Research Article

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Antihyperglycemic effect and phytochemical investigation of *Rubia cordifolia* (Indian Madder) leaves extract

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Abstract: Medicinal plants are used as an important source of medicines in pharmaceutical industry. *Rubia cordifolia* is widely used to cure diabetes mellitus. Present study was aimed to investigate the antihyperglycemic effects of different fractions of *R. cordifolia* leaves and to analyze its antioxidant effect and phytochemical composition. Male albino mice were randomly distributed into seven groups (n = 7). Group-I was normal control, group-II was Alloxan (100 mg/kg)-induced diabetic control, and group-III was standard drug (Glibenclamide 0.5 mg/kg)-treated group. Animals in groups IV–VII were treated with n-hexane fraction, ethyl acetate fraction, n-butanol fraction and aqueous fraction of *R. cordifolia*, orally administered (100 mg/kg) once daily up to 28 days after Alloxan induction, respectively. Methanolic extract (ME) and fractions of *R. cordifilia* were analyzed for antioxidant activity and quantification of total phenolic content and total flavonoid content. HPLC of ME and most active fractions were performed. The results showed that RCEF (G-V) and RCBF (G-VI) have significantly ($P < 0.05$) reduced the increased level of glucose as compared to toxicant control group. It was further revealed that EF and BF have higher antioxidant activity (having IC_{50} 34.9, 36.86 (μg/mL)) owing to phenolic and flavonoid identified by HPLC.

Keywords: diabetes mellitus, polyphenolic compounds, Alloxan, pancreas, *Rubia cordifolia*, antioxidant

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1 Introduction

Diabetes mellitus (DM) is metabolic disorder, caused by insulin deficiency during its production or its action or both [1,2]. Serious complications like muscular tissue damage, retinopathy [3,4], neuropathy [5], nephropathy [6], heart complications [7], and ulceration problems [8] occur if DM is not treated properly. Insulin and glucagon are pancreatic hormones that control normal range of blood glucose level (BGL) according to the body needs. Based on the etiology of the disease, two main types of diabetes are type 1 and type 2 [9]. Factors like dietary supplements, oral hypoglycemic agents, and physical activities lead to decrease BGL in the cases of disease [10]. Loss of pancreatic β-cells or their functions is due to environmental factors and genetics, which results into

hyperglycemia in both type 1 and type 2 diabetes [11]. During the treatment of diabetes; pancreatic β-cells are stimulated by traditional and synthetic medicines that decrease the BGL by inhibiting other hormones which increase the BGL. Other mechanisms to treat DM are reduction in glycogen release, increase in glucose utilization in the body, resisting lipid peroxidation, and blood circulation improvement [12]. Nutritional assessment in medical nutrition therapy is performed to monitor the diabetic patients by food intake, metabolic status, and lifestyle to make changes according to set goals and instructions [13]. Anyhow, there is no proper medicine that can completely cure the DM.

Primary source of diseases treatment had been the medicinal plants [14]. Conventional drugs have been isolated or synthesized from small herbs [15]. In Ayurveda, about 2,000 plant species are considered to have medicinal properties [16]. Chinese Pharmacopoeia shows that there are over 5,700 traditional medicines, most of which are obtained from plants [17]. It is estimated that about 500 herbs are still applied within conventional medicine, although there are some cases where the whole plant is used due to its therapeutic benefits [18]. Modern medicines use the plant's derived compounds based on evidences tested pharmacologically. There are 120 active compounds which are isolated from higher plants and their therapeutic and traditional use shows 80% positive correlation [19]. Active ingredients are obtained from medicinal plants, which are used in health care for drugs discovery and development by modern medicines. Pharmacological uses of medicinal plants are reported to be of 20% of plants in the world [20]. Drug discovery and drug development are based on natural products and their uses for treatment of various diseases [21].

Although synthetic drugs are mainly used to treat diabetes, they exhibit prominent side effects, which cannot be reversed and their approach to common people is also difficult due to high cost. Plant-based medicines have less side effects; that is why they are liked by most of the populations in the world [22]. Different studies on medicinal plants show that antibiotics, anti-inflammatory agents, and antioxidants are derived from plants and drugs derived from plants are nontoxic, safe to use, and cost-effective [23]. Secondary metabolites are derived from plant cell which are considered as bioactive compounds [24] that play an important role in the living plants [25]. Metabolites like alkaloids, saponins, coumarins, glycosides, tannins, phenols, reducing sugars, steroids, and triterpenoids are medicinally important and derived from the plants [26]. Identification and isolation of bioactive metabolites of plants are essential in order to understand their therapeutically active and efficacious nature [27,28]. The most important bioactive metabolites of plants are alkaloids, glycosides, flavonoids as well as resins and lignin [29] and their therapeutic role is due to their antioxidant potential [30]. Medicinal plants are considered an important treasure house of drugs [31]. There are more than 800 plants species which are reported for antihyperglycemic effects [32]. Various plants are used to cure and manage DM in the Native Americans, Chinese, [33], South Americans [34] as well as in Asians [31]. Plants against hyperglycemia from Azad Jammu and Kashmir (AJ&K), Pakistan, are less studied from pharmacological point of view.

Rubia cordifolia (Indian madder) or (Gand) Verdc. of family Rubiaceae is an important medicinal plant of Poonch region of AJ&K Pakistan. It is distributed in China and all South Asian and South East Asian countries. In India, this plant is frequently distributed adjacent to rivers and streams in green forests up to 3,750 m altitude from sea level [35,36]. Active parts of *R. cordifolia* are stem and roots, which are used in Indian folk medicines. Different parts of *R. cordifolia* have been used as blood purifying agent, astringent, antiseptic, and antidysentric agent. It also showed hepatoprotective, anti-rheumatic activity and antiviral activity [37,38]. Traditionally, *R. cordifolia* is said to be used in the treatment of various diseases like diabetes, cancer, acne, allergy, inflammation, and bacterial and viral infections [39,38]. Little work has been done on the leaves of *R. cordifolia* L. regarding the phytochemical studies. Due to increasing rate of diabetes, there is an urgent need of antihyperglycemic drugs. Having minimal side effects and medicinal importance of *R. cordifolia*; present study is carried out to conduct phytochemical investigation on antihyperglycemic effects of traditionally used *R. cordifolia* from Poonch region of Azad Jammu and Kashmir (AJ&K), Pakistan.

