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Original Article

FTIR, HPLC, GC-MS Analysis and Investigation of Hypoglycemic Effects of Leaves Extracts of *Fagonia indica*

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ABSTRACT

Introduction: The present study was aimed for phytochemical analysis, investigations of different functional groups, the hypoglycemic potential of *Fagonia indica* leaves extracts, Reverse phase high performance liquid chromatography (RP-HPLC) and gas chromatography-mass spectroscopy (GC-MS) analysis of the most active hypoglycemic methanol extract. **Methods:** After extraction using cold maceration method, phytochemical screening, Fourier Transform Infrared (FTIR) spectroscopy, investigation of *in vitro* hypoglycemic potential of leaves extracts of the plant by α-glucosidase (sucrase) inhibitory assay were assessed using the standard methods. RP-HPLC and GC-MS analysis of the most active methanol extract was done to identify various biologically active compounds. **Results:** Phytochemical analysis showed various phytochemicals in the plant. FTIR spectral analysis showed diagnostic peaks of various functional groups. Methanol extract of the plant leaves showed the highest sucrase inhibition (37.0±1.4%) at 100 µg/mL concentration. It was followed by aqueous extract (29.1±1%), chloroform extract (27.7±0.5%) and n-hexane extract (25.5±0.9%) at the same concentration. RP-HPLC and GC-MS analysis of methanol extract showed the presence of various compounds with specific retention times. **Conclusion:** The plant leaves possessed *in vitro* hypoglycemic potential with competitive inhibition of enzyme sucrase. Methanol extract is the most effective agent in managing diabetes due to the presence of bioactive phytocomponents.

Key words: *Fagonia indica*, FTIR spectrum, Functional groups, Phytochemicals, RP-HPLC.

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INTRODUCTION

Medicinal plants have been used for thousands of years in folk medicines and are used for their health benefits in developed countries. Approximately 70–95% of the population in developing countries use traditional medicines.[1] Medicinal plants are a significant part of natural wealth and serve as valuable raw material as well as vital therapeutic agents of numerous modern and traditional medicines.[2] The plants have become a treasury house in isolating novel bioactive compounds either as pilot molecules or as drugs for developing new drugs with different mode of actions.[3] Several phytochemicals such as alkaloids, tannins, amino acids, phenols and carboxylic acids have been isolated from the plants. The phytochemicals give definite properties and individuality to the plants.[4] Nowadays a number of phytochemicals obtained from the plants are used as vital drugs worldwide and have the potential of preventing diseases.[5] The extracts prepared from the plants are used as a potential pipeline for developing novel bioactive compounds. Variability in the concentration of substances extracted depends upon the choice of plant part, the extractant used and the extraction procedure.[6,7] The evaluation of phytochemicals present in the plant is the main step during the research on medicinal plants.[8] Phytochemical analysis helps in determining various biological behaviors of plants. Different techniques are utilized for the determination and estimation of phytochemical constituents in plants. Spectroscopic and chromatographic analysis are the most accepted tools used for this purpose. Spectroscopic technique is a powerful analytical tool used for both qualitative and quantitative analysis of biological materials, pharmaceuticals and crude extracts of plants. FTIR is a physico-chemical analytical technique and an established time saving method for characterizing and identifying functional groups present in a plant or other related material.[9] The basis of FTIR is the characteristic peaks within the specific region of the infrared. It measures bonds vibration in functional groups and a spectrum is generated which is called as a biochemical or metabolic "fingerprint" presenting the sample. IR spectra are a source of structural information of the plant extracts for both major/minor constituents.[10]

Diabetes mellitus (DM) is a metabolic disease which is due to relative or complete lack of secretion of insulin, its sensitivity at a lower level, or both. It is characterized by an abnormal levels of glucose in plasma, which leads to complications at major levels, like retinopathy, neuropathy and cardiovascular (CV) disease. It is now becoming a major health problem with an increase in morbidity. Approximately 438 million individuals all over the world suffer from DM and it tends to become much complicated by the year 2030.[11]

There is a need of insulin to uptake glucose from the blood circulation into the cells. DM interferes with this process and causes a disturbance in protein, carbohydrate and lipid metabolism. The control of blood glucose level at an effective level is the key to reversing or preventing diabetic complications and to improve the life quality of diabetic patients.[12]

Alpha-glucosidase is present in the brush-border surface membrane of small intestinal cells, which is capable of metabolizing carbohydrates into absorbable glucose.[13] Its inhibition is a significant way to decrease the postprandial hyperglycemia (PPHG) in diabetic patients.[14] One of the potential mechanism through which inhibitors of alpha-glucosidase blunt postprandrial (PP) glycemic spikes and decreases the concentration of fasting blood glucose levels is by preventing the absorption of intestinal sugar. The alpha-glucosidase inhibitors inhibit the activity of glucosidases within the small intestine and decreases the release of glucose from food. It is the effect commonly used for the identification of potential antidiabetic compounds.

So, the inhibitors of α-glucosidase used as antidiabetic agents having significant therapeutic activity are used to treat DM. Presently,

α-glucosidase inhibitors which are mostly prescribed as hypoglycemic agents like as acarbose, voglibose and miglitol have exhibited GIT side effects. Now there have been the efforts for developing new inhibitors of α-glucosidase from the natural products.

