially exploited in the treatment of Type2DM. However, isolation and characterization of specific chemical constituents responsible for the activity as well as *in-vivo* studies are needed for further affirmation.

KEYWORDS: *Ficus palmata*, Fruits, Phytochemical screening, α-amylase, α-glucosidase.

INTRODUCTION

Diabetes mellitus (DM), a metabolic disorder characterized by increased blood glucose levels is a chronic disease afflicting millions of people worldwide. It is the most common endocrine disorder causing severe tissue and vascular damage leading to serious complications.[1] According to an estimate, about 200 million people worldwide suffered from DM in 2010, and it is expected to reach 300 million by 2025.[2] The prevalence of diabetes in India alone is approximately 40.9 million, which is expected to rise to 60.9 million by 2025.[3] Postprandial hyperglycemia responsible for the development of type 2 diabetes constitutes the majority of all diabetes cases and its complications. Primary goal in the management of diabetes is to regulate the blood glucose concentration as close as to normal physiological level, in order to prevent chronic diabetic complications such as retinopathy, nephropathy, neurologic and cardiovascular diseases.[4] Inhibition of α-amylase and α-glucosidase, the key carbohydrates hydrolyzing enzymes is one of the effectual therapeutic approaches to reduce postprandial increase in blood glucose through obstructing carbohydrate digestion and decreasing the rate of glucose absorption.[5,6] Secreted by pancreas and salivary glands, the digestive enzyme α-amylase is responsible for hydrolyzing dietary starch (maltose), which breaks down into glucose prior to absorption. Inhibition of α-amylase can lead to reduction in postprandial hyperglycemia in diabetic condition.[7] Alpha-glucosidase is a membrane bound enzyme located on the epithelium of the small intestine, catalyzing the cleavage of disaccharides leading to liberation of absorbable monosaccharides such as glucose from the substrate which eventually facilitates the absorption by the small intestine.[8] Inhibitors of the α-glucosidase could be one of the most effective approaches to control diabetes.[9] The two enzymes can retard the uptake of dietary carbohydrates and suppress postprandial hyperglycemia. Hence, α-amylase and α-glucosidase inhibitors are the potential targets for antidiabetic drug development as well as lead compounds for the treatment of diabetes.[10] Conventional drugs used as inhibitors of amylase and glucosidase show gastrointestinal side effects such as bloating, abdominal discomfort, diarrhoea, and flatulence making them less attractive as therapeutic agents.[11-13] Hence the need to identify and

explore natural sources for such inhibitors with less or no side-effects has been realized.

Medicinal plants have been used since prehistoric times worldwide for the treatment of diabetes. Most useful drugs derived from plants have been discovered by follow-up of ethnomedical uses.[14]A number of plants have been pharmacologically investigated for their antidiabetic efficacy.[15-17] Plant derived natural remedies for diabetes are gaining popularity for being effective, inexpensive and safe as compared to their synthetic counterparts.[18] Some of the antidiabetic plants could be important sources of amylase and glucosidase inhibitors.[19] Plants belong to the genus *Ficus* are well known for their therapeutic value in traditional system of medicine. Some of the plants of the genus have ethno-botanically and pharmacologically been investigated for antioxidant[20], antimicrobial[21], anticancer, anti-inflammatory[22] and antidiabetic[23] properties as well as chemical constituents and mechanisms of action of some of the chemical constituents of *Ficus* in management of DM have been studied.[24]

Ficus palmata Forsk. commonly known as Wild Himalayan Fig or Bedu and belonging to the family of Moraceae is a deciduous tree native to North-Western India. The tree produces edible fruits which are consumed as such when ripe while unripe fruits are cooked and eaten as a vegetable. Bark, root, leaves fruit and latex of this plant are pharmacologically regarded as hypoglycemic, antitumour, anti-ulcer, anti-diabetic, lipid lowering and antifungal and have traditionally been used for the treatment of various ailment e.g. gastrointestinal disorder, diabetes, ulcer, tumour, etc.[25,26] Leaves and stem bark of the plants are reported to contain flavonoid while β-sitosterol, tetracyclic tritepene, glaunol acetate were reported from the leaves, bark and heartwood and ceryl behenate, lupeol, α-amyrin acetate from the stem bark.[27] Phytochemical study of the aerial parts of *F. palmata* resulted in the isolation of a new isomer of psoralenoside namely, transpsoralenoside, germanicol acetate, furanocoumarins, (psoralene and bergapten), vanillic acid and the rutin (flavone glycoside).[28] Barks have been pharmacologically examined for antibacterial and antioxidant properties.[28] Antibacterial activity of latex from stems has also been reported.[29] However, no study have so far been reported on α-amylase and α-glucosidase inhibitory action of *F. palmata* fruits. Therefore, the aim of the present study was to examine the phytochemical composition of different solvent fractions of *F. palmata* fruits and evaluate them for their α-amylase and α-glucosidase inhibitory activity.