2 Materials and methods

2.1 Chemicals and instrumentation

All the solvents (methanol, *n*-hexane, *n*-butanol, and ethyl acetate) were of analytical grade, while some solvents (methanol, acetonitrile, and orthophosphoric acid 85%) of HPLC grade were purchased from registered chemical companies (Sigma Aldrich). Alloxan, Standard drug (Glibenclamide) and standards compounds (Mangiferine, Purpurine, Kaempferol, 2-methyl anthraquinone, and Charatine) were purchased from Sigma Aldrich. Equipments used were UV-visible spectrophotometer, glucometer,

mechanical grinder, laboratory centrifuge, rotary evaporator, syringe, nylon filter (0.45 µm), filter papers (Whattman No.1), volumetric flasks, beakers, Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA), Sonicator, oven, digital balance, falcon tubes, Eppendorf tubes, glass vials, cotton, test tubes, etc.

2.2 Plant material

2.2.1 Collection and identification of plant material

Plant samples of *R. cordifolia*, according to their seasonal availability (April to September), were collected. Herbarium sheets of plants were prepared by standard procedure [40]. Dr Azam (Associate Professor, Government Boys Degree College Hajira AJ&K) was consulted for identification of the plants. Correctly, identified specimen (*Rubia cordifolia*) was deposited as voucher specimen (0444) for future references in the herbarium of Medicinal and Aromatic Plants of AJ&K at Government Boys Degree College Abasspur AJ&K Pakistan.

2.2.2 Preparation of methanolic extract (ME)

Plant samples were washed under tap water and dried under the shade at normal temperature for 6 days. Leaves were cut, separated, and grinded into powder form using mechanical grinder. Dried powder of *R. cordifolia* leaves was weighed and packed into zipped polythene bags to avoid moisture and contamination.

ME of *R. cordifolia* were prepared according to well-established protocols [41,42]. Briefly, dried powdered materials (1,000 g) were added to methanol in ratio of 1:3 in flask of 5 L (5,000 mL) and kept for soaking for seven days at room temperature by continuous shaking. The mixture was filtered through filter papers (Whatt man No.1). The process of extraction was repeated in duplicate way. Whole filtrate was concentrated using rotary evaporator at 40°C under reduced pressure and then weighted. This was methanolic extracts of *R. cordifolia* leaves (MERC).

2.2.3 Fractionation process of MERC

The ME of *R. cordifolia* was fractionated with different organic solvents (*n*-hexane, ethyl acetate, *n*-butanol) and water on increasing polarity basis [43]. ME of *R. cordifolia* (100 g) was suspended in the distilled water (200 mL). This formed the water suspension of MERC. The aqueous suspension was partitioned into four fractions, *n*-hexane fraction (HF), ethyl acetate fraction (EF), *n*-butanol fraction (BF), and an aqueous fraction (AF). In this process, *n*-hexane (3 × 200 mL) was added in water suspension and vigorously shaken, which formed two layers. Then *n*-hexane layer was separated by separating funnel. It was dried by rotary evaporator at 40°C under reduced pressure. This was *n*-hexane fraction (HF). Then ethyl acetate (3 × 200 mL) was added to aqueous portion. Two layers were separated by separating funnel; ethyl acetate fraction was dried by rotary evaporator at 40°C under reduced pressure. This was ethyl acetate fraction (EF). Later on, *n*-butanol (3 × 200 mL) was added in the remaining aqueous portion of MERC. Again, two layers were developed in separating funnel, an upper portion of *n*-butanol and lower layer of water suspension of ME. These were separated by separating funnel and then dried by rotary evaporator at 40°C under reduced pressure. There were *n*-butanol fraction (BF) and an aqueous fraction (AF). All obtained fractions were weighed separately and labeled properly. These were labelled as: *n*-hexane fraction of *R. cordifolia* (HFRC), ethylacetate fraction of *R. cordifolia* (EFRC), *n*-butanol fraction of *R. cordifolia* (BFRC), and aqueous fraction of *R. cordifolia* (AFRC), respectively. The dried extracts were kept in refrigerator at 4°C to avoid contamination until further analysis.

2.3 Antihyperglycemic effects of fractions of *R. cordifolia*

2.3.1 Experimental animals

During experimental process, 35 male albino mice (BALBc) of average weight (28 ± 5 g) were purchased from animal house of National Institute of Health (NIH), Islamabad, and were kept in stainless steel cages; providing same environment conditions (25 ± 5°C for 12 h light/dark cycles).

2.3.2 Experimental design

Experiments on mice were performed under the approval of Institutional Bioethics and Biosafety Committee (IBBC) of the University (IIUI) through assigned number No. IIU (BI&BT)/FBAS-IBBC-2016-Dated: 13.07.2016 and according to guideline laid by OECD-423 (adopted on 17th December

2001). Four fractions (HF, EF, BF, and AF) of ME of *R. cordifolia* were tested for their antihyperglycemic effects. Experimental grouping was designed according to standard procedure [44,45] with some modifications. Mice under study comprised seven groups (number = 5 in each) for treatment effect of fractions. Group-I was normal control group (NC), group-II Toxicant control (TC), and group-III was positive control/standard drug control (SD), while the mice in groups IV–VII were treated with *R. cordifolia* fractions. Animals in all groups were provided access with normal feed and water for 28 days.

2.3.3 Treatment procedures

Group-I: (NC): Group-I was allowed for free access for normal feed and water up to 28 days. Group-II: (TC): Alloxan (100 mg/kg body weight) was administered through intraperitoneal injections with 2 days' interval for 6 days. Group-III: standard drug (SD) treated group: Alloxan as in group-II + standard drug (Glibenclamide 0.5 mg/kg) orally administered up to 28 days. Animals in groups IV–VII were treated as:

Group-IV: Alloxan as in group-II + treatment with *n*-hexane fraction of *R. cordifolia* (HFRC); oral administration of dose (100 mg/kg/day) once daily up to 28 days.

Group-V: Alloxan as in group-II + treatment with ethyl acetate fraction of *R. cordifolia* (EFRC); oral administration of dose (100 mg/kg/day) once daily up to 28 days.

Group-VI: Alloxan as in group-II + treatment with *n*-butanol fraction of *R. cordifolia* (BFRC); oral administration of dose (100 mg/kg/day) once daily up to 28 days.

Group-VII: Alloxan as in group-II + treatment with aqueous fraction of *R. cordifolia* (AFRC); oral administration of dose (100 mg/kg/day) once daily up to 28 days.

