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Pharmacological evaluation of *Rhazya stricta* root extract

[Evaluación farmacológica del extracto de raíz de *Rhazya stricta*]

Rashid Mahmood[1,2], Farnaz Malik[2], Shazia Shamas[3], Tanveer Ahmed[1], Mehran Kausar[1], Samina Rubnawaz[1], Muhammad Ashfaq[2], Shahzad Hussain[2], Brian Desmond Green[4] & Bushra Mirza[1]

[1]*Departtment of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan*
[2]*Drugs Control and Traditional Medicines Division, National Institute of Health, Islamabad, Pakistan*
[3]*Department of Zoology, Faculty of Sciences, University of Gujrat, Gujrat, Pakistan*
[4]*Institute for Global Food Security, Queen's University Belfast, Northern Ireland, United Kingdom*
Contactos | Contacts: **Rashid MAHMOOD** - *E-mail address:* rashid_8001@yahoo.com

Abstract: The present study aimed to screen the *Rhazya stricta* Decne root for its antihyperglycemic and antioxidants potential through in-vitro assays along with phytochemical and elemental analyses. The crude extract was prepared through maceration and fractionated using solvent-solvent extraction technique. The spectroscopic studies indicated the presence of various phytochemical classes in the extract and its fractions. The antioxidant assays showed notable results along with a good concentration of phenolic and flavonoid contents. Enzyme inhibition assays demonstrated glucose-lowering effects by inhibiting the enzyme activity which could reduce post-prandial blood glucose level. The Dipeptidyl peptidase-IV (DPP-IV) inhibition assay results showed the novel DPP-IV inhibition activity of the plant extract and all fractions showed noteworthy enzyme inhibition and antihyperglycemic activity. Conclusively, the *Rhazya stricta* root extract displayed its antioxidant and antihyperglycemic potential due to the presence of various classes of phytochemicals and micro-nutrients.

Keywords: *Rhazya stricta;* Antioxidant; Antidiabetic; Phytochemical; DPP-IV inhibitor; Spectroscopic studies.

RESUMEN: El presente estudio tuvo como objetivo examinar la raíz de *Rhazya stricta* Decne por su potencial antihiperglicémico y antioxidante a través de ensayos in vitro junto con análisis fitoquímicos y elementales. El extracto crudo se preparó por maceración y se fraccionó usando una técnica de extracción solvente-solvente. Los estudios espectroscópicos indicaron la presencia de varias clases fitoquímicas en el extracto y sus fracciones. Los ensayos antioxidantes mostraron resultados notables junto con una importante concentración de contenido fenólico y flavonoide. Los ensayos de inhibición enzimática demostraron efectos reductores de la glucosa al inhibir la actividad enzimática que podría reducir el nivel de glucosa posprandial en sangre. Los resultados del ensayo de inhibición de Dipeptidyl peptidase-IV (DPP-IV) mostraron la nueva actividad de inhibición de DPP-IV del extracto de la planta y todas las fracciones mostraron una notable inhibición enzimática y actividad antihiperglicémica. En conclusión, el extracto de raíz de *Rhazya stricta* Decne mostró su potencial antioxidante y antihiperglicémico debido a la presencia de varias clases de fitoquímicos y micronutrientes.

Palabras clave: *Rhazya stricta*; Antioxidante; Antidiabético; Fitoquímico; Inhibidor DE DPP-IV; Estudios espectroscópicos.

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INTRODUCTION

For the treatment of diseases, the use of plants and herbal medicine is very frequent in Asia and developing countries due to their efficiency, fewer side effects and cost-effectiveness (Harinantenaina *et al.*, 2006). In particular, medicinal plants with antihyperglycemic activities are being more desired (Khan *et al.*, 2012) and around 800 plant species have been investigated and reported for their glucose-lowering effects (El-abhar & Schaalan, 2014). However, the exploration of plant material in search of effective antidiabetic agents with less or no side effect is still going on (Arika *et al.*, 2015)

Diabetes mellitus is a group of metabolic disorders in which patients suffer from high blood glucose level as a result of their inability to produce insulin or due to insulin resistance in peripheral tissues. Diabetes is one of the most common diseases in the world and it is estimated that the number of adult sufferers will increase up to 69% and 20% in developing and developed countries respectively till 2030 (Shaw *et al.*, 2010). The disease is reaching epidemic proportions and will account for 12% of the total health expenditure (Zhang *et al.*, 2010). The persistent high blood glucose level in uncontrolled diabetes is associated with a number of complications including retinopathy, neuropathy, nephropathy, and accelerated cardiovascular diseases (Forbes & Cooper, 2013).

High blood glucose levels stimulate the formation of reactive oxygen species and free radical production (Fowler, 2011). Reactive Oxygen Species (ROS) are chemically active derivatives of oxygen including free radicals that pose detrimental effects on cells by enzyme inactivation and damage to vital cellular machinery (Steinberg *et al.*, 1989; Nordberg & Arnér, 2001). As oxidative stress from hyperglycemia may also play an important role in several associated complications (Fowler, 2011), therefore, to maintain health and for the prevention and treatment of diseases, the use of antioxidants is getting the attention of scientists (Halliwell & Gutteridge, 1981).

The chemical composition of the plants determines their biological actions and plants rich in phenolics, alkaloids, terpenoids, coumarins, and glycosides are assumed as pharmacologically active (Grover *et al.*, 2002). Biologically active compounds from plants can be isolated and different spectroscopic techniques like UV-visible spectrometry and infrared spectrometry (IR) can be employed for the identification of these compounds (Ibrahim *et al.,* 2008; Popova *et al.*, 2009).

In the present study, root crude extract of *Rhazya stricta* Decne was prepared through the maceration process and its fractionation was done through solvent-solvent extraction and all fractions were evaluated for their biological activities. Spectroscopic studies like UV-Vis spectrophotometry and FT-IR were done for the detection of biologically active compounds through the identification of important functional groups of the compounds present in the extract and fractions. Then all fractions including crude extract were examined for the presence of phytochemicals and for their antioxidant as well as antihyperglycemic potential. Furthermore, the root of *R. stricta* was also assessed for the presence of micro-nutrients/elements which in minute quantities help the body to maintain the normal functioning.