The phenomenon enzyme inhibition is a decrease in the activity of enzyme which occurs by binding the inhibitor to a regulatory or catalytic site on the enzyme, or to a complex of enzyme-substrate in uncompetitive enzyme inhibition. The enzyme inhibition can be either reversible involving non covalent bonding and irreversible which involves covalent binding. There are a number of mechanisms by which enzymes inhibitors slow enzyme activity as competitive, noncompetitive and uncompetitively. In the competitive type of inhibition, an inhibitor is usually the substrate analogue. It binds to the catalytic site of an enzyme and does not cause catalysis. In uncompetitive enzyme inhibition, an enzyme inhibitor does not show binding to the enzyme, but it shows binding only to the complex of enzyme with substrate. In noncompetitive enzyme inhibition, an inhibitor of enzyme does not show binding to the catalytic site but it shows binding to the 2nd site of the enzyme acting by delaying the turnover rate of the reaction.[15,16]

RP-HPLC and GC-MS techniques are used for the identification of new phytochemical compounds. In RP-HPLC, the separation of compounds depends upon the nature of mobile phase and the stationary phase. So, the selection of mobile phase with right composition is significant. Mobile phase selection depends upon not only on the composition of stationary phase, but also on the nature of the drug. In order to select a solvent as a mobile phase it is necessary to consider its polarity, viscosity and pH.

GC-MS is the most common used methods for the purpose of identification and quantification of new phytochemical compounds. By applying this method unknown organic compounds can be found by matching GC-MS spectra with the reference spectra. Phytochemical, FTIR, HPLC and GC-MS studies are useful for the quality control of selected medicinal important plant used for pharmacological purposes.

Fagonia indica (*F. indica*) Burm.f. variety *indica* is an important medicinal plant distributed in the most parts of Indo-Pakistan sub-continent and westwards to East and North tropical Africa.[17] This is used traditionally in treating fever, urinary discharges, asthma, thirst, dysentery, vomiting, liver trouble, skin diseases, typhoid, stomach troubles and toothache.[18] It is therapeutically used as a diabetes management.[19] Due to the popularity of this plant in traditional medicine, it has become the subject of intense chemical and pharmacological studies.

It is the need of the hour for characterization and identification of compounds present in this plant responsible for hypoglycemic activity. The FTIR spectrum profile of this plant is used as an identification tool. There is no report regarding phytochemical analysis and FTIR studies of various extracts of the plant leaves. No hypoglycemic activity has yet been evaluated using *Fagonia indica* leaves extracts. Also, no investigation reports are present about HPLC and GC-MS analysis of methanol extract of this plant. So, the purpose of this study was to investigate the plant leaves extracts for the presence of phytochemicals having functional groups associated with bioactive components. Also to investigate α-glucosidase (sucrase) inhibitory effects, HPLC analysis and the detection of phytoconstituents using GC-MS analysis of methanol extract of the plant *Fagonia indica* (var. *indica*) leaves.

MATERIALS AND METHODS

Solvents, Chemicals and reagents

Solvents and chemicals of analytical grade were used during the study. n-Hexane, chloroform, HPLC grade methanol (CH_3OH) and distilled water (DW) of E. Merck (Germany) was used. Ferric chloride, sodium hydroxide, potassium acetate, copper sulfate, glacial acetic acid, ethyl acetate, potassium iodide, mercuric chloride, concentrated hydrochloric acid (HCl), concentrated sulfuric acid (H_2SO_4), sodium dihydrogen phosphate and glacial acetic acid of Merck, Darmstadt, Germany and Sigma Aldrich Chemical Co. USA were used. Ninhydrin (BDH, Germany), α-glucosidase (sucrase) enzyme of Sigma Aldrich Chemical Co., USA and acarbose (Bayer, Germany) were purchased. The reagents used were Folin-Ciocalteau reagent, Mayer reagent and Millon reagent.

Instruments

UV/Vis spectrophotometer (Model-UV-1800, Shimadzu Corporation, Kyoto, Japan with 1 cm match cells was used. FTIR spectrophotometer (Agilent Cary 630 FTIR, Agilent Technologies, Inc. California, USA) in the spectral range of 4000 cm^{-1} to 650 cm^{-1}. HPLC (Shimadzu Prominence HPLC instrument), GC-MS (5975C Agilent system), Rotary vacuum evaporator (Heidolph Laboraota 4002, Germany), incubator (Memmert, W. Germany), freeze dryer (Vaco 2 Zirbus technology), oven (Memert, UNB 500, Germany), multichannel pipette (Dragon) and vortex mixer (Biobase Meihua).

Plant material

The leaves of *Fagonia indica* (*F. indica*) plant were collected from district Khanewal (Punjab, Pakistan) and washed carefully in running tap water for the removal of adhering debris and soil particles followed by distilled water. The leaves were shade dried for four weeks. The plant was identified and authenticated by a plant Taxonomist, Department of Botany, GC University, Lahore, Pakistan. A voucher specimen (GC-Herb-Bot. 2967) was deposited in the herbarium of the same University. The plant in dry form was pulverized into smooth powder and stored at 4°C in a tightly covered glass jar for analytical and biological studies.

Extraction procedure

The plant powder was extracted in a sequent manner with solvents (n-hexane, chloroform, methanol and water) in order of increasing polarity using a cold maceration method with stirring frequently. The extracts prepared were filtered, concentrated at 40°C under low pressure using a rotary vacuum evaporator (Heidolph, Laborata 4002, Germany) and were stored at 4°C for further use. It resulted in a total of 4 extracts, i.e., n-hexane, chloroform, methanol and aqueous extract.

Preparation of standard solutions

Stock solutions of the leaves extracts/standard acarbose in a concentration of 10 mg/10 mL were prepared in methanol separately. Dilutions from 20 to 100 µg/mL of extracts/acarbose were prepared using methanol. For the preparation of 20 µg/mL concentration of extracts/acarbose, 20 µL from stock solution of each extract/acarbose was taken in a tube and the final volume was made up to 1000µL. Similarly, other dilutions (40, 60, 80 and 100 µg/mL) for each of extract and standard acarbose were prepared in methanol.

Preparation of mobile phase

The mobile phase used for HPLC analysis was methanol and water (70 : 30, v/v) and it was prepared by adding 700 mL methanol in 300 mL water. This was passed through the membrane filter (Millipore bedfoed MA, USA) with a pore size (0.45 µm) and was subjected to sonication for fifteen minutes to degas the mobile phase.

