ISOLATION, STRUCTURE ELUCIDATION & ANTIDIABETIC POTENTIAL OF *Rosa Brunonii L.* FRUIT – FIGHT DIABETES WITH NATURAL REMEDIES

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ABSTRACT

Diabetes mellitus (DM) is a metabolic disorder that is marked by high blood glucose levels. Members of the Rosaceae family are a good source of antioxidants. Therefore, the current work sought to examine the potential for in-vitro alpha-amylase and alpha-glucosidase inhibition and the antidiabetic activity of *Rosa brunonii L.* fruit chloroform extract (RBFCE) against Alloxan (ALXN) induced diabetes in rats. RBFCE concentrations ranging from 20, 50, 100, 250, 500, and 750 g/mL were used in in-vitro activities, while oral doses of 500 mg/kg, 750 mg/kg, and 1000 mg/kg were given to rats in an in-vivo trial for 21 days. Isolation was carried out through column chromatography and modern spectroscopic techniques were used for characterization and structure elucidation. The isolated compound was identified as catechin. For alpha-amylase and alpha-glucosidase inhibition activity, RBFCE had IC_{50} values of 322.06±17.40 and 248.93±1.62, respectively. The IC_{50} values for acarbose against alpha-amylase and alpha-glucosidase inhibition were 64.64±3.70 and 67.60±4.20, respectively. RBFCE treatment regulated blood glucose levels dose-dependently over a 21-day study period. Histopathological studies revealed that RBFCE has recovered the damaged acinar structures to some extent in pancreatic tissue. Only focal tissue destruction observed. RBFCE treatment displayed normal glomeruli with no signs of inflammation, proliferation, necrosis, cipher of thyroidization and fibrosis. All the extract-treated groups had more protected pancreatic and kidney tissue than control group in dose-dependent manner. The current study results revealed that RBFCE had prominent alpha-amylase and alpha-glucosidase inhibition activity, regulates blood glucose level and normalize histopathological markers in diabetic rats compared to the negative control group.

Keywords: Anti-diabetic activity, alpha-amylase inhibition activity, alpha-glucosidase inhibition activity, *Rosa brunonii* L. fruit, Catechin.

1. INTRODUCTION

Diabetes mellitus (DM) is the most prevalent endocrine disorder affected around (463 million people) 9.3% of the world population in 2019 [1]. In Pakistan, around 11.7% population is living with DM [2]. It is a non-curable hormonal disorder that badly affects the quality of life and increases the risk of mortality [3],[1]. It is a chronic, fatal metabolic disorder that lowers life expectancy and increases mortality risk [3] and have two types, Type-1 (insulin-dependent) and Type-2 (non-insulin-dependent). Most diagnosed cases of diabetes (90–95%) are Type-2 Diabetes Mellitus (T2DM). It is a significant cause of mortality and morbidity and is characterized by abnormal lipid, lipoprotein, and carbohydrate metabolism that results in hyperglycemia [4-6]. In contrast to T2DM, which can be treated without insulin by using only medication, type-1 diabetes can only be treated with insulin injections. The development of T2DM is significantly influenced by oxidative stress [7]. Lipid peroxidation, an excess of free radicals, and a lack of antioxidant protection have all been linked to the development of T2DM [8, 9]. Antioxidants may aid in lowering the risk of developing T2DM [10]. Alkaloids, triterpenoids, peptides, iridoids, glycopeptides, alkyl disulfides, polysaccharides, coumarins, and derivatives of aminobutyric acid all have hypoglycemic effects. Different mechanisms, including the stimulation of insulin secretion, the binding of insulin to receptors, the reduction of insulin resistance, and an increase in glucose consumption followed by different allopathic drugs and herbal remedies to lower blood glucose levels.