2.3.4 Analysis for BGL and body weight of mice on treatment with different fractions of *R. cordifolia*

Glucometer was used to measure the BGL in all the groups with 7 days' interval (0, 7, 14, 21, and 28 days) during the experimental process with standard procedure [46]. On the basis of results of antihyperglycemic activity of different fractions of *R. cordifolia* against Alloxan-induced diabetes in mice, most active fractions were selected for further phytochemical analysis and antioxidant activities.

2.4 Antioxidant activity (DPPH assay)

Radical scavenging effect/antioxidant activity of ME as well as the most active fractions of *R. cordifolia* was performed with procedure discussed earlier [47,48], with some modifications using 2,2 diphenyl-1-picrylhydrazyl (DPPH) as free radical.

For antioxidant activity determination, DPPH (6 mg) was dissolved in 100 mL of methanol to prepare the solution. This DPPH solution (2,800 µL) was mixed in each sample (200 µL) solution by addition in glass vials, leading to the final concentration of 150, 110, 70, 35, 25, and 10 (µg/mL), respectively. All the samples were shaken well and kept at room temperature (25–28°C) for 1 h. Measurement of absorbance was performed at 517 nm using spectrophotometer. DPPH (2,800 µL) solution and mixture of methanol (200 µL) were taken as negative control, while ascorbic acid standard as positive control, while methanol as blank to check the activity of sample. Antioxidant activity (%) was measured according to formula given below and IC_{50} value was calculated by linear regression of standard ascorbic acid by graphic method.

$$\%AA = [(Ab_C - Ab_S) \div Ab_C] \times 100$$

"Ab_C" was absorbance of control and "Ab_S" means absorbance of test sample.

2.5 Phytochemical analysis

2.5.1 Quantitative phytochemical analysis

Quantitative phytochemical analysis of ME and most active fractions *R. cordifolia* were subjected to analysis for total phenolic contents (TPC) and total flavonoids content (TFC) with standard procedure [49].

2.5.1.1 Assay for total phenolic contents (TPC)

Folin-Ciocalteu method was used for estimation of total phenolic contents. Using gallic acid (GA) as standard, the absorbance was measured at 765 nm. GA solution was prepared first and then serial dilution was performed to make the final concentrations of 500, 250, 125, 50, 25, 10, 5, and 2.5 (µg/mL), respectively. The samples solutions were also prepared. All samples and GA solutions were tested with same fashion by measuring the absorbance at

765 nm. During this assay, 4 µL of extract solution + 180 µL distilled water + 4 µL Folin-Ciocalteu reagent afterwards (this was done in three replicates using 96-well plate) for each sample. The plate was shaken vigorously with the addition of reagent. After four minutes, 12 µL of aqueous sodium carbonate (2%) solution was added and then the mixture was allowed to stand in dark for 2 h with the application of intermittent shaking. Blank sample comprising 4 µL extract solution, 180 µL distilled water, and 12 µL of aqueous sodium carbonate (2%) solution was used against the test samples to read the absorbance at 765 nm. Calculation of phenolic compounds concentration was performed according to equation constructed from standard GA calibration curve. Expression of results was done as µg per mg of GAE equivalent (µg/mg GAE) of extract.

$$y = 0.005X - 0.011, \quad (R^2 = 0.987).$$

2.5.1.2 Assay for total flavonoid contents (TFC)

Aluminum chloride method was used to evaluate total flavonoid content (TFC) with described method [50] with little modifications using quercetin as the standard. Different concentrations of quercetin (500, 250, 125, 50, 25, 10, 5, and 2.5 µg/mL) solution were made using methanol. Standard solutions were tested similar to samples using aluminium chloride (2% $AlCl_3$). Absorbance of standard solutions was read at 415 nm against the blank quercetin solution. Quercetin calibration curve was plotted of the values obtained by subtracting the blank values from the values of tested quercetin solutions. During this assay, 100 µL of extracts solution were added in the 100 µL of 2% aluminium chloride in methanol (All this was done in three replicates using 96-well plate). After 40 min, absorbance was read at 415 nm against blanks samples (100 µL extract solution along with 100 µL methanol). The concentration of flavonoid compounds was calculated according to equation obtained from standard quercetin calibration curve. The results were expressed as µg/mg QE of extract.

$$y = 0.005X - 0.054, \quad (R^2 = 0.962).$$

2.5.2 Identification of compounds

2.5.2.1 HPLC analysis of *R. cordifolia* extracts

HPLC analysis of methanolic extract (MERC) and most active fractions of *R. cordifolia* was performed for the identification of bioactive chemical compounds. HPLC analysis of methanolic extracts and most active fractions of *R. cordifolia* were performed by co-elusion with reference standard compounds [51].

2.5.2.1.1 Reference standards

In present investigation, 3 standards of phenolic compounds (Mangiferine, Purpurine, and Charatin) were used for HPLC profiling.

2.5.2.1.2 Plant samples

The samples for analysis were ME of *R. cordifolia* and its most active fractions (EFRC, BFRC).

2.5.2.1.3 Preparation of samples

All samples (plant extract and standards) were prepared in methanol (HPLC (1 mg/mL)) in amber Eppendorf tubes to avoid light effect. These mixtures were subjected to sonication (10 min) for proper mixing. Samples were also filtered using Nylon membrane filters (0.45 µm) in separate Eppendorf tubes.

2.5.2.1.4 Apparatus

The HPLC equipment used was a LC-2010C HT system (Shi-madzu, Kyoto, Japan), having quaternary low pressure gradient pump with an autosampler, degasser, block-heating type column oven, UV detector set at 254 nm, and a Shi-maddu LC-solution workstation. An agilent 1200 HPLC (Agilent technologies Santa Clara CA) was equipped with an online vacuum degasser, a Quat pump, an automated injection valve, and a thermostated column compartment; a DAD and an Agilent Chem Station were selected to analyze samples.

2.5.2.1.5 Conditions of HPLC

HPLC using C_{18} column (250 mm × 4.6 mm i.d., 5 µm, Waters, MA, USA) in tandem with a Phenomenex C_{18} guard cartridge (4.0 mm × 3.0 mm, Phenomenex, Torrance, CA). Column was eluted by using acetonitrile and water in 18 min. The flow rate was set at 1.0 mL/min, while the

column temperature was set at 30°C. Spectra were recorded from 190 to 400 nm, while the chromatogram was acquired at 254 nm.