MATERIALS AND METHODS

Plant collection, identification, and crude extract preparation

Rhazya stricta Decne roots were collected and identification of plant was done by Prof. Dr. Rizwana Aleem, Taxonomist, Department of Plant Sciences, Quaid-i-Azam University Islamabad (Voucher Specimen Ref. No. 130290). After washing, roots were shade dried and ground using laboratory grinder to ease the extraction process. The ground root material was macerated using methanol and ammonical chloroform (1:1) with shaking for three days. The procedure was repeated three times and each soaking took three days. The extract was filtered and concentrated on a rotary evaporator (Buchi, Switzerland) at 40ºC. The filtrate (semi-solid material) was named as root crude extract (RCE) of *R. stricta*.

Solvent-solvent extraction

The fractionation of the crude extract into various portions containing compounds of almost similar polarities is important to proceed further. To extract alkaloids from the root crude extract, a modified method was used in which H_2O, NH_4OH, chloroform, and diluted HCL were used to optimize alkaloid contents. Scheme of solvent-solvent extraction of root crude extract is given in Figure No. 1.

Scheme of extraction

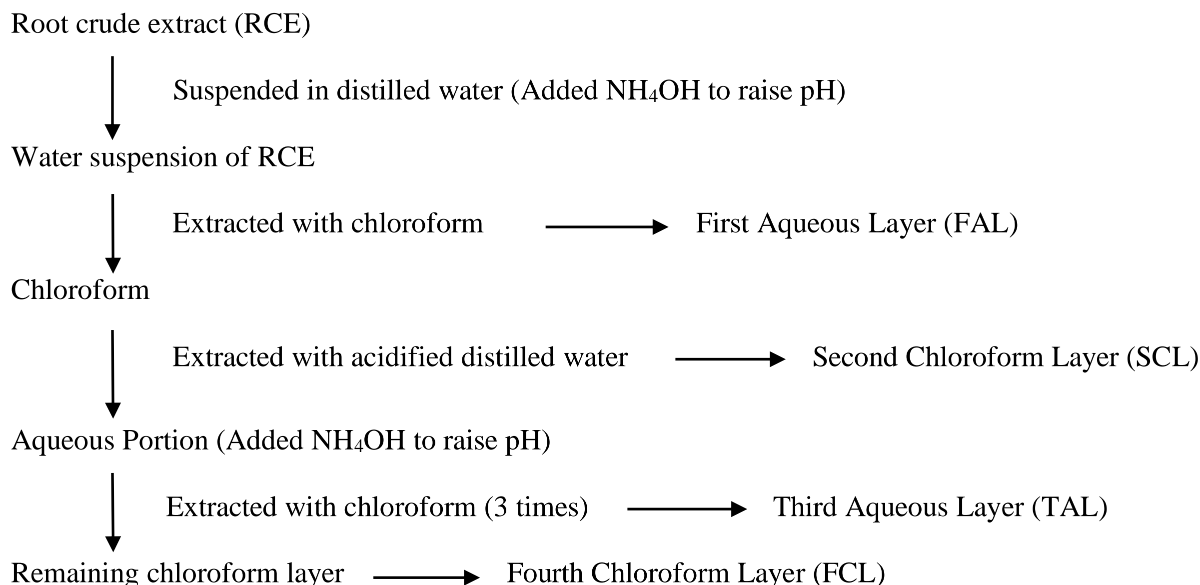

Figure No. 1
Schematic diagram for the extraction and fractionation of root crude extract (RCE) of *R. stricta*

Elemental analysis
Acid digestion and estimation
Acid washed glassware and deionized water was used throughout the analysis to minimize the chances of interferences. For the digestion of the root sample, an earlier reported method of Khan *et al*. (2008) was employed. Powdered root sample was weighed (1gm) and heated in an oven at 110ºC in a china dish for the removal of moisture. The sample was heated in a furnace at 550ºC for 4 hrs and after cooling down; its contents were dissolved by adding 6 M Nitric acid (2.5 ml). The mixture was filtered and transferred to a 20 mL flask and diluted up to the mark. Reference standards of the following elements were procured from Sigma-Aldrich (USA), Fe, Na, K, Ca, Mg, Zn. Cu, Ni, Mn, Cr, Co, and Pb. Estimation of all elements was carried out on Fast Sequential Atomic Absorption Spectrometer (Varian 240 AA FS-Australia). The operating parameters for working elements were set according to the manufacturer's recommendations.

Fourier transform infra-red spectroscopy (FT-IR)
FT-IR analysis was done using the IRTracer-100 Model KRS-5 of Shimadzu Corporation, Japan by ATR (Attenuated Total Reflection) method. Samples were analyzed within the range (400 to 4000 cm^{-1}) with a resolution of 4 cm^{-1} and intensity mode as % Transmittance. The characteristic of chemical bond is the absorption of light of specific wavelength by a particular compound and it can be determined by interpretation of infrared absorption spectrum. Root crude extract and its fractions were analyzed and spectra were prepared and interpreted with the help of reported literature.

UV-Vis spectrophotometric analysis
For the spectrophotometric determination plant extract was prepared in methanol (1 mg/5 ml) and after centrifugation, the supernatant was used for the analysis. Samples were analyzed in the range of 200-800nm wavelength to ascertain absorption peaks in the ultra-violet and visible regions using UV-1900 Series Spectrophotometer of Shimadzu Corporation, Japan. The measuring mode was selected as Absorbance with a slit width of 1.0 nm. Spectrophotometry can be used for the quantification of the specific compound if a specific reference standard is available.

Biological evaluation: qualitative phytochemical screening of root crude extract
Root crude extract of the *R. stricta* and resultant fractions were analyzed qualitatively for the presence of various classes of phytochemicals. Tests were carried out to identify phytochemicals using

procedures earlier described by Baloch *et al*. (2013) and Auwal *et al*. (2014).

Test for tannins
To 2 mL of the aqueous extract solution, 10% Ferric chloride solution was added dropwise. The formation of a blackish-blue color indicated the presence of tannins.

Test for saponins (Frothing Test)
In the aqueous extract solution, 10 ml of distilled water was added, mixed vigorously for 5min and allowed to stand for 30 min. Development of froth/foam is suggestive of the presence of saponins.

Test for alkaloids (Dragendorff's reagent)
Plant extract (1 g) was added with 5 ml of ammonia solution (10%) and the mixture was extracted with chloroform (15 ml). Then the chloroform portion was evaporated to dryness and the residue was dissolved in 15 ml of dilute sulphuric acid. In this acidic solution (2 ml), ammonia solution (10%) was added to neutralize the solution. After that Dragendorff's reagent was added in the test tube. The presence of turbidity or precipitate is indicative of alkaloids.