MATERIALS AND METHODS

Chemicals and reagents

All the organic solvents used for extraction and fractionation were of analytical grade and refer to Merck, and Sdfinechem while the water used was triple-distilled in a glass distillation apparatus. Chemicals including α-glucosidase (*Saccharomyces cerevisiae*), α-amylase (procaine pancreas), 3,5, di-nitro salicylic acid (DNSA), p-nitro-phenyl-α-D-glucopyranoside (p-NPG), sodium carbonate (Na_2CO_3), sodium dihydrogen phosphate, di-sodium hydrogen phosphate, Dimethylsuphoxide (DMSO) were purchased from Hi-Media, Mumbai. For qualitative phytochemical screening, readymade available or freshly prepared reagents like Dragendorff's Reagent, Ferric chloride, Liebermann Burchard Reagent, Ehrlich Reagent, Ninhydrine etc. were used. Analytical sodium sulphate (Na_2SO_4) was normally used for dehydrating organic solvents and extracts. UV/Vis double beam spectrophotometer (Chemito 1700, Thermo Fisher) was used for measuring absorbance during assays.

Plant material

Fully grown and ripen fruits of *Ficus palmata* (FP) were collected from the tree located in the campus of Forest Research Institute, Dehradun, India (GPS data: elevation 437 m; longitude 77099′79″ E and latitude 30034′41″ N) during June/July 2016. The collected fruit samples were authenticated by Botanical Survey of India (Northern Regional Centre), Dehradun under Accession Nos. 115594. A voucher specimen of the fruit is preserved in the Chemistry and Bioprospecting Division for future reference. Collected fruits were cleaned properly under running tap water and then dried in shade at room temperature. Fruits of FP were cut into small pieces, dried in shade and then ground to coarse powder using an electronic grinder. Fruit powder was stored in airtight containers at room temperature till further use.

Extraction and fractionation

Dried and powdered fruits of FP (100g) were at first defatted with petroleum ether and then extracted with methanol: water (4:1 v/v) at room temperature for 72 hrs. The FP fruit extract (FPFE) were filtered through Whatman No.1 filter paper and was concentrated under reduced pressure using rotavapor (Buchi) and finally dried to a constant weight. The yield of FPFE was calculated on moisture free basis. FPFE was further fractionated with n-butanol (BuOH), ethylacetate (EtOAc), methanol (MeOH) and Water (Aqueous) to obtain BuOH, EtOAc, MeOH and aqueous fractions. Each of the fractions was concentrated under vacuum to obtain respective dry extracts.

Phytochemical screening

Phytochemical screening of BuOH, EtOAc, MeOH and aqueous fractions of FPFE was carried out following standard to detect presence the various phytochemicals such as phenols, flavonoids, alkaloids, coumarins, tannins, saponins, terpenoids, steroids, alkaloids, etc. according to the standard protocols.[30-33] Changes in colour and/or formation of precipitate with addition of specified detecting reagents to the test solutions of various fractions was observed and, results were recorded as present (+) or absent (–) based on the observations. All the qualitative phytochemical tests were replicated thrice for confirmation.

Test for alkaloids
Each of the fractions of FPME was mixed with ammonia solution in a test tube and after chloroform was added after some time. The mixture was shaken, filtered and evaporated over water bath to remove chloroform. To this 2 ml of Mayer's reagent was added which resulted in appearance of cream coloured precipitate which indicated the presence of alkaloids. Furthermore each of the fractions was treated with a few drops of Dragendorff's reagent. Formation of brick red colour or precipite evidenced the presence of alkaloids.