2.6 Statistical analysis

Data were expressed as mean values ± S.E with five animals in each group. Comparison between the groups was performed by one-way analysis of variance (ANOVA) using Statistix 8.1, followed by Tukey HSD test with $P < 0.05$ considering statistically significant. Calibration curve was constructed to calculate mean and standard deviation for DPPH assay and analysis of TPC and TFC by excel 2010.

Ethical approval: Institutional Bioethics and Biosafety Committee (IBBC) approved the study through assigned number No. IIU (BI&BT)/FBAS-IBBC-2016-Dated: 13.07.2016.

3 Results

3.1 Antihyperglycemic effect of different fractions of *R. cordifolia*

The effect of treatment of different fractions of *R. cordifolia* on BGL of alloxan-induced diabetic mice is represented in Figure 1, while on body weight is represented in Figure 2 below. The BGL in the group I (NC) remained 95.80 ± 1.190, 98.10 ± 0.852, 99.300 ± 1.315, 98.40 ± 1.219, and 99.36 ± 0.880 (mg/dL) from day zero up to 28th day, respectively. The BGL of group II (TC) was significantly increased 97.86 ± 1.322, 253.01 ± 1.215, 263.52 ± 1.127, 283.20 ± 1.530, and 313.44 ± 1.272 (mg/dL) with the treatment of alloxan (100 mg/kg) as compared to normal control (NC) group I. BGL with the treatment of standard drug (Glibenclamide 0.5 mg/kg) and different fractions of *R. cordifolia* (HFRC, EFRC, BFRC, and AFRC) with the dose of 100 mg/kg in the treatment groups (G-IV–G-VII), after alloxan-induced diabetes, was significantly reduced as compared to diabetic control group generally. This effect was highly significant ($P < 0.05$) on time-dependent manner, i.e., after 14th day treatment as shown in Figure 1. The BGL was almost same before the treatment of standard drug and *R. cordifolia* fractions, respectively, on day seven. The comparison of blood glucose level among treatment groups (IV–VII) of *R. cordifolia* fractions showed that G-V and G-VI highly reduced the BGL which is comparable to standard drug (Glibenclamide) treatment (G-III), while the G-IV and G-VII have shown the similar effect with less reduction in BGL which was increased due to Alloxan induction.

The body weight in the Group-I (NC) significantly increased 28.10 ± 0.714, 28.90 ± 0.748, 31.30 ± 0.860, 32.62 ± 0.924, and 35.30 ± 0.768 (g) from day zero up to 28th day, respectively. The body weight in group II (Diabetic control) was significantly decreased 29.00 ± 1.225, 28.50 ± 0.1.025, 27.10 ± 0.833, 25.32 ± 0.393, and 23.62 ± 0.543 (g) with the treatment of alloxan (100 mg/kg/day) as compared to normal control group I (NC). Body weight with the treatment of SD (Glibenclamide 0.5 mg/kg) and different fractions of *R. cordifolia* (HFRC, EFRC, BFRC, AFRC with the dose of 100 mg/kg) in the treatment groups (III–VII), after Alloxan-induced diabetes, was significantly increased as compared to diabetic control group generally. This effect was highly significant ($P < 0.05$) on time-dependent manner, i.e., after 14th day treatment as shown in Figure 2 below. The body weight was almost same before the treatment of standard drug and different fractions, respectively, on day seven. The comparison of BGL among treatment groups (IV–VII) of selected plants extract showed that, in G-V and G-VI, the body weight increases with passage of time which is comparable to standard drug (Glibenclamide) treatment (G-III), while the G-V and G-VII have shown similar effect with reduction in body weight like group two (G-II).

3.2 DPPH radical scavenging activity of *R. cordifolia*

The scavenging effect (%) of *R. cordifolia* is increased with increasing concentration of sample as shown in Table 1. Concentration-dependent potential against DPPH was exhibited by samples and standard antioxidant ascorbic acid (AA). Methanolic extract (MERC) as well as its different fractions (HFRC, EFRC, BFRC, and AFRC) showed effective free radical scavenging effect (% inhibition). There is highest scavenging activity (%) of AA (126.3 ± 0.70) followed by EFRC (123.11 ± 0.85), BFRC (120.53 ± 1.36), MERC (119.00 ± 0.87), AFRC (109.10 ± 0.85), and HFRC (106.77 ± 1.66) as indicated in the Table 1. The IC_{50} calculation was performed by calibration curve with standard ascorbic acid concentrations (10, 25, 35, 70, 110, and 150 (μg/mL)). In case of antioxidant effects of above samples, MERC, HFRC, EFRC, BFRC, AFRC, and AA have the IC_{50} value of 38.19, 48.52, 34.9, 36.86, 44.88, and

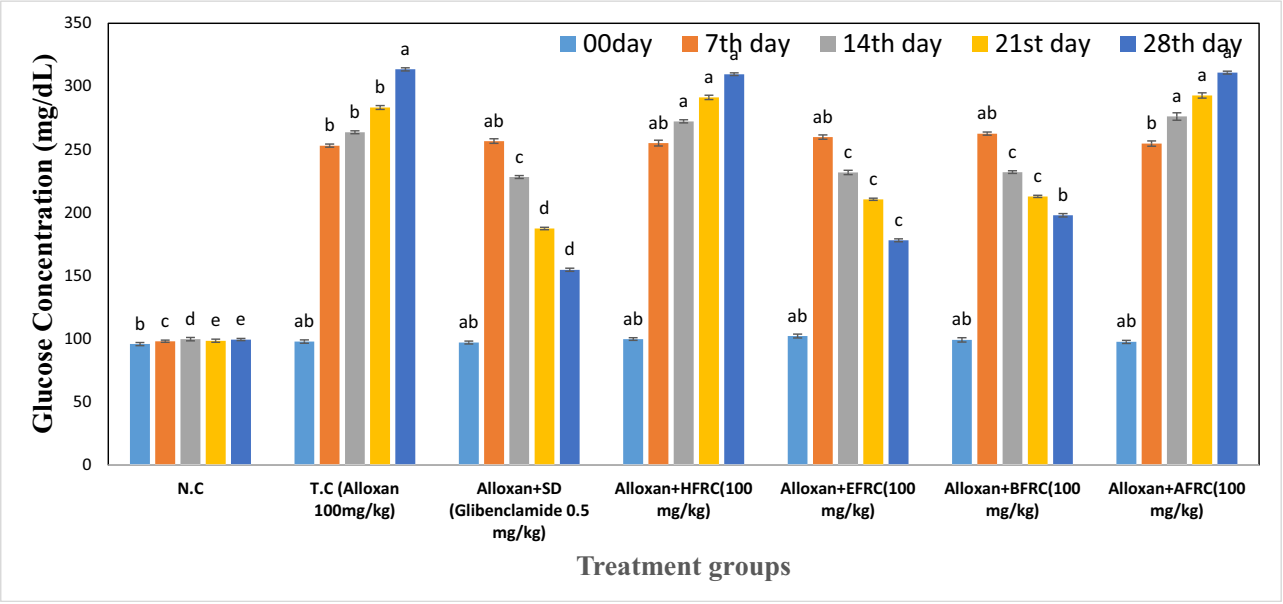

Figure 1: Effect of different fractions of *R. cordifolia* on BGL of alloxan-induced diabetic mice. Values are expressed as mean ± S.E ($n = 5$) letters a, b, c… show the significant ($P < 0.05$) difference and same letters show nonsignificant ($P > 0.05$) differences.