Phytochemical investigations

Qualitative phytochemical analysis

It was carried out to identify different phytochemicals present in various extracts following the standard methods.[20-22]

Quantitative phytochemical analysis

The extracts were subjected to quantitative analysis to estimate phytochemicals (alkaloids, saponins and sterols) by following the standard procedures.

Estimation of alkaloids

The extract (2 g) was treated with 10% of acetic acid (100 mL) in a beaker and waited for 4 h. The solution was concentrated to 1/4th of its volume on water bath (Memert, Germany). Later, conc. ammonium hydroxide (NH_4OH) was added drop by drop to form the precipitates. The precipitates separated on a pre-weight (W_1) Whatmann filter paper were washed with a solution of dilute NH_4OH. The filter paper along with residue was dried and weighed (W_2). Alkaloids were estimated in mg/g as well as in %age.[21]

Estimation of saponins

The extract (2 g) was treated with 20 mL DW and transferred to a separating funnel to which diethyl ether (20 mL) was added. The contents were shacked vigorously. After, ether layer was separated and the remaining aqueous layer was added to 60 mL of n-butanol where saponins were precipitated. The saponins obtained were separated at pre-weighted Whatmannn filter paper (W_1) that was washed with 10 mL of an aqueous solution of sodium chloride (5%). The precipitates were then dried at 40°C in an oven (Memert, UNB 500, Germany) for final weight (W_2) and the content of saponins was estimated.[23]

Estimation of sterols

The extract (2 g) was mixed with 75 mL DW and 25 mL of KOH (10%) was added to this mixture. The solution was transferred to a separating funnel and was extracted three times with 75 mL of pet. ether. The fraction separated in ether was then added into a pre-weighted (W_1) flask. After concentrating the solution on a water bath (Memert Germany), it was dried and finally cooled using desiccator. The flask was again weighted and sterols content (mg/g, %age) was calculated.[24]

FTIR spectroscopic analysis

Using FTIR spectrophotometer (Agilent Cary 630 FTIR, Agilent Technologies, Inc. California) all the FTIR spectra were taken within the spectral range (4000 to 650 cm^{-1}) using Thermo Nicolet Omnic software. Characteristic peaks of various nature and functional groups were taken using a diamond attenuated total reflectance (ATR) and IR values were taken by comparing with the readings IR frequencies.[25-27]

Antidiabetic activity

Alpha-glucosidase (sucrase) inhibitory assay of various leaves extracts

In vitro hypoglycemic potential of various extracts of the plant leaves was evaluated by α-glucosidase (sucrase) inhibition assay by following Dahlqvist[28] standard method with minor modification. An enzyme solution consisted of 1g rat intestinal powder in 10 mL sodium phosphate buffer (pH 7.0, 0.1 M) and was sonicated (sonicator, Jinyuanbao) for half minute with a break of 15 sec to prevent heat. Later, it was centrifuged (10,000 g × 10 min × 4°C) using centrifuge (Sigma 2-16 k,Germany). It resulted into a supernatant which was collected and labeled as rat intestinal α-glucosidase (sucrase enzyme). The reaction solution consisted of a solution (20 µL) of an enzyme, phosphate buffer (100 µL, 0.1 M) at pH 7.0, a solution of sucrose (37 mM) as a substrate and various concentrations (20-100 µg/mL) of the leaves extracts of the plant. Acarbose (a reference standard) at various concentrations (20-100 µg/mL) was used as standard α-glucosidase (sucrase) inhibitor (positive control). The solution of phosphate buffer served as negative control. The reaction solution was then incubated at 37°C for 30 min in a water bath (Memert Germany). The reaction was then stopped by placing in boiling water for 5 min. Following Bergmeyer and Bernt method[29] of glucose oxidase, the amount of glucose released was estimated. Sucrase inhibition and IC_{50} values were determined.

$$\%\text{inhibition} = [(Ac-As)/Ac] \times 100$$

Where Ac = the absorbance of control without sample and As = the absorbance of the sample.

Machanism of enzyme α-glucosidase (sucrase) inhibition

Enzymatic kinetic investigation of α-glucosidase (sucrase) inhibition of various extracts was done using the standard experimental conditions.[30] Experiments were performed by increasing the concentrations of the extracts from 20 to 100 µg/mL and the mechanism of enzyme inhibition was checked graphically from Michaelus-Menton equation.

The Michaelis-Menton presented Michaelis and Menton equation[31] which is important fundamentally to enzyme kinetics.

$$v = V_{max}[S]/(K_m + [S]\text{Where}$$

[S] = the concentration of substrate

K_m = Michaelus constant. It is an inverse measure of the strength or affinity of binding between the enzyme and its substrate.

V_{max} = the max velocity that an enzyme could achieve

The data obtained from α-glucosidase (sucrase) inhibition was plotted in Michaelus-Menton graph by using GraphPad Prism (version 5) software. This showed K_m, V_{max} values and the type of enzyme inhibition.

HPLC analysis

Chromatographic conditions

HPLC analysis was done using HPLC (Shimadzu Prominence HPLC instrument), liquid chromatograph (LC 20A), a quaternary solvent pump unit (LC 20AT-VP), column C_{18} (250 × 4.6 mm, 5 µm) (Shimadzu Prominence), a universal loop injector (Rheodyne 7725) with an injection capacity of 20 µL, and UV-Vis spectrophotometer detector. Column oven (CTO-20A), Degasser (DGU-20A-5R) and the software used during HPLC analysis was Shimadzu Lab Solution Version 6.43 SPI.

