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HPLC analysis and antidiabetic effect of Rheum ribes root in type 2 diabetic patients

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Abstract

Background and objective: Rheum ribes (polygonaceae) roots are used traditionally to treat diabetes, hypertension, obesity, ulcer, diarrhea, anthelmintic and expectorant. The purpose of this study was to investigate clinically the antidiabetic activity of Rheum ribes in type 2 diabetes mellitus patients and phytochemical study for the correlation of antidiabetic activity with the active constituents in the plant.

Methods: The antidiabetic activity of Rheumribes given alone and in combination with standard oral hypoglycemic agents was investigated in type 2 diabetes mellitus. The study was conducted on 120 patientswith type 2 diabetes mellitus for a period of 12 weeks. Preliminary phytochemical screening was carried out followed by high performance liquid chromatography analysis for the identification of flavonoid constituents in the root of Rheumribes.

Results: Rheumribes root showed significant blood glucose reduction ($P < 0.01$) on 12^{th} weeks of the observation period with 39.63% percent blood glucose reduction. The combination treatment of Rheumribes root and glibenclamide showed a significant difference ($P < 0.05$) with the treatment group of glibenclamide and metforminwith maximum percent of reduction in blood glucose level of 48.91%.The results of preliminary phytochemical screening showed the presence of alkaloids, tannins, flavonoids, anthraquinones, and quinones. Quercetin was identified by high performance liquid chromatography. The proposed high performance liquid chromatography method was validated for linearity, accuracy, precision and limit of quantitation.

Conclusion: Rheum ribes root was found to reduce significantly blood glucose levels in type 2 diabetes mellitus patients.Quercetin was identified by high performance liquid chromatography as an important flavonoid constituent.

Keywords: Rheum ribes, T2DM,Quercetin, HPLC.

Introduction

Diabetes mellitusis a metabolic disorder which arises from complex interactions between multiple genetic, environmental and lifestyle factors.[1]According to the recent data from World Health Organization and International Diabetes Federation, the number of people affected with diabetes worldwide has increased dramatically over recent years. Currently there are over 366 million diabetics worldwide and this is likely to increase to 552 million more by the year 2030.[2]The limitation of currently available oral anti-diabetic agents either in terms of efficacy/safety coupled with the emergence of the disease into global epidemic have encouraged alternative therapy that can manage diabetes more efficiently and safely.[3]Literature reports more than 800 plants have been utilized as empirical treatment for diabetes. One tenth of them have been characterized as hypoglycemic plants with active compounds such as mucilage gum, glycans, flavonoids, triterpenes and alkaloids.[4]Rheum ribes belonging to Polygonaceae family is known as Rhubarb (by English), Rawand (by Arabic), and Rewas (by Kurdish). It is distributed in the temperate and subtropical regions of the world, chiefly in Western

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Asia (Turkey, Syria, Lebanon, Iraq, Iran, Azerbaijan, Armenia to Afghanistan and Pakistan). Stem, leaf and stem form of flowers of this plant are edible raw and cooked.[5,6] The roots are used to treat diabetes, hypertension, obesity, ulcer, diarrhea, as anthelmintic and expectorant.[7,8] Literature survey reveals antidiabetic activity of Rheum ribesin animals[9,10] but no such studies were performed clinically. High performance liquid chromatography (HPLC) is the preferred analytical tool for fingerprints and quantification of marker compounds in herbal drugs because of its simplicity, sensitivity, accuracy and suitability for thorough screening.[11] Previously recorded data indicated the identification of emodin, aloe emodin, chrysophanol, physion anthraquinone constituents in the root of Rheum ribes by HPLC.[12] Hence, the present study was planned to investigate clinically the antidiabetic activity of Rheum ribes in type 2 diabetes mellitus (T2DM) patients and phytochemical study by HPLC for the correlation of antidiabetic activity with the active constituents in the plant.

Methods

Plant materials

Rheum ribes root were collected from the mountain of Hajiomaran, Kurdistan region, Iraq. The roots were cleaned, cut into small pieces, air dried under shade for 5-7days and stored in bottles until used. The identity of the plant was confirmed by assistant professor Al-Khayat AH from the College of Education, University of Salahaddin, Iraq. A voucher specimen is stored at the Department of Pharmacognosy, Hawler Medical University, Iraq (Voucher No.A3).