Test for steroids (Salkowski's test)
Concentrated sulphuric acid was added carefully in the second portion of the solution prepared above for alkaloids and after the formation of a lower acidic layer, the interface was observed for a red-brown color suggestive of steroid ring.

Test for flavonoids (pew's test)
Aqueous extract solution (5 ml) was mixed with 0.1 g of metallic zinc and 8 ml of concentrated sulphuric acid. The formation of red color is indicative of flavonoids.

Tests for carbohydrate (Molisch's test)
To the 2 ml aqueous extract solution, Molisch's solution was added dropwise and a small volume of concentrated sulphuric acid was allowed to go down along the side of the test tube to form a layer without shaking. The purple color development is suggestive of the presence of carbohydrates.

Test for cardiac glycosides
Glacial acetic acid (2 ml) was added into 0.5 ml of extract followed by a few drops of ferric chloride (5%). After that sulphuric acid (conc.) was added gently to form an upper layer. Brown ring formation at the interface will be a sign of cardiac glycosides.

Test for quinones
Extract (1 ml) was added to sulphuric acid (1 ml). Development of red color pointed toward the presence of quinones.

Test for glycosides
Chloroform (3 ml) was added to 2 ml of extract followed by the addition of 10% ammonia solution. Pink color development will be suggestive of glycosides presence.

Test for terpenoids
2 ml of chloroform was added to the 0.5 ml of the extract followed by concentrated sulphuric acid carefully. The red-brown color at the junction will be a sign of the presence of terpenoids.

Test for phenols
1 ml of the extract was mixed with 2 ml of distilled water followed by the addition of a few drops of 10% ferric chloride. Green color development will indicate the presence of phenols.

Determination of Antioxidant Potential
DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical scavenging assay
DPPH free radical scavenging activity was measured spectrophotometrically (Ismail *et al*., 2015) at 1000, 500, 250 and 125 µg/ml final concentrations of the extract. Briefly, the assay was performed using transparent 96 wells plates. Freshly prepared DPPH solution (195 µl) was poured into each well of the 96 wells plate. Extracts were prepared in DMSO at a concentration of 200 mg/ml and 5 µl of each extract was poured into the respective wells of the plate and mixed well to get final concentrations of 1000, 500, 250 and 125 µg/ml. Ascorbic acid and DMSO were used as positive and negative controls and the reaction mixture was incubated at 37ºC for 30 minutes time interval. The change in color of DPPH solution was measured by taking absorbance at 515 nm using microtiter plate reader (Elx800-BioTek). Percent activity of the sample was calculated by the following formula. The experiment was run in triplicate and IC_{50} was also calculated.

$$\textbf{Scavenging activity (\%)} = \left[1 - \frac{As}{Ac}\right] \times 100$$

Where:
"Ac" is the Absorbance of control
"As" representing sample absorbance.

Total antioxidant capacity

Total antioxidant capacity was measured by the phosphomolybdenum method as performed earlier by Prieto *et al*., (1999). The assay is based on the reduction of Molybdenum by the extract to a green phosphate complex at acidic pH. Extract sample (0.1 ml) was mixed with 1 ml of reagent solutions (28 mM sodium phosphate, 0.6 sulfuric acid, and 4 mM ammonium molybdate) and the mixture was incubated at 95ºC for 90 minutes. The reaction mixture was cold down and absorbance was taken at 695 nm against DMSO (blank). The antioxidant capacity of each sample was articulated as the number of gram equivalent of ascorbic acid through the calibration curve of the standard.

Reducing power assay

The assay is based on the capacity of samples to reduce iron (III) to iron (II) and was measured according to the method of Jafri *et al.* (2017). A 200 µl of each sample extract (prepared in DMSO) was mixed with 500 µl of 2 M phosphate buffer and 500 µl of 1% potassium ferricyanide and incubation was done for 20 minutes at 50ºC. After incubation, 500 µl trichloroacetic acid (10%) was added and centrifuged at 3000 rpm for 10 min. The same volume of distilled water was mixed with the upper layer of the mixture (500 µl) and 0.1% ferric chloride (100 µl) was added to the mixture and absorbance was taken at 700 nm against a blank (DMSO). The results were calculated through the calibration curve of the standard and were expressed as ascorbic acid equivalents.

Determination of total phenolic contents

The total phenolic content of the extracts was measured as described by Baba & Malik, (2015). In brief, all extracts were prepared (1 mg/ml) and 200 µl of samples were mixed with 0.5 ml of Folin–Ciocalteu reagent. After 5min at room temperature, 20% sodium carbonate (2 ml) was added and absorbance was measured at 650 nm in the dark after 90 min using a spectrophotometer. Gallic acid was used as a standard for the development of calibration curve and results are showed as gallic acid equivalents.

Determination of total flavonoid contents

For the estimation of total flavonoid contents, the aluminum chloride method was followed as described by Jafri *et al*. (2017). In the experiment, from each extract fraction (1 mg/ml in Methanol) 0.5ml was taken and mixed with 1.5 ml of methanol and 0.1 ml of 10% aluminum chloride was added into it. After thorough mixing, 0.1 ml of 1 M potassium acetate was added followed by 2.8 ml of distilled water. After half an hour at room temperature absorbance was measured at 415 nm using a spectrophotometer. DMSO in distilled water (0.5 + 4.5 ml) was used as blank, DMSO as the negative control and quercetin as standard and for the calibration curve.

Enzyme inhibition assays

α-Glucosidase inhibition assay

Alpha-glucosidase inhibition activity of *R. stricta* root crude extract and its fractions was estimated using 96-well microtiter plate by an earlier described method (Mushtaq *et al*., 2017) with modifications according to system suitability. Each well of the microtiter plate contained 5 µl test sample, phosphate buffer 65 µl, 5 µl α-glucosidase enzyme solution and 25 µl PNPG (*p*-nitrophenyl-α-D-glucopyranoside) substrate solution (20 mM in phosphate buffer). After thorough mixing, it was incubated at 37ºC for 30 min. The reaction was stopped after 30 min by adding 100 µl sodium bicarbonate solution (0.5 mM). Acarbose 1mg/ml was used as a positive control and DMSO as a negative control. The absorbance was taken at 405 nm wavelength using BioTek Elx-800 microtiter plate reader. The experiments were carried out in triplicate and percent inhibition was calculated by the following formula;