The column was equilibrated by running the pure solvent for ½ h through the column before inserting the injection of the solution of methanol extract. The standard solution of methanol extract (10 µL) was passed through a reverse phase (RP) C_{18} column by the use of isocratic solvent and maximum wavelength (λ_{max}) of 190 nm. UV (Photo diode array) detector was used during the analysis and the temperature of the column (CTO-20A) for operating was adjusted at 30°C. Total injection volume was 10 µL and the flow rate of the mobile phase was adjusted at 1 mL per minute. Total run time of the mobile phase was 10 min. The software used during HPLC analysis was Shimadzu Lab Solution Version 6.43 SPI.

GC-MS analysis of methanol extract of the plant leaves

It is used to identify various active natural chemical components which are biologically active. It was carried out by the use of GC-MS (5975C Agilent system). GC was coupled with a mass spectrometer, MS model (7890A). This was worked with the conditions mentioned: capillary column (30 m Long, 50 µm internal diameter and 0.25 mm film thickness). An injection volume (I µL) of helium as a carrier gas with a flow rate (0.7 mL/min) was used. Column temperature (60°C-310°C) was maintained at a rate of 10°C/minute. The temperature of both injector and detector was set at 250°C. A sample volume of 4 µL was injected in splitless mode. May spectra were taken with a scan range (50-

650 *m/z*) with an ionization energy of 70ev. GC-MS software used was a Mass hunter with NIST library.

Identification of chemical compounds

The chemical compounds were identified by the comparison of GC-MS spectral data with NIST (National Institute of Standard Technology) having more than 62000 patterns. The chemical compounds with their molecular weight and chemical structures of methanolic extract of *F. indica* leaves were identified.

Statistical analysis

Tests were performed in triplicate. Data was presented as mean±SD (n=3). The results were tabulated and put for statistical analysis. Data was analyzed using One Way ANOVA using the software (Graph Pad Prism 5). Dunnett's Multiple Comparison Test was applied for finding the significance difference between various extracts.

RESULTS

Phytochemical analysis

The results of phytochemical analysis of various extracts are given in Table 1. The extracts showed the presence of various phytocomponents like carbohydrates, flavonoids, sterols, proteins, tannins, saponins and alkaloids.

Quantitative analysis

The plant showed a different degree of phytochemicals present in various extracts (Table 2). Among three components saponins content was higher in the extracts followed by alkaloids and sterols. The amount of sterols was very low in n-hexane extract. Other extracts had no sterols content.

Identification of functional groups

The results of FTIR analysis of various extracts are given in Figure 1 (A to D) and Table 3. The observed absorption band ranged from weak to medium, sharp and strong absorption bands.

Antidiabetic activity

Alpha glucosidase (sucrase) inhibitory activity of Fagonia indica leaves extracts

The inhibitory effect (%inhibition) with IC_{50} values of *Fagonia indica* leaves extracts and standard (acarbose) to α-glucosidase (sucrase) are given in Table 4 and Figure 2. The methanol extract of the plant leaves exhibited the highest inhibitory activity measured 37.0±1.4% inhibition at 100 µg/mL. The results of % inhibition for the other extracts were lower; aqueous extract 29.1±1.0%, followed by chloroform extract 27.7±0.5% and n-hexane extract 25.5±0.9%. A linear correlation was found between the antidiabetic activity and the concentrations of extracts.

Machanism of α-glucosidase (sucrase) inhibition of various extracts and the standard acarbose

The extracts showed competitive inhibitory activity against α-glucosidase (sucrase). The value of K_m was found to increase in α-glucosidase inhibition. Michaelus-Menton graph showing enzyme inhibition has been given in Figure 3. The values of V_{max} and K_m are given in Table 5.

HPLC analysis of methanol extract for the determination of various compounds

HPLC analysis of methanol extract of *F. indica* leaves showed various peaks of compounds having different retention times (RT). The values of specific area and height of HPLC peaks are given in Table 6.

GC-MS analysis

Six therapeutic important compounds were identified from the methanol extract of *F. indica* leaves through GC-MS analysis. Chemical compounds with molecular formula and molecular weight at various retention times are given in Table 7. GC-MS spectra with structural representation of compounds (A-F) identified in *F. indica* leaves by GC-MS is given in Figure 4.

DISCUSSION

The presence of phytochemicals in the leaves of *F. indica* justifies the local use of this plant in treating various disorders. Secondary metabolites such as alkaloids, tannins, saponins and flavonoids present in the plant are considered as active and are mainly responsible for free radical scavenging and antidiabetic activities.[32] As per literature various phytochemicals are present in this plant. Variety *indica* of the aerial parts of this plant contains carbohydrates, flavonoids, saponins, tannins, alkaloids, triterpenoids, cardiac glycosides, cynogenic glycosides, anthraquinones, coumarins and irodoids.[33] The plant contains saponins, triterpenoids, flavonoids, alkaloids, glycosides, cardiac glycosides, cynogenic glycosides, sterols,

Table 1: Phytochemical profile of leaves extracts of *F. indica*.

Phytoconstituents	n-Hexane extract	Chloroform extract	Methanol extract	Aqeous extract
Alkaloids	-	-	+	+
Flavonoids	+	+	+	+
Glycosides	+	+	+	+
Tannins	+	+	+	+
Terpenoids	+	+	+	+
Phenols	+	+	+	+
Saponins	+	-	+	+
Coumarins	+	+	+	+
Carbohydrates	-	+	+	-
Fats and oils	+	+	-	-
Anthraquinones	-	-	-	-
Proteins	-	+	+	+
Sterol	+	-	-	-

Key: (-) the absence and (+) the presence of phytoconstituents.

Table 2: Phytochemical contents in mg/g and %age in various extracts of *F. indica*.