34.41 (µg/mL), respectively. EFRC showed lowest IC_{50} value 29.92 (µg/m L). The order of decreasing IC_{50} values of MERC and its different fractions is HFRC > AFRC > MERC > BFRC > EFRC (having IC_{50} values of 48.52, 44.88, 38.19, 36.86, and 34.9 (µg/mL), respectively. The IC_{50} value of standard antioxidant AA is 34.41 (µg/mL). Lowest is the IC_{50} value, higher is its antioxidant activity.

3.3 Phytochemical analysis

3.3.1 Total phenolic contents (TPC) and total flavonoids contents (TFC)

TPC (µg/mg GAE) and TFC (µg/mg QE) were calculated according to standard calibration curve of gallic acid

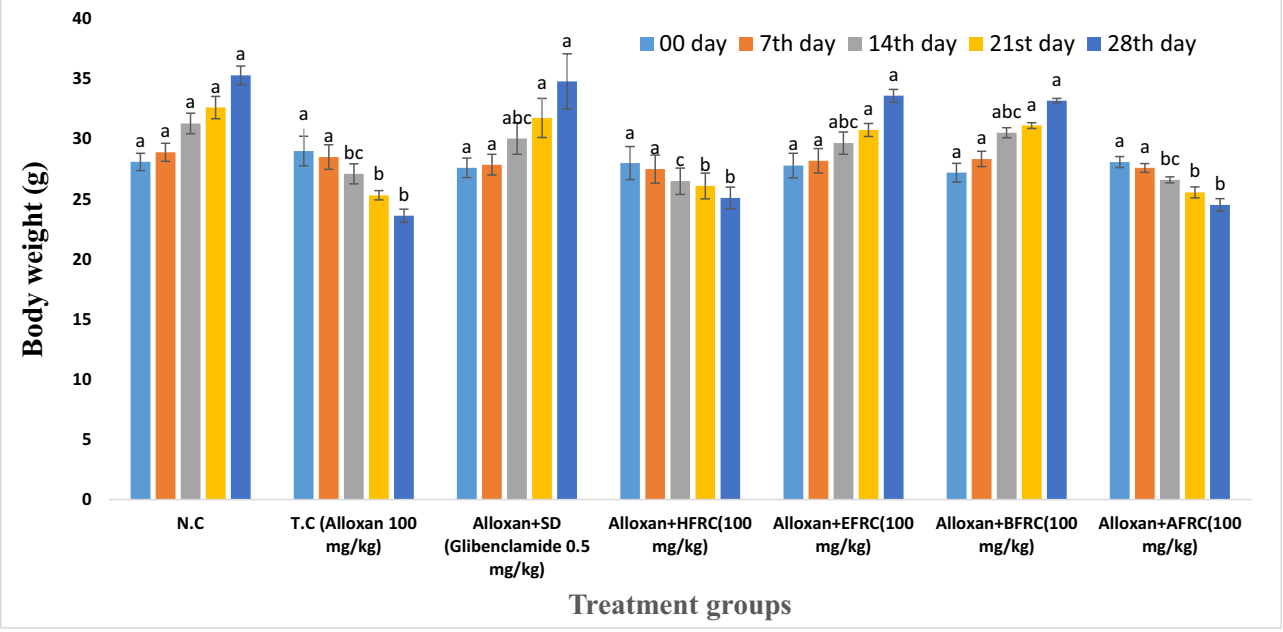

Figure 2: Effect of different fractions of *R. cordifolia* on body weight (BW) of alloxan-induced diabetic mice. Values are expressed as mean ± S.E ($n = 5$) letters a, b, c… show the significant ($P < 0.05$) difference and same letters show nonsignificant ($P > 0.05$) differences.

Table 1: Percent (%) DPPH radical scavenging activity of *R. cordifolia* extract/fractions and ascorbic acid (AA) with IC_{50} values

S. no.	Plant extract/ fraction	Radical scavenging effect (%) at different concentrations (µg/mL) ± S.D						IC_{50} (µg/mL)
		10	25	35	70	110	150	
1	MERC	24.40 ± 0.55	35.73 ± 1.50	48.27 ± 0.06	88.00 ± 0.92	103.67 ± 1.47	119.00 ± 0.87	38.19
2	HFRC	19.73 ± 0.31	30.27 ± 1.14	43.47 ± 1.29	76.50 ± 1.32	92.20 ± 0.92	106.77 ± 1.66	48.52
3	EFRC	26.11 ± 1.11	36.20 ± 0.80	52.20 ± 1.01	91.57 ± 1.25	107.00 ± 1.80	123.11 ± 0.85	34.9
4	BFRC	25.10 ± 1.15	36.10 ± 1.05	49.87 ± 1.21	89.13 ± 2.00	105.10 ± 1.15	120.53 ± 1.36	36.86
5	AFRC	23.13 ± 1.03	32.30 ± 1.21	44.27 ± 1.32	78.47 ± 1.55	96.50 ± 1.32	109.10 ± 0.85	44.88
6	AA	26.12 ± 0.98	42.04 ± 0.01	52.2 ± 1.11	85 ± 1.00	106.02 ± 1.03	126.3 ± 0.70	34.41

(GA µg/mL) and quercetin (µg/mL). The results show the presence of TPC and TFC of *R. cordifolia* in Table 2 below. The results show that TPC in MERC, EFRC, and BFRC have 99.732 ± 0.938, 207.306 ± 0.730, and 119.120 ± 0.893 (µg/mg GAE), respectively. The results show that TFC in MERC, EFRC, and BFRC have 96.564 ± 0.996, 195.224 ± 0.940, and 76.848 ± 0.519 (µg/mg QE), respectively

3.3.2 HPLC *R. cordifolia*

HPLC of *R. cordifolia* is presented below in Figure 3(d–f). The chromatograms of standard compounds are shown in Figure 3(a–c) below. Mangiferines and Purpurine were two standard compounds used whose retention time is 9.102 and 12.400 (min), respectively. There is shown the presence of mangiferine in all the samples, while the purpurine is present in methanol extract and ethylacetae fractions.