Inhibition (%) = [Abs. of control – Abs. of sample / Abs. of control] x100

α-Amylase inhibition assay

The crude extract of *R. stricta* and its fractions were subjected to determine alpha-amylase activity according to the method reported earlier (Channar *et al*., 2017). For the assay, extract (20 µl), starch (40 µl) and potassium phosphate buffer (30 µl-pH 6.8) were added in all wells of microtiter plate followed by the addition of 10 µl of the enzyme (0.2 U) in phosphate buffer (0.1 M). The plate was incubated for 30 min at 50ºC and 20 µl 1 M HCl was added in the mixture as stopping reagent.100 ul Iodine reagent (5 mM KI and 5 mM I_2) was appended for the color

development and absorbance was measured at 540 nm by a microplate reader (BioTek, Elx800). The experiments were run in triplicate using Acarbose (1 mg/ml) as positive control while DSMO as a negative control. The enzyme inhibition (%) was calculated by the following formula;

Inhibition (%) = [Abs. of control – Abs. of sample / Abs. of control] x100

Dipeptidyl peptidase-IV inhibition Assay

The *R. stricta* root crude extract and its fractions were investigated for their potential against DPP-IV enzyme by following a method reported by Saleem *et al*. (2014). The assessment of DPP-IV inhibition was done fluorometrically using Gly-Pro-aminomethylcoumarin substrate, purified porcine DPP-IV enzyme (1U/ml) and Berberine (Al-Masri *et al*., 2009) as a positive control. All samples were prepared in HEPES buffer and run in triplicate using 96 well microtiter plate. Briefly, 20 µl sample, 30 µl AMC substrate and 20 µl DPP-4 enzyme (1 U/ml) were added in each of the respective well and reaction mixture was incubated at 37ºC with gentle agitation for one hour. After incubation, 100 µl Acetic acid (3 mM) was added in each well to stop the reaction. The amount of free AMC after liberation from the substrate is monitored using Excitation and Emission wavelength at 351 and 430 nm respectively with the help of Tecan Safire fluorometer (Reading, England). Extracts were tested at different concentrations like 2.0, 1.0, 0.5 and 0.25 mg/ml. HEPES buffer was used as negative control and percentage inhibition was calculated by the given formula. Finally, IC_{50} values were calculated which represents the 50% inhibition of DPP-IV activity by each fraction:

$$\% \text{ inhibition} = \frac{(Fc - Fs)}{Fc} \times 100$$

Where:
“Fc” is fluorescence of the negative control
“Fs” is fluorescence of the sample.

Statistical analysis

GraphPad Prism software version 5.01 was used for statistical analysis. All values are expressed as mean ± SEM of three experiments using one-way ANOVA with Tukey's post-test for comparison. The $p \leq 0.05$ was considered significant. The IC_{50} values were determined by linear regression curve.

RESULTS

Elemental Analysis

Trace elements always play an important role in the cure and prevention of diseases. Total twelve elements were determined in the root of *R. stricta* and all are present in the root in varying concentrations. The element present in the highest concentration is K followed by Na and Ca. Fe is also abundantly present in the root. Results of the elemental analysis are shown in Table No. 1 and each result is an average of three independent measurements.

Table No. 1
Elemental mean value (Mean ± SEM) determined in the root of *R. stricta* collected from district Karak (KPK)

Elements	Concentration (ppm)	Elements	Concentration (ppm)
Fe	9.213 ± 0.12	Zn	0.1088 ± 0.02
Na	18.66 ± 0.64	Cu	0.1317 ± 0.01
K	33.23 ± 0.47	Ni	0.1383 ± 0.01
Ca	17.41 ± 0.88	Mn	1.395 ± 0.03
Mg	2.958 ± 0.05	Cr	1.269 ± 0.11
Co	0.026 ± 0.01	Pb	0.1633 ± 0.01

Qualitative phytochemical screening

Qualitative phytochemical analysis of the *R. stricta* root crude extract and its fractions exhibited the presence of tannins, saponins, flavonoids, alkaloids, steroids, quinone, terpenoids, phenols, and carbohydrates in varying concentrations while cardiac glycosides and glycosides were completely absent (Table No. 2). From all of the phytochemicals, the alkaloids and steroids are present in highest concentration in the crude extract fraction as well as in the second chloroform layer while in forth chloroform layer these are exhibited to be present in good quantity. Flavonoids and terpenoids are the second most abundant constituent present in the RCE and some of its fractions along with carbohydrates while phenols, tannins, saponins, and quinones are also revealed to be present in a reasonable amount as given in Table No. 2.

Table No. 2
Qualitative phytochemical screening of *Rhazya stricta* crude extract and its fractions

	RCE	FAL	SCL	TAL	FCL
Tannins	+	-	-	-	-
Saponins	+	+	+	+	-
Alkaloids	+++	+	+++	+	++
Steroids	+++	-	++	+	+
Flavonoids	++	+	++	+	+
Glycosides	-	-	-	-	-
Cardiac Glycosided	-	-	-	-	-
Quinones	+	-	+	-	-
Terpenoids	++	+	+	+	+
Phenols	+	-	+	-	-
Carbohydrates	++	+	+	+	+

In the table above
+++ Highest amount; ++ Moderate level presence, + Represents the availability of less amount,
- Represents the absence of particular ingredients
RCE (Root crude extract), FAL (First Aqueous Layer), SCL (Second Chloroform Layer), TAL (Third Aqueous Layer), FCL (Fourth Chloroform Layer)

UV-Vis spectrophotometric analysis

UV-visible spectroscopy determined the presence of various classes of compounds in the crude extract by showing the major bands at 221, 284, 291, 413 and 778 nm wavelength (Figure No. 2) with different absorbance value. Similarly, all extracted fractions also showed some peaks at same wavelengths with minor shifts and varying absorbance but only fraction SCL has shown all peaks which were present in parent fraction with minor shift and varying absorbances (Table No. 3). Some additional peaks were also eluted in all resulting fractions even in visible range but their absorbance values were very lower which can be negligible.