Phytochemicals	n-Hexane extract (mg/g ± SD, % content)	Chloroform extract (mg/g ± SD, % content)	Methanol extract (mg/g ± SD, % content)	Aqeous extract (mg/g ± SD, % content)
Alkaloids	---	0.4±0.1, 0.04	11.6±0.1, 1.2	3.2±0.6, 0.3
Saponins	25.7±2.2, 2.6	4.1±0.5, 0.4	16.5±1.1, 1.6	5.2±0.2, 0.5
Sterols	10.5±0.8, 1.0	---	--	--

The data are presented as mean±SD. Non-significant difference at P<0.05 was found between the solvents by applying Dunnett's Multiple Comparison Test.

coumarins and irodoids.[34] Preliminary phytochemical screening may be useful for the detection of the bioactive principles which leads to the drug discovery. According to the phytochemical constituents found in this plant, it can be proposed that this plant may show various biological activities. So, this plant is used pharmacologically for developing health benefitting novel compounds.

The extracts prepared from this plant exhibited different degrees of phytochemicals with respect to various solvents used. The leaves are rich in alkaloids and saponins with considerable amount of sterols. Among the four solvents, methanol was found to be the most efficient with respect to quantitative and qualitative extraction of both primary and secondary metabolites. It was observed that n-hexane is comparatively an inefficient solvent for extracting the phytochemicals under the investigation. Methanol extract having the highest content of metabolites may reflect the polarity of methanol solvent.

FTIR spectroscopy is a sensitive and reliable method for determining the composition of molecules. It is done to screen, identify and characterize quality attributes assuring the quality of different extracts prepared from

Table 3: FTIR characteristic absorption peak values with vibrational mode and respective functional groups with group frequency of various extracts of *F. indica*.

Characteristic Absorption (cm^{-1})	Vibrational Mode	Functional Group	Group Frequency (cm^{-1})
3283.8, 3291.2, 3298.7	-OH stretch broad	Alcohol, Polymer	3400-3200(s)
2929.7	-CH stretch usually two bands	Ac-H	2950-2700(w)
2918.5	Antisym stretch	Alkanes, $-CH_2-$	2935-2915(s)
2851.4	Sym stretch	Alkanes, $-CH_2-$	2865-2845(s)
2124.6	C≡C stretch	Alkynes C≡C, Monosub	2140-2100(m)
2109.7	C≡C str, Monosub	Alkynes	2140-2100(m)
2098.5	O-H stretch, very broad	Alcohol and Phenol	3200-2500(w)
1736.9, 1733.0	C=O stretch	Aldehyde saturated	1740-1730(s)
1621.4, 1625.1	NH def	Amide II Primary	1650-1620(s)
1617.7	NH def	Amines Secondary	1650-1550(m)
1543.1	NH deformation	Amide II, Secondary, solid	1570-1515(s)
1513.3	-CH3 antisym def	Alkanes	1470-1430(m)
1461.1	Scissoring	Alkanes, $-CH_2-$	1485-1445(m)
1408.9	O-H in-plane def	Phenol	1410-1310(s)
1379.1	CH3, Sym def	Alkanes	1380-1370(m)
1375.4	-CH3 sym def	Alkanes	1380-1370(m)
1315.8	-OH in plane def	Phenol	1410-1310(v)
1263.6	OH in-plane def	Alcohol, secondary	1350-1260(v)
1241.2	Ar-O stretch	Ether, alkyl aryl	1270-1230(s)
1233.7	-C-O stretch	Ethers	1250-1150(s)
1192.7	C-O stretch	Ether, diaryl	1250-1150(s)
1155.5	C-O stretch	Ethers, diaryl	1250-1150(s)
1121.9	C-O stretch	Ether, dialkyl	1150-1060(s)
1096.8	C-O stretch	Ethers, dialkyl	1150-1060(s)
1088.4	C-N stretch	Aliphatic amines	1200-1000(m)
1036.8	R-O stretch	Ether	1075-1020(s)
1021.3	C-N stretch	Aliphatic amines	1200-1000(m)
1017.6	C-N stretch	Aliphatic amines	1200-1000(m)
1013.8	C-F stretch	Halogen compound	1100-1000(s)
872.2	CH out of plane def	Aromatic compound	900-860(w)
861.0	C-H out of plane, def	Aromatic compounds m-disub	900-860(w)
719.4	C-Cl stretch	Halogen, chloroform	750-700(s)
667.2	C-Br stretch	Halogen, Bromo	690-515(s)

Key: Def= Deformation, Monosub= Monosubstituated, Disub= Disubstituated, Stretch= Stretching, Fr= Frequency, Sym= Symmetrical

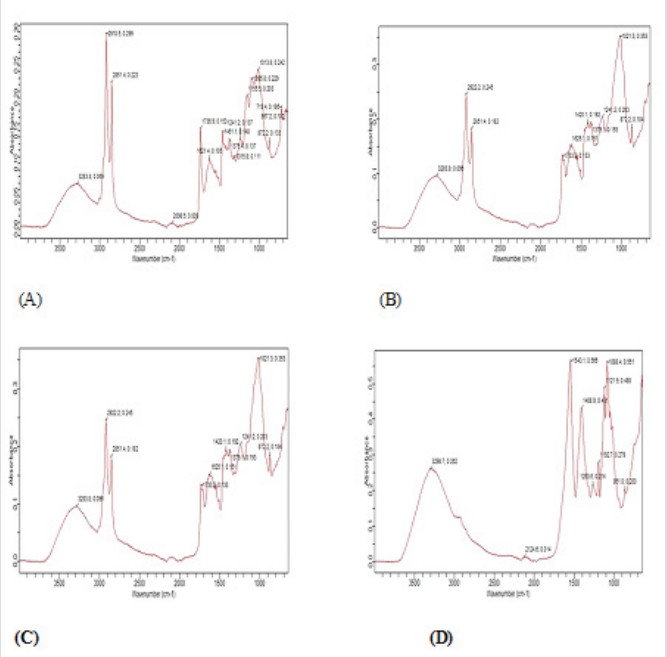

Figure 1: FT-IR Spectrum of various extracts of *F. indica* leaves. (A) n-hexane extract, (B) chloroform extract, (C) methanol extract, and (D) aqueous extract.