4 Discussion

The management of diabetes is much cost-effective and hectic in poor population of the underdeveloped countries. The synthetic drugs also impair the body metabolites and cause serious side effects. Plants-based medicines to treat diabetes are practiced nowadays by pharmaceutics and health care professionals all over the world and possible management of diabetes is urgent to reduce the mortality rate [52].

For testing of plants for antidiabetic effect, mouse model is more accepted and has resemblance to human being [53]. *R. cordifolia* root extract exhibited significant antihyperglycemic activities on streptozotacin (STZ)-induced hyperglycemic rats by 2 weeks of treatment. The beneficial effect of *R. cordifolia* root extract treatment might be due to different types of active principles with diverse range of biological activities [54]. To investigate

Table 2: TPC and TFC of *R. cordifolia*

S. no.	Samples	TPC (µg/mg GAE), mean ± S.D	TFC (µg/mg QE), mean ± S.D
1	MERC	99.732 ± 0.938	96.564 ± 0.996
2	EFRC	207.306 ± 0.730	195.224 ± 0.940
3	BFRC	119.120 ± 0.893	76.848 ± 0.519

GAE: gallic acid equivalent, S.D: standard deviation.

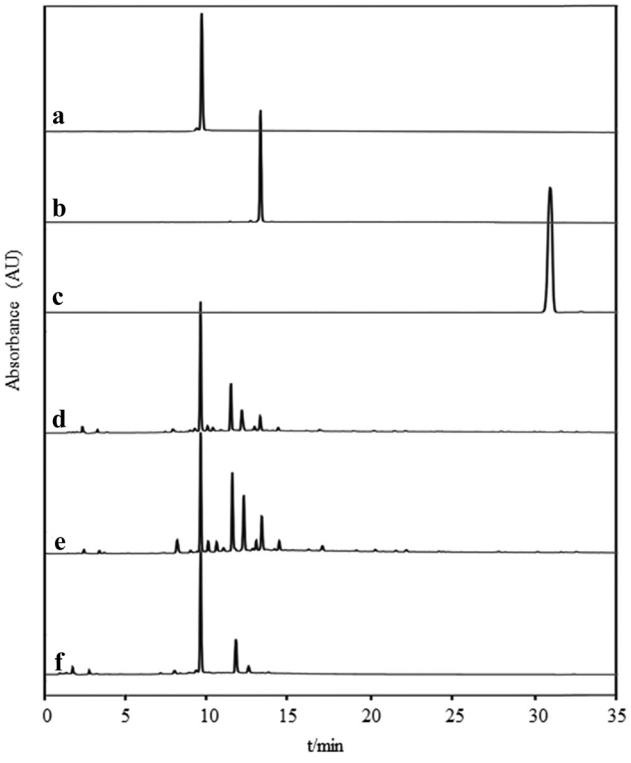

Figure 3: HPLC chromatograms of Standard-1 (Mangiferine) (a). Standard-2 (Purpurine) (b). Standard-3 (Charatine) (c). ME of *R. cordifolia* (d). EF of *R. cordifolia* (e). *n*-Butanol Fraction of *R. cordifolia* (f).

the pharmacological activities of plants extracts, there is systematic approach which best evaluates any biological activity. In present study of analysis, solvent-solvent fractionation was applied to separate the phytoconstituents into respective fractions based on increasing polarity of solvents. The antidiabetic plant (*R. cordifolia*) fractions, i.e., *n*-hexane fraction, ethyl acetate fraction, *n*-butanol fraction, and aqueous fraction of *R. cordifolia* (HFRC, EFRC, BFRC, AFRC), separated the constituents of ME of *R. cordifolia* leaves. Solvent-solvent fractionation technique on the basis of polarity of solvents from the methanolic extract of plants showed strong antioxidant activity by *n*-hexane, ethylacetate, *n*-butanol, and aqueous fractions [55]. Our results about antihyperglycemic effect of fractions of *R. cordifolia* (HFRC, EFRC, BFRC, and AFRC) have shown significant ($P < 0.05$) reduction in blood glucose level (BGL).

The results of *R. cordifolia* fractions against alloxan-induced diabetes show that all the fractions (HFRC, EFRC, BFRC, AFRC) treatments (100 mg/kg) have shown the significant reduction effect on BGL (Figure 1) which was elevated due to Alloxan induction in mice as compared to toxicant control group (T.C-G-II) on time-dependent manner (14, 21, and 28 days). The highest effect in treatment groups (G-V and G-VI) was shown by polar fractions (EFRC and BFRC) on day 28, showing the highly significant reduction of the elevated level of BGL nearly similar to standard antihyperglycemic drug (Glibenclamide 0.5 mg/kg) treatment (G-III). The more significant antidiabetic effect of these fractions to reduce the elevated level of BGL is due to separation of phytochemicals in these solvent fractions as compared to other fractions (HFRC and AFRC) during solvent-solvent partition. The antidiabetic effect of these fractions is also supported by measurement of body weight (BW) of mice during the whole treatment period (Figure 2). The normal BW (31.30 ± 0.860, 32.62 ± 0.92, and 35.30 ± 0.76 g) on 14, 21, and 28th day was decreased (27.10 ± 0.833, 25.32 ± 0.393, and 23.62 ± 0.543 g) on Alloxan induction. On treatment with standard drug (Glibenclamid) and *R. cordifolia* fractions (HFRC, EFRC, BFRC, and AFRC), BW (g) was increased in treatment groups (G-III, G-V, and G-VI) significantly ($P < 0.05$) on day 14, 21, and 28, respectively, as compared to toxicant control group (G-II) and comparable to SD (G-III), but was nonsignificant ($P > 0.05$) in increase in body weight of groups under treatment (G-IV and G-VII). These results showed that the EFRC and BFRC have strong antihyperglycemic effect against alloxan-induced toxicity in mice. The effect of these fractions (EFRC and BFRC) has detoxified the effects of Alloxan-induced toxicity and kept the BW increased in normal way. The fractions (HFRC and AFRC) have less effect on Alloxan-induced toxicity and BW (g) remained decreasing during the treatment.