Test for steroids
To 5 ml of the extract fraction, 2 ml of acetic anhydride and 2 ml conc. sulphuric acid (H_2SO_4) was added. The colour changed from violet to blue or green indicated the presence of steroids.

Test for terpenoids
Each fraction (0.5 mg) was mixed in 2 ml of chloroform, and then 3 ml of concentrated sulphuric acid was carefully added to form a layer. Formation of reddish brown colouration at the junction indicated the presence of terpenoids.

Test for phenolics
Each fraction (50 mg) was dissolved in 5 ml of distilled water and few drops of 5% ferric chloride were added. Bluish black colour indicated the presence of phenolic compounds. Further a small part of extract fractions (50 mg) were dissolved in little amount of distilled water and 3 ml of 10% lead acetate solution was added. Appearance of a bulky white precipitate indicated the presence of phenolic compounds.

Test for flavonoids
A few drops of diluted sodium hydroxide solution were added to the solution of each fraction (0.5 ml); appearance of an intense yellow colour which disappeared upon the addition of a few drops of dilute H_2SO_4 acid showed the presence of flavonoids. Presence of flavonoids were also tested following Shinoda test. The extract fractions were dissolved in alcohol and a piece of magnesium followed by concentrated hydrochloric acid was added and heated. Appearance of magenta colour shows the presence of flavonoids.

Test for tannins
A small quantity of each fraction (0.5 ml) was dissolved in chloroform (5 ml) and acetic anhydride (1 ml) was added. Finally H_2SO_4 (1 ml) was added carefully to the solution along the wall sides of the vessel. Formation of green colour indicated the presence of tannins. Further each of fractions were mixed with water; heated, filtered and ferric chloride was added. Presence of tannin was indicated by appearance of blue or greenish black or dark red colour with potassium ferrocyanide and ammonia.

Test for anthraquinones
Dried extract fractions were disolved in benzene for 10 minutes and then filtered. To this filtrate 10% ammonia solution was added and shaked vigorously for 30 seconds. Formation of pink, violet, or red color in the ammonia phase indicated the presence of anthraquinones.

Test for coumarins
Dried extract fractions were dissolved in hot distilled water, cooled and divided into two parts to be used as test sample and reference. To the test sample, 0.5 ml of 10% ammonium hydroxide was added then examined under UV light (λmax= 365 nm). The occurrence of an intense fluorescence suggested the presence of coumarins and derivatives.

Test for saponins
Each fraction of the FPFE (0.5 ml) was mixed with distilled water (20 ml) in a test tube and then the shaken for 15 min. The formation of a foam layer on the top of the test tube showed the presence of saponins.

Test for glycosides
A small portion of extract was dissolved in alcohol and a few fragments of magnesium ribbon or zinc powder and concentrated HCL acid were added drop wise. Development of pink to crimson colour suggested the presence of flavonol glycosides. Further presence of steroidal glycosides was detected by adding 2ml concentrated H_2SO_4 to the extract fractions and resulting appearance of reddish brown colour.

Test for carbohydrates
Molisch test was adopted to detect presence of carbohydrate. To 2 ml of the extract fractions, 2 drops of alcoholic solution of α-napthol was added in a test tube with shaking. Then conc. H_2SO_4 was added from the sides of test tube. Formation of violet ring was indicative of carbohydrate.

Test for proteins
Biuret test was performed to detect the presence of protein. To 3 ml of extract fractions, 4% NaOH solution and few drops of 1% $CuSO_4$ solution was added. Formation of violet or pink colour indicated the presence of proteins.

***Test for* amino acids**
To the extract fractions few drops of phenolphthalein were added and then dilute solution of sodium hydroxide was added drop-wise until the solution turns pink. Formation of pink coloration indicated the presence of free amino acids.

Alpha-amylase inhibitory assay
Alpha-amylase inhibitory activity of BuOH, EtOAc, MeOH and aqueous fractions of FPFE was determined by standard method with minor modification.[34] The reaction mixture containing 50 µl phosphate buffer (100

mM, pH 6.8), 10 µl of α-amylase (2 U/ml), and 20 µl of varying concentrations (50-500µg/ml) of extract fractions were preincubated at 37^0C for 20 min. Then, the 20 µl of 1% soluble starch (100 mM phosphate buffer pH 6.8) was added as a substrate and incubated further at 37^0C for 30 min. Then 100 µl of the DNSA colour reagent was added and boiled for 10 min. The absorbance of the resulting mixture was measured at 540 nm. Acarbose at various concentrations was used as a standard. The percentage inhibition was calculated using the formula –

Inhibitory activity I (%) = (Ac − As/Ac) ×100.