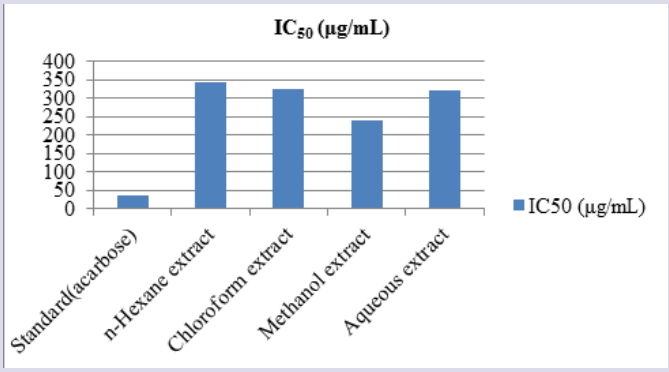

Figure 2: IC_{50} values of sucrase inhibition of standard and various extracts of *F. indica* leaves. Significant difference at ***P<0.001 was found when the values were compared to standard.

the plant. The FTIR peaks showed the number of functional groups having a great significance towards the medicinal properties of *F. indica*. This information first is used for the identification (authentication), evaluation and standardization of the plant. Using macroscopic fingerprint characteristics of FTIR spectrum, it can be judged for the origin of various extracts and to identify medicinal materials. The bands between wavenumbers (4000-650 cm^{-1}) revealed the presence of various phytochemical compounds extracted with various solvents. Bonds in various compounds can also be determined by interpreting infrared absorption spectra.[27] Functional groups of various compounds appear on different wavelengths in FTIR studies. The biochemical composition of proteins, lipids, carbohydrates and polyphenols give the absorption bands in the range of 1880-900 cm^{-1}.[35]

FTIR results showed the presence of functional groups of various phytochemicals i.e., phenol, alkanes, alkenes, alkynes, aldehydes, ketones, alcohols, esters, carboxylic acids, amines and ethers in the plant which may be responsible for various pharmacological activities. The confirmation for the identification of various classes of compounds in the extracts is done through various chromatographic (column chromatography and thin layer chromatography) and spectral (UV, FTIR, GC-MS and NMR) techniques. These phytochemicals have various functional groups like O-H, C-O, C-H, C=C, CH_3 and N-H. No bond was found in the region of 2220 to 2260 cm^{-1} indicating that no cyanide group was present in the samples. This implies that the plant has no toxicity due to cyanide group. As there is an absence of cyanide group in the extracts, but still toxicological studies of the extracts are to be required for *in vivo* studies.

The bioactive compounds present in leaves extract show certain biological functions. To determine whether the function might be antidiabetic, α-glucosidase inhibition of *Fagonia indica* leaves extracts was tested. *In vitro* analysis showed that the methanol extract exhibited the highest α-glucosidase (sucrase) inhibitory activity as compared to other extracts. So, the hypoglycemic effect analysis of *Fagonia indica* leaves extract was carried out using only the methanol-maceration extract. It is suggested that α-glucosidase inhibition may be due to saponins and alkaloids present in *Fagonia indica* extracts (Table 2). It has been reported that flavonoids and alkaloids have shown α-glucosidase inhibition properties.[36,37]

Fagonia indica leaves extracts may inhibit α-glucosidase (sucrase) enzyme within the small intestine that showed *in vitro* digestion of sucrose and such a way decrease glucose absorption rate. So, the normal blood glucose level can be controlled even by treating with sucrose dose that would normally cause a diabetic condition.

HPLC analysis of methanol extract was used for estimating compounds in the extract. In literature, *F. indica* extract was found to contain several compounds in varying amount. HPLC analysis of methanol extract

Table 4: % inhibition of α-glucosidase (sucrase) by *F. indica* leaves extracts.

Extract conc. (µg/mL)	%inhibition by Standard± SD	%inhibition by n-hexane extract±SD	%inhibition by chloroform extract±SD	%inhibition by methanol extract±SD	%inhibition by aqueous extract±SD
20	47.0±1.7	17.9±1.02	20.2±2.0	28.6±1.8	21.9±1.7
40	49.5±1.0	18.1±1.5	21.2±1.0	31.6±2.1	22.8±2.4
60	54.2±1.3	23.0±1.0	23.1±1.4	32.4±1.5	24.1±1.4
80	62.7±2.0	23.2±1.4	26.0±1.0	34.1±1.0	27.3±0.6
100	64.8±0.4	25.5±0.9	27.7±0.5	37.0±1.4	29.1±1.0

Data are expressed as mean±SD (three reading). Significant results at ***$P < 0.001$ when the values are compared to the diabetic and positive control (acarbose).

Table 5: Enzymatic kinetics of standard (acarbose) and various leaves extracts with Michaelus-Menton V_{max} and K_m values in α-glucosidase (sucrase) enzyme inhibition.

Standard/ extracts	V_{max} (Mean±SEM) with 95% confidence intervals	K_m (Mean±SEM) with 95% confidence intervals	Enzyme inhibition mode
Standard (acarbose)	69.4±5.6 51.7 to 87.0	11.7±4.8 0.0 to 27.01	Competative inhibition
n-Hexane extract	27.5±2.5 19.5 to 35.5	13.3±5.7 0.0 to 31.5	Competative inhibition
Chloroform extract	29.1±2.2 21.9 to 36.0	10.7±4.4 0.0 to 24.8	Competative inhibition
Methanol extract	35.7±1.18 31.9 to 39.4	3.8±1.5 0.0 to 8.5	Competative inhibition
Aqueous extract	37.0±2.4 29.4 to 44.6	15.8±4.3 2.2 to 29.4	Competative inhibition

Table 6: HPLC analysis of methanol extract.Various peaks of compounds with specific area and height are given at different retention times.