Another study showed the similar findings by Arika et al. [56] and Karau et al., [57] who reported that *Lippia javanica* and *Pappea capensis* exhibited antihyperglycemic effect. The possible mechanism of antidiabetic activity of *R. cordifolia* is regenerating ability to damaged pancreatic β-cells and stimulated the secretion of insulin from regenerated or remnant beta cells [58]. Our findings about antidiabetic activity of most active fractions of *R. cordifolia* (EFRC and BFRC) are strongly supported by these findings discussed in earlier studies.

Biological activities of medicinal plants are correlated to their antioxidant activity. Therapeutic potential of plants is contributed to their antioxidant activities. DPPH assay is an important tool for the analysis of plant extracts [55]. The results of ME as well as its different fractions (HFRC, EFRC, BFRC, and AFRC) showed effective free radical scavenging effect (% inhibition). The antioxidant scavenging effect of extract samples of *R. cordifolia* was increased with increase in concentration (Table 1). There is highest scavenging activity (%) of EFRC (123.11 ± 0.85) followed by BFRC (120.53 ± 1.36), MERC (119.00 ± 0.87), AFRC (109.10 ± 0.85), and HFRC (106.77 ± 1.66) as indicated in the Table 1. Our results of antioxidant effects of samples (MERC, HFRC, EFRC, BFRC, AFRC) having order of decreasing IC_{50} values of MERC and its different fractions are HFRC > AFRC > MERC > BFRC > EFRC (having IC_{50} values of 48.52, 44.88, 38.19, 36.86, and 34.9 μg/mL), respectively. The IC_{50} results of BFRC and EFRC are very close to IC_{50} value of standard antioxidant AA which is 34.41 μg/mL. Results prove that lower is IC_{50} value; highest is antioxidant activity. The fractions (HFRC, EFRC, BFRC, and AFRC) tested for DPPH assay showed that EFRC and BFRC have the strong antioxidant activity.

These findings showed that the phytoconstituents separated in ethylacetae fractions (EF) and *n*-butanol fraction (BF) of *R. cordifolia* have strong antioxidant activity. These are intermediate polar fractions as compared to aqueous fraction (AF) and *n*-hexane fraction (HF) of these investigated plants. Earlier studies on antioxidant activity of phenolic and flavonoid fractions of *Cleome gynandra* and *Maerua angolensis* showed radical (DPPH) scavenging activity by different fractions (*n*-hexane, dichloroform, acetonitril, ethyle acetate, methanolic, and *n*-butanol fraction) of each plant. The highest activity was shown by *n*-butanol fraction (BF) and ethyl acetate (EF), while lowest activity was exhibited by dichloromethane and *n*-hexane fraction [59]. Similar study about

antioxidant activity by DPPH assay showed the strong antioxidant activity by *n*-butanol (BF) of both plants [60]. Our estimation of *R. cordifolia* fractions by DPPH assay is similar in which results are closely correlated to findings performed earlier [60]. In the study about antioxidant activity of *Conocarpus erectus* L. leaves, *n*-butanol extract (BF) showed the scavenging activity for DPPH which was comparable to antioxidant activity of ascorbic acid [61].

During the investigation on leaves extracts of *Gaultheria procumbens* L., it was concluded that systematic phytochemical evaluation and compounds profiling by different fractions (diethyl ether, ethyl acetate, *n*-butanol, and water fractions) showed the dose-dependent effect of ethyl acetate and *n*-butanol fractions showed highest activities at concentration of 100 µg/mL [62]. These findings of antioxidant activity strongly support our investigations about antioxidant activity of fractions of *R. cordifolia*. *Carissa opeca* leaves were evaluated for antioxidant profile in which chloroform, *n*-hexane, *n*-butanol, ethylacetate, and aqueous extract were investigated by DPPH assay in which all the fractions have strong antioxidant scavenging effect with highest activity of methanolic fraction followed by chloroform fraction, *n*-hexane fraction, aqueous fraction, ethyl acetate, and *n*-butanol fraction [55], which is of little contrast to our results in which order of antioxidant activity is EFRC > BFRC > MERC > AFRC > HFRC by DPPH assay. This might be the nature of phytoconstituents and part of plant used as well as the climatic effect and geographic locality of plants.

Standardized procedure is of significance for the crude extraction of plants to get desired portion for therapeutic purpose and remove the unwanted parts by treating with selective solvent [63]. Biological activities are associated to quality of phytochemicals like phenolic and flavonoids, tannins, terpenoids, saponin, and alkaloids. Phytochemicals like alkaloids, phenols, flavonoids, tannins, saponins, and terpenoids are very valuable and they play important physiological actions in the body being used for treatment of diseases [63]. Previously, screening of biologically active compounds like glycosides, anthraquinones, saponins, steroids, flavonoids, and phenols from various solvent extracts of root, stem, and leaf in *R. cordifolia* was performed by Pendli et al. [63]. Screening of similar phytochemicals also in our investigation showed that ME, ethyl acetate, and *n*-butanol have highest potential to separate the required biologically important constituents from medicinal plants. These phytochemicals have strong antibacterial, anti-inflammatory, antidiarrheal, and anti-cancerous activities [64,65]. Flavonoids are important for their antidiabetic activity along with alkaloids [66]. The results of *R. cordifolia* revealed that this plant with ME and its fractions (EFRC and BFRC) are important for the preparation of drugs to treat the different diseases including the diabetes.

The important phytochemicals of plants are phenolic and flavonoids which play important role in pharmaceutical development. The quantitative analysis of polyphenolic compounds is essential to know about their total content in the respective part of plants. Our results about TPC (µg/mg GAE) of *R. cordifolia* ME and its most active fractions (EFRC, and BFRC) showed 99.732 ± 0.938, 207.306 ± 0.730, and 119.120 ± 0.893 (µg/mg GAE) (Table 2). This showed that highest TPC (µg/mg GAE) were present in EFRC (207.306 ± 0.730) followed by BFRC (119.120 ± 0.893 µg/mg GAE) and MERC (99.732 ± 0.938 µg/mg GAE) in *R. cordifolia* (Table 2). The order of presence of TPC in *R. cordifolia* is EFRC > BFRC > MERC. The highest content in EFRC and BFRC showed that phenolic compounds are more separated in ethyl acetate fraction (EF) and *n*-butanol fraction (BF) of *R. cordifolia*.