Hypoglycemic study

Study Population

The patients who visited Layla Qassim diabetic center, Erbil, Iraq were included in this randomized controlled study. After assessment of eligibility of the participants, they were randomly allocated to receive one or other of the alternative treatments under study. The groups received the experimental treatments were compared with a positive-control treatment. A total of 120T2DM patients were selected and divided randomly into three groups. Group I received orally Rheum ribes root powder 350mg as a capsule dosage form three times daily.[13] Group II received orally glibenclamide 5mg once daily and Rheum ribes root powder 350mg three times daily. Group III received orally metformin 500 mg three times daily and glibenclamide5mg once daily. The study was conducted for a period of 12 weeks. All patients (male and female) were put on same dietary regimens. Initial postprandial blood glucose level was measured at time enrolment in the study then at the end of the study. Blood glucose was measured using ACCU-CHEK Active test strips (Roche).

Ethical Clearance

The study protocol was duly submitted to the ethics committee of the College of Pharmacy, Hawler Medical University and approval was taken before starting the study (Approval no. 2/2013). The study was performed under the supervision of the physicians. All the procedure was informed to the patients in their native language and informed written consents were taken from them. The patients were followed up weekly by physicians at Layla Qassim center for evaluation of blood glucose level, any inadequate treatment regime, or any side effect or problem may the treatment regimes cause.

Criteria for selecting patients

For T2DM, the following criteria of selection were considered: postprandial blood glucose level equal to or greater than 180-200mg/dl of blood without any detectable and visible complications. T2DMpatients taking oral hypoglycemic agent with history of inadequate control of blood glucose with this agent, patient of either sex (male or female) between ages of 35-55 years, not pregnant or nursing patients, not smokers or consuming alcohol, not suffering from type1 diabetes mellitus, and not taking medicines for other health condition.

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Statistical analysis

Blood glucose levels were expressed in mg/dl as mean ± SEM. Data were statistically analyzed using the statistical package for the social sciences (version 10.0). Data within the same treatment group were compared using paired-samples t-test. Comparison between the groups was made by two-way analysis of variance (ANOVA) followed by post hocLSD test. Values were considered to be significantly different when the P value was equal or less than 0.01 and 0.05. Percent reduction of blood glucose concentration was calculated with the following formulation;[14]

$$\frac{(G0 - G1)}{G0} \times 100$$

where G0 is blood glucose level before administration, G1 is blood glucose level after administration

Preliminary Phytochemical screening

Twenty grams of dried powdered Rheum ribes root were extracted with 200 ml of 80% ethanol using ultrasonic assisted extractor for 1hr at 40C°, the residual then filtered and air-dried.[15] Root extract analyzed qualitatively for their phytoconstituent natural product groups (alkaloids, anthraquinones, flavonoids, quinones, saponins, tannins, steroid and terpenoids) using standard procedures.[16-19]

HPLC Analysis

Chemicals and materials:

All chemical reagents used for analysis HPLC were analytical or HPLC Grade (99.99%). HPLC-grade acetonitrile, methanol and acetic acid were purchased from Scharlauchemie, S.A., Europian Union. Ultra purewater was used for sample preparation and preparation of mobile phases for HPLC analysis. Standards of quercetin, emodin, aloe emodin, chrysophanol, physion purchased from Chroma Dex, USA.

Preparation of sample solution:

Ten grams of the dried powdered roots was extracted with 100 ml petroleum ether using ultrasonic assisted extractor for 1hr at 40 C°.[15] The residual plant materials were dried and re-extracted with 100 ml (80%) ethanol for one hour that yielded an extract, which was after drying dissolved in 10 ml (5%) HCl and refluxed for one hour. Liquid-liquid fractionation using chloroform (10 x 3 ml) resulted an organic fraction on drying in vacuum that used for evaluation of flavonoid constituents. The organic extract was filtered through a 0.45-µm membrane prior to injection into the HPLC system.