Peak No.	Retention Time	Area	Height
1	1.19	2373363	182811
2	1.50	4061210	321130
3	1.83	2397973	184862
5	2.1	826256	72342
6	2.3	537968	43002
7	2.55	380995	40462
8	3.18	19223214	503849
9	6.16	161888	16538
10	6.52	123747	14580
11	7.03	93941	10424

Mobile phase run through column (methanol : water, 70 : 30, v/v)

Table 7: The identified compounds in the methanolic extract of *F. indica* leaves by GC-MS analysis.

Compounds	Retention time (Minute)	Name of the chemical compounds	Molecular formula	Molecular weight	Compound nature
A	17.87	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$	330	Ester
B	17.87	Hexadecanoic acid, 1-[[[(2-aminoethoxy) hydroxyphosphinyl]oxy]methyl]-1,2-ethanediyl ester	$C_{37}H_{74}NO_8P$	691	Ester
C	20.91	Octadecanoic acid, 4-hydroxybutyl ester	$C_{22}H_{44}O_3$	356	Ester
D	23.15	Piperine	$C_{17}H_{19}NO_3$	285	Alkaloid
E	25.13	α-Tocopherol-β-D-mannoside	$C_{35}H_{60}O_7$	592	Tocopherol
F	26.57	17-(1,5-Dimethylhexyl)-10,13-dimethyl-4-vinylhexadecahydrocyclopenta[a] phenanthren-3-ol	$C_{29}H_{50}O$	414	Steroid

of whole plant of *F. indica* var. *Schewinfurthii* was found to contain several phenolic compounds like vanillic acid, caffeic acid, p-coumaric acid, sinapic acid, chlorogenic acid, 4-hydroxy-3-methoxy benzoic acid and ferulic acid in varying amounts.[38] It indicated that methanol solvent has more extractive potential for the compounds. The reported HPLC chromatogram of methanol extract also helped in determining functional groups present in the extract. Phytochemical analysis showed the presence of secondary metabolites which was also shown by HPLC analysis having different peaks with retention times.

Many researchers identified various therapeutic compounds by GC-MS.[39-41] Such compounds possess antibacterial, anti-inflammatory, antioxidant, antifungal, anticancer and anti-diabetic activities. It was confirmed from the previous studies that n-hexadecanoic acid is an important chemical component with hypocholesterolemic, lubricant, pesticide, antiandrogenic, antioxidant, anti-inflammatory, nematicide, antibacterial and haemolytic 5-alpha reductase inhibitor characteristics.[42] Among 6 compounds, Octadecanoic acid, 4-hydroxybutyl ester, Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethylester and Hexadecanoic acid1-[[[(2-aminoethoxy) hydroxyphosphinyl]oxy] methyl]-1,2-ethanediyl ester have pontential glucose reduction capacity.[43] As per literature various phytoconstituents from the plant have been isolated and characterized. Fagonilin was isolated from the plant aerial parts along with β-sitosterol, lupeol, and β-amyrin.[44] A new triterpenoid saponin 3-O-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl]-kaempferol was isolated from this plant.[45] Sulphated triterpenoid glycosides (Nayabin A-G) such as β-D-glucopyranosyl-3β-hydroxy-23-β-D-glucopyranosyloxy-lup-20 (29)-en-28-oate (Nayabin-A) and β-D-glucopyr-anosyl 3β-O-sulfo-23,30-dihydroxy-olean-12-en-28-oate (Nayabin-B) along with a known triterpenoid glycoside were also isolated from the whole plant.[46] Flavonoid glycoside along with known glucopyranosyl ester was also obtained from this plant.[45] This property insists the traditional use of *F. indica* for diabetes. The above studies showed hypoglycemic activity and other bioactive compounds separated in the methanol extract of *F. indica* leaves. Hypoglycemic activity of the

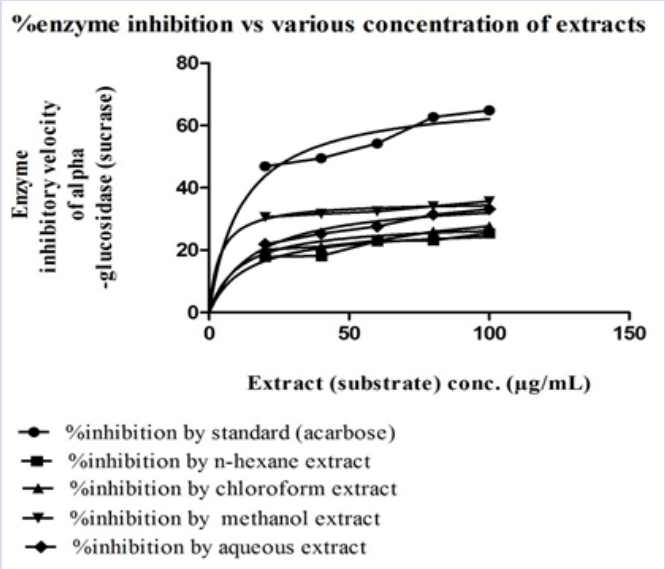

Figure 3: Michaelus-Menton graph of standard acarbose and various extracts of *F. indica* showing α-glucosidase (sucrase) inhibition.