Results of TFC (µg/mg QE) of *R. cordifolia* (MERC, EFRC, and BFRC) showed 96.564 ± 0.996, 195.224 ± 0.940, and 76.848 ± 0.519 (µg/mg QE), respectively (Table 2). There were highest TFC (µg/mg QE) in EFRC (195.224 ± 0.940) followed by MERC (96.564 ± 0.996) and BFRC (76.848 ± 0.519) of *R. cordifolia*, showing that flavonoids are more separated in ethyl acetate solvent during the fractionation of methanolic extract. The variations in TPC of same plant in different solvents may be due to the nature of solvents' polarities and also the nature of phenolic compounds. Different plants have different phytochemicals present, and in our studied plant, the contents are different in different solvents [50]. In another study, same method of calculation of TPC and TFC was performed by Arya et al. [21] in vegetables from Nepal (*Basella alba, Cassia tora, Alternanthera sessilis, Digera muricata, Leucas cephalotes, Portulaca oleracea, Ipomoea aquatic,* and *Solanum nigrum*) in methanolic extract in which *A. sessilis* had highest phenolic contents with smallest in *B. alba,* but flavonoids were high in *P. oleracea* while the least was recorded for *I. aquatica* which showed that there was higher contents of these phytochemicals as compared to methanol extract for *R. cordifolia* in our investigation. This difference is quiet representation that geographical regions, type of plants, and extraction methods have effect on plants' secondary metabolites contents. The solvents and extraction procedure are important factor for dissolving the plant compounds [67]. The plant has valuable compounds in polar solvent extract than nonpolar solvents due to hydroxyl group, so the presence of TPC and TFC in ME of *R. cordifolia* is due to its polarity and partition of these compounds on

their polarity basis having different extractive capacities [68].

Identification of phytochemicals is an important parameter for knowing the exact role of plants metabolites with biological activities. Pure compounds have more prominent biological activities than crude extract or complex compounds when they are not separated. In our results about analysis of polyphenolic compounds from *R. cordifolia*, HPLC tests confirmed the presence of mangiferine (Figure 3a) in all samples of *R. cordifolia* (MERC, EFRC, and BFRC, i.e., Figure 3d–f), respectively, while purpurine (Figure 3b) is present in methanol extract and ethyl acetate fraction of *R. cordifolia* only (Figure 3d and e). Phytochemicals like carbohydrate, alkaloids, amino acids, saponin, glycosides, phenolic compound, and tannins were found as major constituents in *R. cordifolia* Linn. Root, which play important role for biological activities like antitoxin, antiseptic, anti-mutagenic, anti-carcinogenic, antidiabetic, and antioxidant agents [69]. Physiochemical characterization of any drug is important before any pharmaceutical activity. Chemical characterization of *R. cordifolia* showed that it has proteins, glycosides, phenols, flavonoids, and tannins in an earlier investigation [70]. According to Kumar et al. [71], Purpurin is also present in traditionally used plants in Himalayan region and these plants are used for treating varieties of diseases. The oral administration of aqueous root extract (1 g/kg/day for 8 weeks) of *R. cordifolia* showed antihyperglycemic effect in mice due to presence of cordifoliol, cordifodiol, rubiacordone, purpurin, and alizarin compounds [72,54]. In our investigation, purpurin is identified from ethyl acetate fraction and methanol extract of *R. cordifolia*, showing the evidence that antihyperglycemic effect is due to chemical compound (purpurin) in association with other phenolic and flavonoids. Naturally, mangiferin is predominant in *Mangifera indica*. Daily oral administration of 400 mg/kg *M. indica* extract in laboratory animals resulted in decrease of serum glucose by some 60 mg/dL compared to control in 15 days [73]. In our investigation about identification of compounds, it is evident that mangiferin identification in both the fractions *R. cordifolia* (EFRC and BFRC) and its ME exerted the antihyperglycemic effect by 100 mg/kg orally administered once daily in mice up to 28 days' treatment after Alloxan-induced diabetes in mice for 6 days. *R. cordifolia* is traditionally used as an anti-inflammatory, antiseptic, and galactopurifier, and during the investigation of its anti-cancerous activities, it was proved that its methanolic extract has strong anticancer effect [74]. Kaempferol, mangiferine, purpurine, and anthraquinones are commercially available compounds having antidiabetic potential. These are used as standards for detecting the compounds in methanolic extract, ethyl acetate, and *n*-butanol fractions of *Lespedeaza cuenetta* plant, which is traditionally used for the treatment of type-2 diabetic. Antihyperglycemic effect of our investigated plants is associated to synergistic effect of phytochemicals such as phenolic and flavonoids present in *R. cordifolia*.

5 Conclusion

The active antidiabetic plant (*R. cordifolia*) in our investigation showed that the solvents fractions (ethylacetate fraction and *n*-butanol fraction) have significant antidiabetic effect on Alloxan-induced diabetic mice. This effect is due to the antioxidant activity of these solvents fractions that have been demonstrated by DPPH assay. The antidiabetic activity of *R. cordifolia* is associated to antioxidant activity and phytochemical contents like phenolic and flavonoids, specifically the mangiferine and purpurine in *R. cordifolia* as identified by HPLC profiling (Figure 3d–f), respectively. The other phytochemicals such as tannins, terpenoids, alkaloids, and saponins may have synergistic effect against alloxan-induced diabetes in mice. Furthermore, the results obtained from this study confirmed that *R. cordifolia* have antidiabetic and antioxidant activities which support or justify the reported folkloric use of *R. cordifolia* to treat the diseases of diabetes in northern region of AJ&K, Pakistan. In brief conclusion from our investigation, we say that *R. cordifolia* has the most active antihyperglycemic effect in alloxan-induced (100 mg/kg) mice due to synergistic effect of phytochemicals like purpurin and mangiferin present in its fractions and methanolic extract.

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Data availability statement: Data is available and is the property of main investigator and can be provided on demand/request.

Informed consent: There is no informed consent

Abbreviations

DM	diabetes mellitus
NC	normal control group
RC	*Rubia cordifolia*
MERC	methanolic extract *Rubia cordifolia*
EFRC	ethyl acetate fraction *Rubia cordifolia*
BFRC	*n*-butanol fraction *Rubia cordifolia*
DPPH	2,2 diphenyl-1-picryl-hydrazyl
TPC	total phenolic content
TFC	total flavonoid content

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