Where, As is the absorbance of test sample and Ac is the absorbance of control.

Alpha-glucosidase inhibitory assay

The α-glucosidase inhibitory activity of BuOH, EtOAc, MeOH and aqueous fractions of FPFE was carried out according to the standard method with slight modification.[35] Reaction mixture containing 50 µL phosphate buffer (100 mM, pH = 6.8), 10 µL α-glucosidase (1 U/ml), and 20 µL of varying concentrations of extract fractions (50-500µg/ml) were preincubated at 37^0C for 15 min. Then, 20 µl of 5 mM p-NPG was added as a substrate and further incubated at 37^0C for 20 min. Further, 50 µL of 0.1M Na_2CO_3 was added to the reaction mixture and absorbance of the released p-nitrophenol was measured at 405 nm. Acarbose at various concentrations was taken as a standard. The results were expressed as percentage inhibition, which was calculated using the formula –

Inhibitory activity (%) = (Ac − As/Ac) ×100.

Where, As is the absorbance of test sample and Ac is the absorbance of control.

STATISTICAL ANALYSIS

All data presented are mean values of triplicate measurements (n= 3), obtained from three separate tests/measurements; unless stated otherwise. Results are expressed as means±standard deviations (SD). The statistical significance was evaluated by the analysis of variance (ANOVA) followed by Duncan Multiple Range Test (DMRT) using SPSS ver.16.0. $P \leq 0.05$ was considered as a statistically significant.

RESULTS AND DISCUSSION

Phytochemical screening

Extraction of FP fruits with methanol: water (4:1 v/v) yielded 22.71% of FPFE on dry weight basis. The qualitative phytochemical screening of BuOH, EtOAc, MeOH and aqueous fractions of FPFE revealed the presence of various phytochemicals as summarized in Table 1.

Table 1: Phytochemical analysis of FPFE fractions.

Phytochemicals	FPFE Fractions			
	BuOH	EtOAc	MeOH	Aqueous
Phenolics	+	+	+	+
Tannins	-	+	+	+
Flavonoids	+	+	+	+
Terpenoids	+	+	+	-
Steroids	+	+	+	-
Alkaloids	+	+	+	-
Anthocyanins	-	+	+	+
Coumarins	-	+	+	-
Glycosides	+	+	+	+
Saponins	-	-	+	+
Carbohydrates	-	-	+	+
Protein	-	-	+	+
Amino acids	-	-	+	+

(+) Present (–) Absent

The results of phytochemical screening of different fractions of FPFE as presented in Table 1 showed that varied range of phytochemical groups such as alkaloids, terpenoids, steroids, flavonoids, phenolics, tannins, anthocyanins, coumarins, glycosides, saponins, carbohydrates, protein and amino acids were present in different fractions. The MeOH fraction recorded the highest number different phytochemicals followed by EtOAc and aqueous fractions. This suggested that these solvents are effective to isolate active bioctive phytochemicals due to their high polarity. Among all the tested fractions, BuOH fraction showed the lowest number of phytochemicals. All the fractions showed positive tests for phenolics, flavonoids and glycosides. Tannins and anthocyanins were present in all fractions except BuOH whereas terpenoids, steroids and alkaloids were absent in aqueous fraction. Whereas saponins, carbohydrate, proteins and amino acids were present in MeOH and aqueous fractions; EtOAc and MeOH fractions showed positive test for coumarins only.