Chromatographic condition

The qualitative analysis of quercetin was performed on Knauer HPLC instrument equipped with ChromGate HPLC software provided by Knauer was use with Eurospher 100, C18 column (4.6 mm i.d. x 250 mm, 5 mm) and UV/Visible detector. The mobile phase was methanol: acetonitrile: water (60:20:20 v/v/v). The mobile phase was filtered through a 0.45 µm membrane filter, then deaerated ultrasonically prior to use. HPLC separation of standard quercetin at 262 nm and run time 6 min. Flow rate and injection volume were 1.1 ml/min and 20 µl. All chromatographic operations were carried out by the integration of the peak using external standard method and at ambient temperature. The chromatographic peaks of the analytes were confirmed by comparing their retention time with those of the reference standards.

Method Validation[20,21]

Linearity

One mg of quercetin, was weighed into a 5 ml volumetric flask, dissolved in acetonitrile: methanol (1:1) filled up to volume for preparing stock solutions. Standard solutions were prepared for each compound at three different concentration (50, 100, and 150 µg/ml) levels in 5 ml volumetric flasks for the establishment of calibration curves. The prepared dilutions were injected in series, peak area was calculated for each dilution, and concentration was plotted against peak area.

x

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Accuracy

Accuracy was determined by the standard addition method for the three concentrations (50, 100, and 150 µg/ml) and the recovery was calculated by comparison of the found amounts with the added ones. The experiment was performed in triplicate. Recovery (%) was calculated for each concentration.

Precision

Precision was determined as both repeatability and intermediate precision, in accordance with ICH recommendations. Repeatability of sample injection was determined as intra-day variation and intermediate precision was determined by measurement of inter-day variation. For both intra-day and inter-day variation, standard solutions at three different concentration (50, 100, and 150 µg/ml) were determined in triplicate.

Detection (LOD) and Quantification (LOQ) Limits

LOD and LOQ were determined by the standard deviation (Sy/x) method. Blank samples were injected in triplicate and the peak area of the blanks were calculated. LOD and LOQ were determined from the slope (S) of the calibration plot and the standard deviation of the response for the blank sample, Sy/x, by use of the formulae LOD= 3.3 × Sy/x/S and LOQ= 10 × Sy/x/S.

Results

The hypoglycemic activity of Rheum ribes root powder was investigated on T2DM The hypoglycemic activity of Rheum ribes root powder was investigated on T2DM patients individually at the dose of 350 mg three times daily and collectively with metformin 500 mg three times daily and glibenclamide 5 mg once daily. The results are summarized in Table 1 and Figure 1.

Table 1: Postprandial blood glucose levels for different study groups.

Treatment group	Blood Glucose Concentration (mg/dl)		P value*
	Week 0	Week 12	
Rheum ribes	315.4 ± 18.8	190.4 ± 19.7	0.00026
Rheum ribes & Glibenclamide	313.6 ± 17.4	160.2 ± 18.6**	0.000003
Glibenclamide & Metformin	359.8 ± 26.1	208.8 ± 20.3	0.00018

The values are given as mean ± SEM (N = 40), * significant ($p < 0.01$) as compared with week 0 (initial data), **significant ($p < 0.05$) as compared with glibenclamide& metformin group within the same time period.