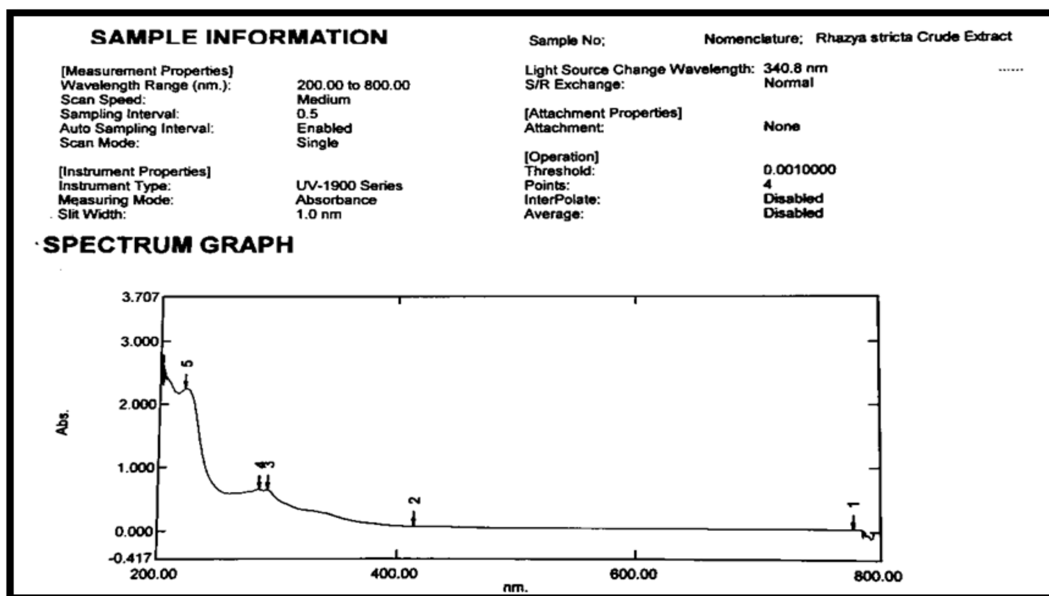

Figure No. 2
UV-visible spectrum of *R. stricta* root crude extract

Table No. 3
λ_{max} with absorbance obtained in all fractions of *Rhazya stricta* root

RCE λ_{max}	RCE Abs.	FAL λ_{max}	FAL Abs.	SCL λ_{max}	SCL Abs.	TAL λ_{max}	TAL Abs.	FCL λ_{max}	FCL Abs.
490	0.001			497	0.027				
413	0.071			407	0.003				
291	0.631			292	0.529	289	1.306		
281	0.641			284	0.539			285	3.034
221	2.237	221	2.717	224	2.048	221	3.770	233	3.932

RCE (Root crude extract), FAL (First Aqueous Layer), SCL (Second Chloroform Layer), TAL (Third Aqueous Layer), FCL (Fourth Chloroform Layer)

Fourier transform infrared spectrophotometry (FTIR)

The FT-IR analysis of root crude extract of *R. stricta* and its resulting fractions was done to determine the functional groups of phytochemicals present in all fractions. From all fractions of root crude extract, SCL and FCL showed close resemblance with its parent crude extract indicating that only these fractions contained almost all major compounds just like crude extract. The other two aqueous fractions showed some spectra in some specific regions with low intensity but many bands of important functional groups were missing in these fractions exhibiting an absence of major functional groups (Table No. 4). It is important to mention here that the region 1500-400 cm^{-1} is known as fingerprint region and peaks in this region mostly arise from complex deformations of the molecule while the region 4000-1500 cm^{-1} is called functional group region and peaks in this region are characteristics of specific kind of bonds of the functional group. The ATR spectra of the crude extract are given as Figure No. 3.

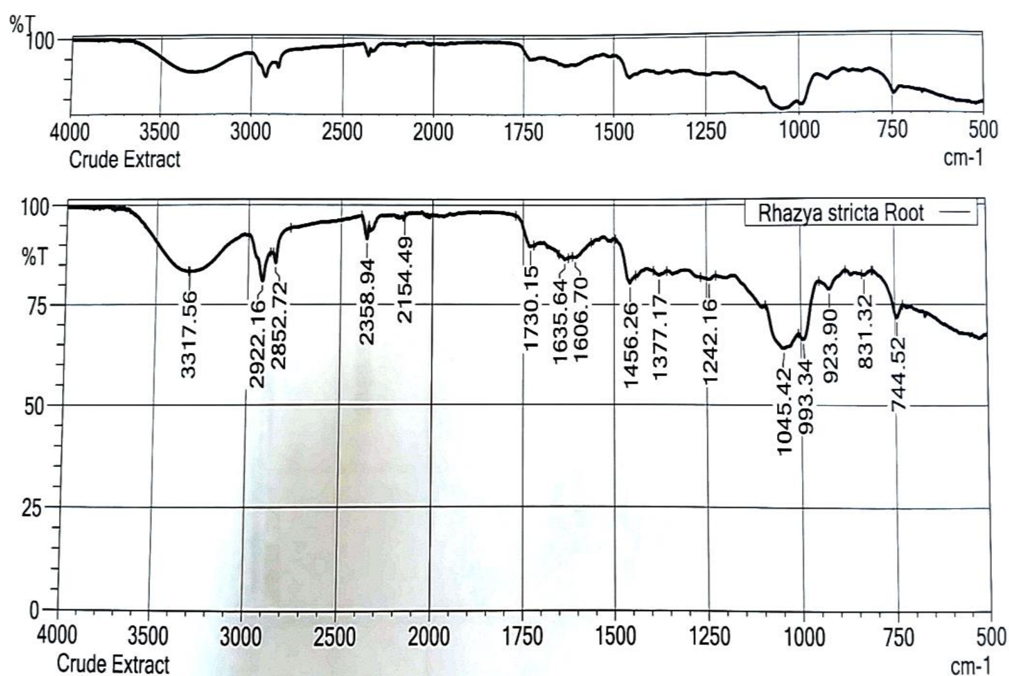

Figure No. 3
ATR spectra of *Rhazya stricta* root crude extract

Table No. 4
FTIR Results: Peaks obtained in all fractions along with their Functional Groups (Coates *et al.*, 2000; Kumar, 2006)

Peaks (RCE)	Peaks (FAL)	Peaks (SCL)	Peaks (TAL)	Peak (FCL)	Bonds	Functional Group
				520		Non identified
	669	669	669	669	C-H	Alkyne/Sulfate Ion
744	748	740		742	C-H	Aromatic
831	829		831		C-H	Aromatic/Nitrate Ion