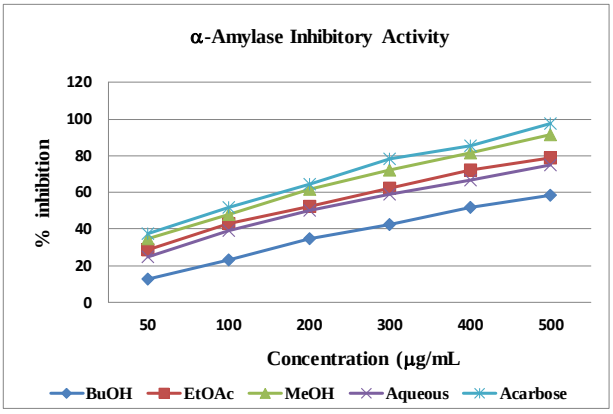
Chemical constituents of plants are known to be biologically active and are responsible for diverse pharmacological properties of plants.[36,37] The results of phytochemical screening of FPFE fractions revealed the presence of phenolic and flavonoid compounds in all

fractions suggesting that these solvents are apt for isolating phenolic compounds due to their high polarity. The phenolic compounds, owing to their antioxidant property[38] are associated with reduced risk of heart and cardiovascular diseases.[39,40] Flavonoids belonging to polyphenolic group are accredited for health promoting properties such as antioxidant, anti-allergic, anti-inflammatory, antimicrobial and anticancer properties.[41-43] Several studies have shown that elevated extra- and intra-cellular glucose concentrations result in oxidative stress.[44] Phenolics including flavonoids, tannins and their derivatives considered to be antioxidants or free radical scavengers[45-49] thus beneficial in DM and related complications. Saponins involved in defense mechanism of plant are known for their antimicrobial activity.[45,46] Terpenoids have been pharmacologically referred as antibiotics, insecticidal, anthelmintic and antiseptic.[50,51] Alkaloids found to be present in all fractions except aqueous are reported to have analgesic, antispasmodic and bactericidal, antimalarial and analgesic activities.[52,53] Glycosides detected in all the fraction of FPFE are said to be beneficial in cases of congestive heart failure and cardiac arrhythmia.[54] Among all the tested fractions of FPFE, BuOH has the lowest number of phytochemicals present. Whereas, all fraction found to be good source of phenolic compounds; the MeOH fraction contained the highest number of different phytochemicals as compared to the other fractions.

Alpha-amylase inhibitory activity

The inhibitory effects of different FPFE fractions on α-amylase enzyme were evaluated by *in-vitro* method. All the fractions exhibited inhibitory action against the enzymes in a concentration dependant manner. The BuOH, EtOAc, MeOH and aqueous fractions exhibited the highest α-amylase inhibitory activity of 61.77, 85.41, 90.63, and 78.25 % respectively at a concentration of 500µg/ml (Figure 1).

Fig. 1: Alpha-amylase inhibitory of activity of FPFE fractions.



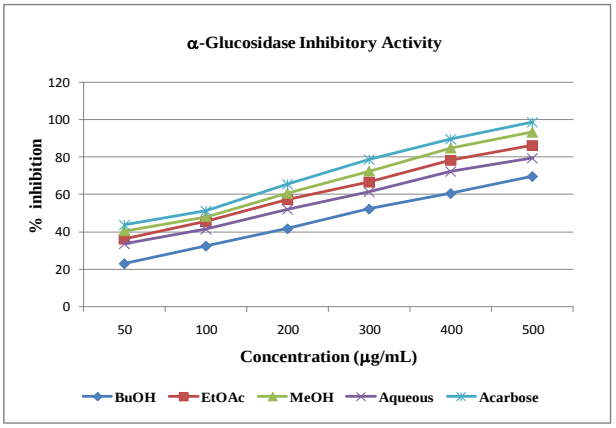
The values are Mean ± S.D. of triplicates (P≤ 0.05)

In the present study, the methanol fraction of FPFE showed the most potent α-amylase inhibitory activity as compared to other fractions at all tested concentrations.

Alpha-glucosidase inhibitory activity

The alpha-glucosidase inhibitory activity of different fractions of FPFE were evaluated by *in-vitro* method. All the fractions exhibited inhibitory action against α-glucosidase in a dose dependant manner. The BuOH, EtOAc, MeOH and aqueous fractions exhibited the highest α-glucosidase inhibitory activity of 69.81, 86.39, 93.43, and 79.55% respectively at a concentration of 500µg/ml (Figure 2).

Fig. 2: Alpha-glucosidase inhibitory of activity of FPFE fractions.



The values are Mean ± S.D. of triplicates (P≤ 0.05)

Results of the study showed that the methanol fraction of FPFE showed the strongest α-glucosidase inhibitory activity as compared to other fractions at all tested concentrations.