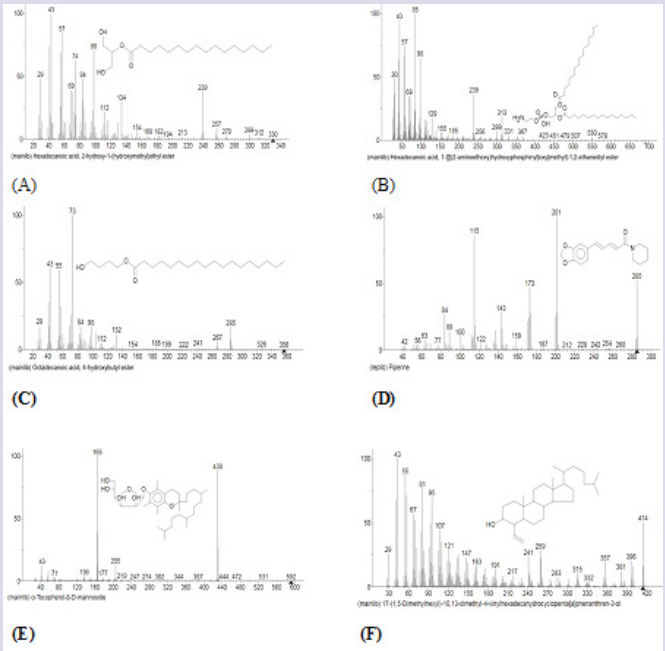

Figure 4: GC-MS spectra with structural representation of compounds (A-F) identified in *F. indica* leaves by GC-MS.

whole plant of *F. indica* (var. *Schewenfurthii*) was investigated by *in vitro* α-amylase inhibitory assay.[38] But these are no reports on hypoglycemic activity of methanol extract of *F. indica* (var. *indica*) leaves using the sucrase inhibitory assay. Therefore, this study was done to investigate the plant leaves extracts for hypoglycemic activity HPLC and GCMS analysis of the most active methanolic extract. The present study has shown the presence of various compounds in the plant responsible for hypoglycemic activity.

This study will be useful for the characterization purposes of various phytoconstituents. So our study may be helpful in the discovery of new drugs. This is a useful tool used analytically for the quality and identification of medicinally important plant.

CONCLUSION

The methanol extract *of Fagonia indica leaves* may be used as an antidiabetic agent which decreases the levels of blood glucose through α-glucosidase (sucrase) inhibition. Enzymatic kinetic studies showed that this extract has less competitive inhibition in comparison to standard acarbose. HPLC and GC-MS analysis showed the presence of antidiabetic compounds. *F. indica* has promising medicinal and physiological properties owing to the presence of these compounds. The presence of natural chemical compounds in the plant may be responsible for the inhibition of α-glucosidase (sucrase) enzyme. Therefore, further investigation is needed to isolate, purify and characterize these compounds to be used as lead compounds for developing drugs responsible for various biological activities. Further studies are required on bioactive compounds to evaluate their efficacy by *in vivo* studies and to demonstrate their efficacy and safety in clinical trials.

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CONFLICT OF INTEREST

The authors declare that they have no competing interest.

ABBREVIATIONS

FTIR: Fourier Transform Infrared; **DM:** Diabetes mellitus; **PPHG:** postprandial hyperglycemia **RP-HPLC:** Reverse phase high performance liquid chromatography; **ATR:** attenuated total reflectance; **GC-MS:** Gas chromatography mass spectroscopy; **CAN:** acetonitrile; **UV:** UltraViolet; **SD:** Standard deviation, **NIST:** National Institute of Standard Technology.

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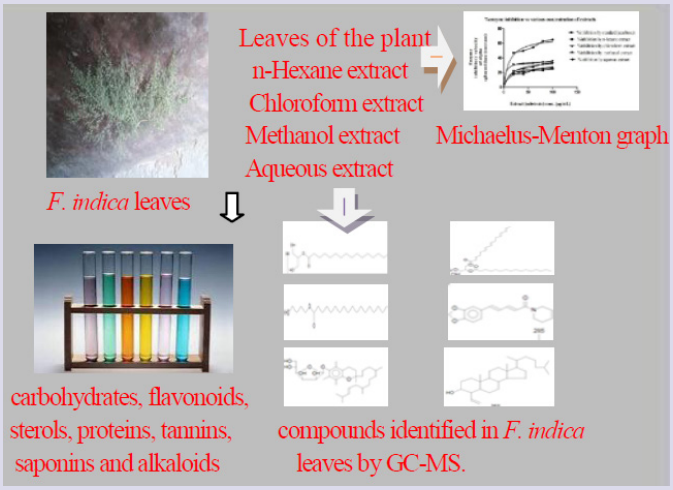
PICTORIAL ABSTRACT



SUMMARY

The present study was aimed for phytochemical analysis, investigations of different functional groups, the hypoglycemic potential of Fagonia indica leaves extracts, Reverse phase high performance liquid chromatography (RP-HPLC) and gas chromatography-mass spectroscopy (GC-MS) analysis of the most active hypoglycemic methanol extract. After extraction using cold maceration method, phytochemical screening, Fourier Transform Infrared (FTIR) spectroscopy, investigation of *in-vitro* hypoglycemic potential of leaves extracts of the plant by α-glucosidase (sucrase) inhibitory assays were assessed using the standard methods. RP-HPLC and GC-MS analysis of the most active methanol extract was done to identify various biologically active compounds. Phytochemical analysis showed various phytochemicals in the plant. FTIR spectral analysis showed diagnostic peaks of various functional groups. Methanol extract of the plant leaves showed the highest sucrase inhibition (37.0±1.4%) at 100 µg/mL concentration. It was followed by aqueous extract (29.1±1%), chloroform extract (27.7±0.5%) and n-hexane extract (25.5±0.9%) at the same concentration. RP-HPLC and GC-MS analysis of methanol extract showed the presence of various compounds with specific retention times. The plant leaves possessed in vitro hypoglycemic potential with competitive inhibition of enzyme sucrase. Methanol extract is the most effective agent in managing diabetes due to the presence of bioactive phytocomponents.

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