	866		866		C-H	Aromatic/Carbonate Ion
923	925	925	925	923	C-H	Alkene
993	997		995		C-H	Vinyl
	1028			1033		Non identified
1045			1045		C-O	Primary Alcohol
	1099				C-O	Secondary Alcohol/Sulfate Ion
		1159		1159	C-O	Tertiary Alcohol
		1192		1193	C-O	Phenol
1242		1236		1236	C-O/O-H	Phenol/Aromatic Ether
			1263		O-H	Alcohol/Aromatic Ether
		1338		1338	O-H	Alcohol
1377					N=O	Nitro/Nitrate Ion
			1417		C-H	Vinyl/Carbonate Ion
	1435				N=O	Nitro/Carbonate Ion
1456		1460		1460	C=C	Aromatic/Carbonate Ion
1606		1604		1604	C=C/N-H	Aromatic/ Primary Amine
1635	1631		1631	1629	N-H	Primary Amine
		1656			N-H	Amide
1730		1728		1728	C=O	Carbonyl (Aldehyde)
2154					C≡C	Alkyne
2358	2360	2360	2360	2360	C-H	Aromatic
2852		2852		2850	C-H	Alkyl
2922	2931	2924	2929	2922	C-H	Alkane
	3271		3263		C-H	Alkyne
3317					C-H	Alkyne

RCE (Root crude extract), FAL (First Aqueous Layer), SCL (Second Chloroform Layer), TAL (Third Aqueous Layer), FCL (Fourth Chloroform Layer).

Antioxidant Assays

DPPH Free Radical Scavenging Activity

DPPH assay was used to measure free radical scavenging activity of the plant extract and its fractions. Initially, all fractions were analyzed at 1mg/ml concentration, then for the determination of IC_{50} all fractions were reanalyzed at lower concentrations. The obtained IC_{50} values ranged from 313.5 µg/ml to 776.1 µg/ml (Table No. 5). The root crude extract has shown good scavenging activity while the SCL fraction exhibited as the second most potent fraction after RCE. The overall results showed the potential of *R. stricta* root as a free radical scavenger.

Table No. 5
DPPH percentage inhibition by root crude extract and fractions

Fractions	DPPH Percentage (%) Inhibition				IC_{50} µg/ml
	1000 µg/ml	500 µg/ml	250 µg/ml	125 µg/ml	
FAL	56.0 ± 1.65	45.9 ± 1.90	28.9 ± 1.44	18.5 ± 1.15	776.1
SCL	73.9 ± 0.82	62.2 ± 0.95	44.0 ± 0.91	32.3 ± 1.33	400.0
TAL	58.5 ± 1.06	49.4 ± 1.21	30.7 ± 0.89	18.5 ± 1.56	714.2
FCL	68.0 ± 1.12	59.0 ± 0.81	40.7 ± 1.78	27.6 ± 1.53	477.0
RCE	78.0 ± 1.92	64.1 ± 1.04	48.4 ± 1.85	37.2 ± 1.44	313.5
Ascorbic Acid	--	--	--	--	101.2

All the above data is the mean of three determinations.
± = Standard Error Mean
IC_{50} = Concentration at 50% inhibition

Total antioxidant capacity

Total antioxidant capacity of *R. stricta* root extract and fractions was determined by the phosphomolybdenum method by spectrophotometrically. Highest TAC was observed by the SCL fraction while RCE also exhibited remarkable activity (Figure No. 4)

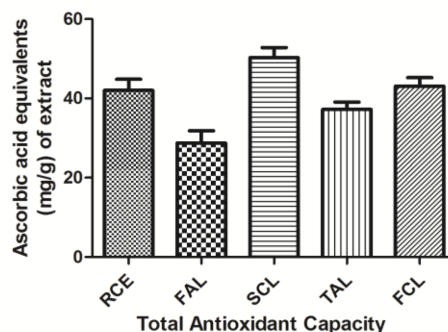

Figure No. 4
Total antioxidant capacity of *R. stricta* root extract and fractions showing maximum activity by SCL fraction. Bars are representing Standard Error. Data are expressed as the mean ± SEM of triplicate investigations ($p < 0.05$)

Total Reducing Power Assay

The root crude extract and its fractions were screened for their reducing power ability which is an indicator of plants antioxidant capacity. The results showed that all fractions exhibited good reducing power capacity that is expressed as ascorbic acid equivalents per gram of extract. Both chloroform layers have shown good results as compared to the aqueous layers of root crude extract. Of all the fractions, RCE was found to have maximum reducing power followed by SCL (Figure No. 5).

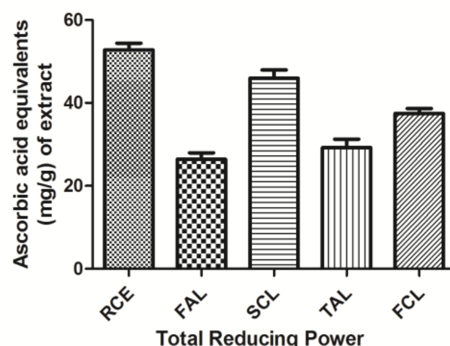

Figure No. 5
Total Reducing Power Capacity of *R. stricta* root extract and fractions showing maximum activity by RCE and SCL fractions. Data are expressed as the mean ± SEM of triplicate investigations ($p < 0.05$)

Total Flavonoid Content (TFC)

The total flavonoid content was estimated as quercetin equivalent at 415 nm wavelength by a colorimetric method and a calibration curve was made with standard solutions of quercetin (0.1 to 25 μg/ml). Interestingly, the highest flavonoids concentration was found in SCL fraction rather than its parent fraction. Other fractions also possessed a good level of flavonoids as shown in Figure No. 6. The flavonoids content was expressed as milligram equivalents of quercetin per gram of extract.

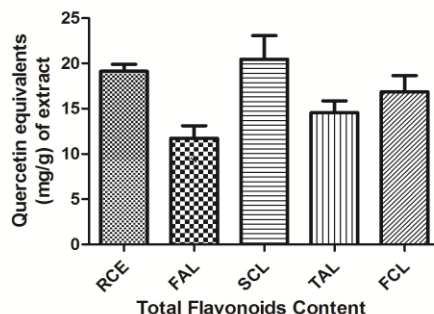

Figure No. 6
Total flavonoids content (TFC) determined in the *R. stricta* root crude extract and its fractions. Results are expressed as mean ± SEM of triplicate analysis ($p < 0.05$)

Total Phenolic Content (TPC)

Total phenolic content was determined in the extract and fraction using Folin-Ciocalteu reagent and expressed in term of gallic acid equivalents per gram of extract. The results showed that the highest level of phenolics was found in SCL fraction while the crude extract was the fraction containing the second-highest amount of phenolics. The FCL and TAL were found to have an almost equal amount of the content while FAL was the fraction with least phenolic content (Figure No. 7).