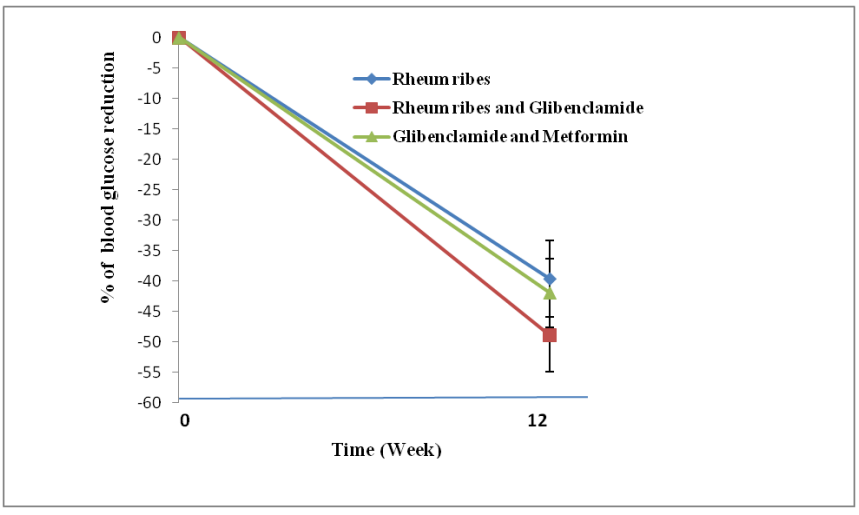

Figure 1: Percent reduction in postprandial blood glucose levels for different study groups, values are expressed as % Means reduction ± SEM

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After 12 weeks of drugs administration, group I showed a significant reduction of 39.63% in blood glucose compared to week 0. The highest reduction in blood glucose was for group II (48.91%). The phytochemical characteristics of Rheum ribes root extractare summarized in Table 2. The results revealed the presence of a number of important natural product groups such as alkaloids, anthraquinones, flavonoids, quinines and tannins. The identification and validation study for flavonoids was performed on Knauer HPLC instrument, C18 column and UV/ Visible detector recording at 262 nm from root extract. An isocratic system was chosen to minimize the variation of the baseline and ghost peaks. Representative chromatograms are shown in Figure 2 which indicated the presence of quercetin. The HPLC method was sensitive with LOD of 20.801 µg/ml.

Table 2: Preliminary phytochemical screening of Rheum ribes root extract.

Phytochemical test	Result
Alkaloids	+
Anthraquinones	+
Flavonoids	+
Quinones	+
Saponins	-
Steroid and Terpenoids	-
Tannins	+

Positive result: + ; Negative result: -

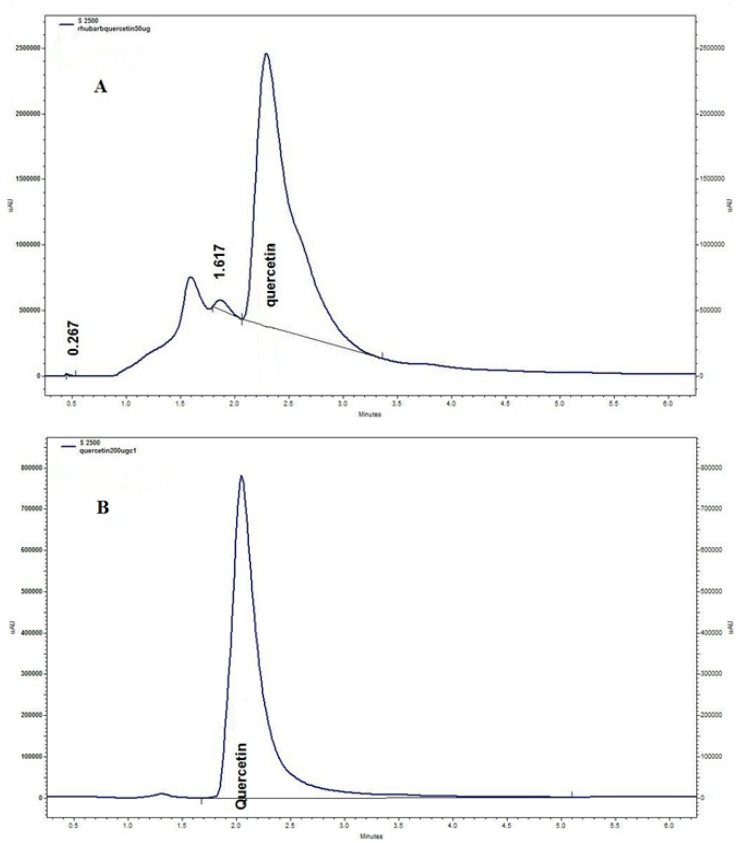

Figure 2: HPLC chromatograms of A: Flavonoid extract; B, quercetin standard compound.

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The validation data are presented in Table 3. The intraday and interday precision are expressed in term of relative standard deviation RSD (n = 3) at concentrations of 50, 100, 150 µg/ml, while the accuracy of the proposed method is expressed as the recovery of standard compound (Table 4).