Acarbose was used as a standard reference drug for both α-amylase and α-glucosidase enzyme inhibitory assay. The standard enzyme inhibitor showed α-amylase inhibitory activity with an IC_{50} value of 154.87±2.33 µg/ml and α-glucosidase inhibitory activity with an IC_{50} value of 105.63±1.71 µg/ml. Among all, methanol fraction of FAFE has shown the highest α-amylase and α-glucosidase enzyme inhibitory activity with an IC_{50} value of 166.91±2.73 and 118.73±0.67 µg/mL respectively which were comparable with that of acarbose (Table 2). Lowest IC_{50} values are indicative of highest inhibitory activity.

Table 2: α-amylase and α-glucosidase inhibitory effects of FAFE fractions and Acarbose.

Fafe Fractions	IC_{50} values of α-amylase inhibition (g/ml)	IC_{50} values of α-glucosidase inhibition (g/ml)
BuOH	365.43±3.57	316.77±1.51
EtOAc	194.06±1.69	137.53±1.03
MeOH	166.91±2.73	118.73±0.67
Aqueous	259.74±1.09	226.47±2.89
Acarbose	154.87±2.33	105.63±1.71

The values are Mean ± S.D. of triplicates (P≤0.05)

Increased postprandial glucose level may result into micro-vascular damage through oxidation of low density

lipoprotein (LDL) and pro-atherogenic mechanism. Carbohydrate rich diet causes sharp rise in the blood glucose level due to rapid absorption of dietary carbohydrate in the intestine aided by action of α-amylase and α-glucosidase enzymes. Inhibitors of these carbohydrate hydrolyzing enzymes which delay carbohydrate digestion and prolong overall carbohydrate digestion time have been used as hypoglycemic agents to control hyperglycemia in T2DM.[55,56] On account of the adverse side effects of synthetic hypoglycemic drugs like acarbose, miglitol and voglibose used in conjunction with other antidiabetic drugs, there has been growing interest during the past few years in exploring plant for safe and effective α-amylase and α-glucosidase inhibitors. Despite, known traditional therapeutic uses of some *Ficus* species, antidiabetic property of FP fruits in terms of their α-amylase and α-glucosidase inhibitory activity has not been reported so far. ent fractions of hydro-methanolic (methanol: water- 4:1) extract of *F. palmata* (FPFE). In the present study, BuOH, ETOAc, MeOH, and aqueous fractions of FPFE have demonstrated α-amylase and α-glucosidase inhibitory activities in a dose-dependent manner. Methanol fraction showed the highest inhibitory potential at all the tested concentrations. In phytochemical screening, MeOH fraction showed the highest number of phytochemicals followed by EtOAc and aqueous fractions. A number of bioactive phytochemicals particularly phenolics, flavonoids and terpenoids from different plants have been recognized for their antidiabetic action.[57-59] The considerable α-amylase and α-glucosidase inhibitory activity of FPFE fractions might be due the presence of such compounds. Further, highest inhibitory activity shown by MeOH fraction of FPFE comparable to that of acarbose may be attributed to greater concentration of such compounds in the methanol fractions.

CONCLUSION

The results of the present study ample demonstrated the potent α-amylase and α-glucosidase inhibitory activity *F. palmata* fruits which could be exploited in the management of postprandial hyperglycemia in T2DM. Different fractions of the fruit extracts showed considerable enzyme inhibitory action in a dose-dependent manner and methanol fraction demonstrated the highest inhibitory efficacy as compared to other fractions which may be due the presence of bioactive phytochemicals like phenolics, flavonoids, and terpenoids as detected in the phytochemical screening in higher concentration. This therapeutic potentiality of the specific fraction of FPFE could be beneficially exploited in the in treatment of Type2DM. The study further validates the ethnomedicinal uses of *F. palmata* fruits in the management of diabetes. The study, however directs further research on isolation and characterization of specific chemical constituents responsible for the enzyme inhibitory activity of *F. palmata* fruits as well as *in-vivo* study for further confirmation of the obtained results.

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AUTHORS CONTRIBUTION STATEMENT

Dr. Y.C. Tripathi conceptualized the work, designed all experiments and guided analytical works, data analysis and interpretation and drafting the manuscript. Ms Nishat Anjum carried out the experimental works, recorded data, evaluated the results and drafted the manuscript.

CONFLICT OF INTEREST

Conflict of interest declared none.

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