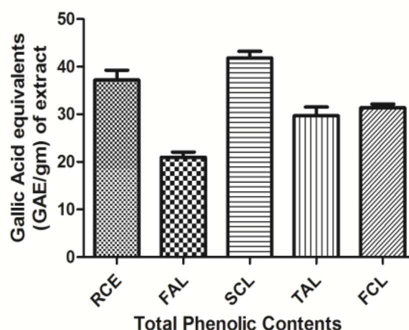

Figure No. 7
Total Phenolic Content (TPC) determined in the *R. stricta* root crude extract and its fractions. Results are expressed as mean ± SEM of triplicate analysis ($p < 0.05$)

α-Amylase inhibition activity

The root crude extract and its fraction showed good alpha-amylase inhibition activity. All fractions including crude extract were examined for their activity against enzyme initially at 1mg/ml concentration and acarbose was used as a positive control. All fractions exhibited reasonable activity against the enzyme and all were then re-analyzed with lower concentrations for the calculation of IC_{50} value (Figure No. 8). At 1 mg/ml concentration the RCE inhibited 52% enzyme (IC_{50} 1.175 mg/ml) while SCL was the second most potent fraction with 45% inhibitory activity (IC_{50} 1.327 mg/ml) in comparison with the aqueous extract fractions FAL and TAL which showed moderate activity with IC_{50} 2.762 and 3.038 mg/ml, respectively.

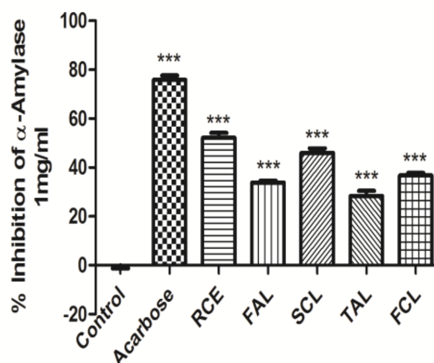

Figure No. 8
α-Amylase inhibition by *R. stricta* extract and fractions at 1 mg/ml concentration.
Results are expressed as mean ± SEM (* $p < 0.001$)**

α-Glucosidase inhibition

The alpha-glucosidase inhibition activity of the root extract and fractions was not good as alpha-amylase inhibition and the crude extract inhibited 47% alpha-glucosidase at 1 mg/ml. The SCL and FCL again proved to be the second and third most successful fractions after crude extract by inhibiting 44% and 28% enzyme activity at the same concentration (Figure No. 9). The RCE, SCL, and FCL were analyzed again at different concentrations for the calculation of IC_{50} which were 1.596, 2.266 and 3.075 mg/ml respectively. The chloroform fractions (SCL and FCL) have exerted better inhibitory effects in comparison with the aqueous fractions FAL and TAL which displayed very little activity like 10.2% and 13.6% respectively, hence aqueous layers were not further evaluated with lower concentrations.

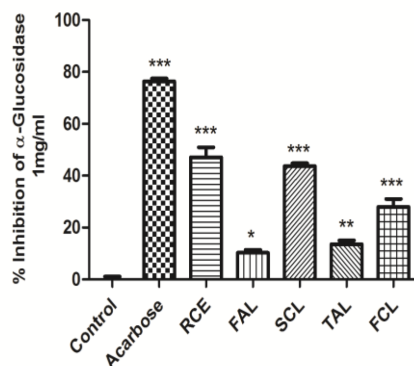

Figure No. 9
α-Glucosidase inhibition by *R. stricta* root extract and fractions at 1 mg/ml concentration. Results are expressed as mean ± SEM (*$p < 0.001$, **$p < 0.01$, *$p < 0.05$)**

Dipeptidyl peptidase-IV (DPP-IV) inhibition assay

Root extract and fractions were evaluated for their potential against DPP-IV enzyme which has the ability to degrade incretin hormone in the gut resulting in a high glucose level in the blood. All fractions were examined at 1mg/ml and then re-examine at lower concentrations for IC_{50} value. The root crude extract presented 70% DPP-IV inhibition activity while SCL and FCL fractions displayed good results by inhibiting 64% and 40% enzyme activity at 1 g/ml concentration (Figure No. 10). The aqueous fractions FAL and TAL also inhibited enzyme activity by 27% and 31% respectively. For the calculation of IC_{50}, all fractions including the root crude extract were evaluated at 2.0, 1.0, 0.5 and 0.25 mg/ml concentrations. Of all tested fractions, the RCE and SCL exhibited good inhibitory results with IC_{50} 0.520 and 0.853 mg/ml while the FCL, FAL, and TAL showed IC_{50} as 1.804, 2.797 and 2.394 mg/ml, respectively.

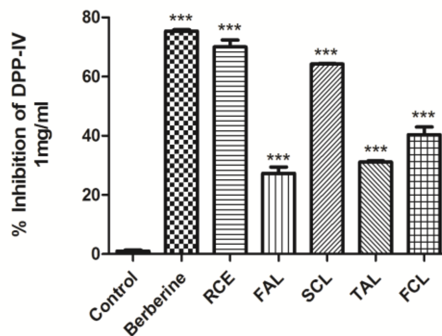

Figure No. 10
DPP-IV percentage inhibition by *R. stricta* root extract and fractions at 1 mg/ml concentration Results are expressed as mean ± SEM (*$p < 0.001$, **$p < 0.01$, *$p < 0.05$)**

DISCUSSION

Natural products are the valuable endowment of nature and development of human pharmaceuticals is considerably grateful for nature as these are the most significant source for modern medicine discovery (Corre & Challis, 2009). Biological properties of the plants are due to the presence of specific kinds of phytoconstituents which gives distinctive properties to plants (Parekh & Chanda, 2007). Secondary metabolites are the chemical substances produces by the plants which have been used to maintain the health of human being as well as animals. These include alkaloids, terpenoids, flavonoids and many other compounds which possess particular importance, for example, phenolic compounds have been known as antioxidants and exhibited cholesterol-lowering activities with the potential to prevent chronic diseases (Boyer & Liu, 2004)

Rhazya stricta, a member of the Apocynaceae family is widely used to treat various diseases in different regions of the world especially in South Asia and the Middle East as folk medicine (Baeshen *et al*., 2014). This plant is reported to contain many phytochemicals like alkaloids, flavonoids, phenols, saponins, steroids, and tannins (Reddy *et al*., 2016). In the present study, *Rhazya stricta* root crude extract was analyzed through UV-Vis spectrophotometer which determined the presence of different components in the extract and its resulting fractions. The main peaks obtained in crude extract were at λ_{max} 221, 284, 291 and 413 nm while another peak in the visible range was also seen at 778 nm. Our results are in compliance with the earlier reports which stated that most of the terpenoid indole alkaloids (TIA) showed λ_{max} at around 217, 221, 230, 283, 290 nm (Pawelka & Stöckigt, 1986; Akhgari *et al*., 2015a;) and *R. stricta* is already reported to be rich in TIAs (Akhgari *et al*., 2015b).