Discussion

Hyperglycemia is involved in the etiology of development of diabetic complications. Hypoglycemic herbs increase insulin secretion, enhance glucose uptake by adipose or muscle tissues and inhibit glucose absorption from intestine and glucose production from liver.[22] At the end of observation period, group I which received Rheum ribes root showed a significant change in blood glucose level (P <0.01) when compared by paired-samples t-test with its initial blood glucose level with 39.63% percent blood glucose reduction. The hypoglycemic activity of Rheum ribes root can be more observed on week 12 when the results of group I, II, and III were compared by two-way analysis of variance (ANOVA) which showed significant difference (P <0.05) among the groups, and this was followed by post hoc LSD test that confirmed a significant difference (P <0.05) between the combination treatment of Rheum ribes root and glibenclamide in group II with that of group III and the maximum percent of reduction in blood glucose level was recorded for group II on week 12 as (48.91%). No serious side effects were recorded during the period of the study. Rheum ribes is a safe plant and it has a long traditional use that confirms its safety. According to result of Fallah et al[23] Rheum ribes reduce fasting blood glucose concentration with no side effects on blood biochemical factors related to liver and kidney.

Table 3: Validation parameters of the proposed HPLC method.

Parameters	Quercetin
Linearity range (µg/ml)	50-150
Correlation coefficient(r)	0.9989
Slope	0.118
Intercept	0.591
SE of intercept	0.432
SD of intercept	0.748
LOD (µg/ml)	20.801
LOQ (µg/ml)	63.248
Retention time (min)	2.050

Table 4: Precision and recovery data of HPLC method.

Compound	Amount added (µg/ml)	Amount recovered (µg/ml)[a]	Recovery (%)[a]	RSD (%)	
				Intra day[b]	Inter day[c]
Quercetin	50	50.97 ± 0.63	101.95 ± 1.27	1.24	0.92
	100	98.04 ± 1.68	98.04 ± 1.68	1.72	1.44
	150	150.97 ± 2.12	100.65 ± 1.41	1.4	1.36

[a]Mean ± SD (n=3) mean the sample analyzed three times
[b]Samples were analyzed three times a day
[c]Sample were analyzed once a day over three consecutive days

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For the pharmacological as well as pathological discovery of novel drugs, the essential information's regarding the chemical constituents are generally provided by the qualitative phytochemical screening of plant extracts. Phytochemical screening of root extract of the Rheum ribes showed the presence of some important phytoconstituents including alkaloids, flavonoids, and anthraquinones, which were supported bypreviously recorded data.[6,24-27] The presence of quinones in Rheum ribes has not been reported previously. Quercetin was identified by HPLC in the root of Rheum ribes. The calibration curve was linear over the concentration range 50 to 150 µg/ml for standard solutions. Correlation coefficients (r) was 0.9989 showing a good linearity response for the method. LOD and LOQ were calculated and the results indicated that the proposed method exhibits a good sensitivity. A repeatability test was performed in order to estimate intra-day variation in the peak areas and retention times. The highest value for RSD was 1.72 % (n = 3) proving that repeatability reproducibility of the method is satisfactory. Recovery ranged between 98.04–101.95% showing that the presented methods had good accuracy. A validated simple HPLC method for the qualitative determination of quercetin has been developed. The method was precise and sensitive. Anthraquinones could stimulate insulin release from pancreatic β-cells.[9] According to the results obtained either in cell cultures or in animal models, flavonoids, among which quercetin has been reported to improve diabetic status. Further studies are needed to better characterize the mechanisms of action underlying the beneficial effects of this flavonoid on diabetes mellitus.[28]

Conclusion

Rheum ribes root was found to reduce significantly the blood glucose levels in T2DM patients individually and collectively with glibenclamide and metformin. A number of important phytoconstituent natural product groups were detected on preliminary phytochemical screening. Quercetin was identified by HPLC. Rheum ribes may be an effective addition in the family of antidiabetic agents.

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Conflicts of interest

The authors report no conflicts of interest.

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