The results of FTIR observed to be containing various functional groups like alcohol, phenol, carbonyl, vinyl, aromatic, alkyne, alkane, alkene, alkyl, and amines (Coates *et al*., 2000; Kumar, 2006). Presence of these functional groups is pointing towards the biological activities conferred by the plants. The hydroxyl group is an integral part of various phenolic compounds such as flavonoids and tannins and plants enriched in these kinds of phytoconstituents are considered to be containing antioxidant and anti-inflammatory properties (Poojary *et al*., 2015). In view of above said functional groups identified in the root extract of *R. stricta*, we are convinced that the plant is rich in various alkaloids, flavonoids, polyphenols, tannins and many other phytochemicals (Chandrashekar *et al*., 2018). Additionally, earlier reported data claimed that the *R. stricta* is a toxic shrub however, we have not observed any band in between the region 2260-2200 cm^{-1} indicative of the absence of any cyanide group in this extract.

Antioxidant activity

It is believed that oxidative damage to cells caused by free radicals may act as a crucial factor in the normal process of aging as well as in the pathogenesis of several clinical disorders including diabetes (Apel & Hirt, 2004; Tang *et al*., 2004). To prevent this process, the intake of antioxidants is highly recommended which may help the immune system to inactivate these reactive species (Abdel-Hameed, 2009). The assessment of antioxidant potential was done on the root fractions of *R. stricta* using different antioxidant assays. Fractions obtained through solvent-solvent extraction of root crude extract of *R. stricta* exhibited significant ($p < 0.001$) free radical scavenging activity with IC_{50} ranged from 400 to 776 μg/ml. Regarding the results of TAC and TRP assays, the SCL fraction examined to be the most potent among other fractions along with crude extract. The results of antioxidants assays are supported by previous reports in which *R. stricta* extract was examined for its antioxidant potential and found to have significant antioxidants activity (Ali *et al*., 2000; Iqbal *et al*., 2006; Al-busafi *et al*., 2007).

The total flavonoid and total phenolic contents of *R. stricta* extract and fractions were also determined in the study and the crude extract along with its fractions were observed to be rich in these phytochemicals. A direct correlation has been observed between the presence of polyphenols and antioxidant capacity as well as the reducing power of certain plants extracts, that is in agreement with the results of our study (Abdel-Hameed, 2009). Hence, it is suggested that the polyphenols might be major contributors to the antioxidants potential of this plant.

Antidiabetic potential

The efficient anti-diabetic drugs are always in great demand because of an increasing number of diabetic patients worldwide (Thring & Weitz, 2006) and medical science is still facing a challenge of management of diabetes mellitus with fewer side effects (Ortiz-Andrade *et al*., 2005). *R. stricta* is an

important medicinal plant of Apocynaceae family that is little investigated for its biological activities although it produces a large number of terpenoid indole alkaloids (Akhgari *et al*., 2017). Root crude extract of *R. stricta* and its fractions obtained through the solvent-solvent extraction were evaluated for their antihyperglycemic potential using different enzyme inhibition assays and all were found to be effective in lowering glucose level by inhibiting α-amylase, α-glucosidase, and DPP-IV enzymes in a dose-dependent manner.

α-Amylase and α-Glucosidase inhibition activity

The present study determined that the root of this plant has the potential to bring down the glucose level in many different ways. Root crude extract and its fractions inhibited α-amylase and α-glucosidase enzymes significantly ($p < 0.05$). Root extract and chloroform fractions showed good enzymes inhibition while aqueous fraction inhibited enzyme activity moderately. Another member of Apocynaceae, *Picrolima nitida* (ethanolic extract) is also reported to be containing α-glucosidase inhibition activity with IC_{50} value 6.15 mg/ml (Kazeem *et al*., 2013).

Inhibitory effects against dipeptidyl peptidase-IV (DPP-IV)

Recent advancements in the diabetes therapeutics have resulted in the development and clinical use of incretin-based therapies. These therapies stimulate insulin secretin either by increasing circulating level of Glucagon-like Peptide-1 (GLP-1) or by inhibiting the dipeptidyl peptidase-IV enzyme that is involved in the degradation of incretin hormones. Therefore, as an alternative to drug therapies, DPP-IV inhibitors from natural sources should be sought (Robinson *et al*., 2016).

In the initial screening, the RCE and its fractions were able to inhibit DPP-IV significantly ($p < 0.01$). Of all the fractions, the crude extract and its SCL fraction exhibited potent DPP-IV inhibition activity while all other fractions also inhibited enzyme activity significantly ($p < 0.05$) with varying percentages. All fractions were further evaluated at lower concentrations for the calculation of IC_{50} value and RCE exhibited remarkable DPP-IV inhibition with IC_{50} 0.520 mg/ml.

Our study revealed that root of *R. stricta* has an excellent antihyperglycemic activity to cope with diabetes with different modes of action as it inhibited the carbohydrate digesting enzymes on one side and on the other side it inhibited DPP-IV enzyme activity. The inhibition of DPP-IV helps incretin hormone (functional form) to be available in the bloodstream for the release of insulin from the pancreas. *R. stricta* is earlier reported to be insulin secretagogue as in many of the animal studies done by different groups, an increase in insulin concentration after treatment with *R. stricta* and insulin-dependent glucose disposal has been reported (Tanira *et al*., 1996; Ahmed *et al*., 2016). Hence, the DPP-IV enzyme inhibition might be one of the reasons for the insulin secretion through incretin hormone stimulation.

CONCLUSION

It is concluded that the root extract of *Rhazya stricta* proved to be containing strong antioxidant potential and the presence of flavonoids and phenolics is also effective in quenching of free radicals. The results of *in-vitro* antihyperglycemic enzyme inhibition assays confirmed the presence of antidiabetic compounds in the plant root. Especially, the plant has not been investigated earlier for DPP-IV inhibition activity and the results of this study demonstrated that it can be a good source of DPP-IV inhibitors for the treatment of diabetes mellitus. Moreover, our results indicated that the extraction procedures adopted in the study are effective in the extraction of biologically active portion of the plant extracts.

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