Herbal medications are increasingly being used to treat *DM* and its complications worldwide. Controlling postprandial hyperglycemia is critical in the treatment of T2DM. Several synthetic oral hypoglycemic drugs with potential adverse effects are being used to control raised blood glucose levels around the globe. Finding new, more effective, and safer alternatives has become a top priority in the modern era due to potential side effects of the hypoglycemic agents currently in use. *Rosaceae* plants, which include trees, herbs, and shrubs, are well known for their significant medicinal and cosmetic importance due to their strong anti-oxidant potential. Members of the *Rosaceae* family have been used for a very long time to treat numerous comorbidities all over the world. *Rosa brunonii* L., also referred to as the "Himalayan musk rose," is an important member of the *Rosaceae* family and can be found in the western Himalayas. *Rosa brunonii* L. is a plant that is high in antioxidants and may be essential for managing oxidative stress-related type 2 diabetes as well as protecting the gastric mucosa [11]. The plant has a potent antioxidant capacity that may also squelch reactive oxygen species (ROS), which is crucial for cell dysfunction and insulin resistance leading to T2DM [12].

Keeping in view the previous research work on chemical content and folk use of *Rosa brunonii* L. fruit, *in-vitro* bioactivities must be carried out in order to study the inhibition of alpha amylase, alpha glucosidase enzymes. Moreover, it is also needed to conduct an *in-vivo* anti-diabetic activity using *Wistar albino* rats followed by isolation and structure elucidation of the isolated compound.

2. MATERIALS AND METHODS

2.1. Materials

Potato starch, α-amylase enzyme, Sodium potassium tartrate, 3, 5 di-nitro-salicylic acid, α-glucosidase enzyme, p-nitrophenyl-α-glucopyranoside (pNPG), Dimethyl sulphoxide (DMSO), GBM (lot no. G00100, 99.7%, Sanofi), CMC (lot no. # 38591180306, India), ALXN, silica gel, chloroform, n-hexane, ethyl acetate, methanol, n-butanol (Sigma Aldrich).

2.2. Equipment and apparatus

Weighing Balance Sartorius (TE214S), pH meter (Thermo / Orion 3), UV-Vis Spectrophotometer UV-2450 (Shimadzu, Japan), Routine chemistry analyzer AU480 Beckman Coulter USA, Glass chamber (25 x 12 x 25 cm), Cage, ACCU CHEK Glucometer (China), Gastric gavage, Centrifugation machine, Eppendorf glass tubes (edentate & non edentate), Syringe, Measuring cylinder, Funnel, Filtration assembly, Beaker, Nylon filter 0.25µm, Mortar and pestle, Incubator, glass column.

2.3. Ethical approval

The Animal Ethical Committee, College of Pharmacy, University of Punjab (AEC/PUCP/1077), developed by National Institute of Health, authorized the research protocol for animal data.

2.4. Animals

The present investigation employed *Wistar albino* rats (184–210 grams) from the local animal house facility in polypropylene cages with rice husk bedding.

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Animals were housed at 21–23°C and 60–70% relative humidity in a 12-hour light–dark cycle. They were given a normal feed and water *ad libitum* for a week before the study.

2.5. Collection of plant material

Rosa brunonii L. fruits were collected from Murree hills Punjab, Pakistan. After identification, a voucher specimen was deposited in the herbarium of Government College University Lahore for *Rosa brunonii* L. (GC.Herb.Bot.3316) for future reference.

2.6. Preparation of plant extract

Fruits from the *Rosa brunonii* L. plant were harvested, rinsed with distilled water, dried in the shade, and ground into powder using a machine. The crude extract was obtained by macerating 5 kg of powdered *Rosa brunonii* L. fruits in 20 L of methanol. The filtered liquid extract was weighed and stored in an airtight container after concentration in a rotary evaporator (Laborota 4000-efficient, Heidolph Germany) at 40°C. The methanolic fraction was separated into the n-hexane, chloroform, ethyl acetate, n-butanol, and aqueous fractions. A rotary evaporator was used for drying and weighing each fraction.

2.7. Isolation and purification of compound

Chloroform ($CHCL_3$) fraction of *Rosa brunonii*. L fruits were selected for isolation of secondary metabolites which was then purified by column chromatography. For this purpose, silica gel (60 mesh) was dissolved in n-hexane to form slurry and loaded into the column. The column was packed carefully by minimizing the bubble interruption. Sample of $CHCl_3$ fraction of *Rosa brunonii*. L fruits weighing about 187 g was carefully loaded onto the column packed with silica gel. Separation was started with non-polar solvent, n-hexane as an eluent. Polarity of the eluent was raised by mixing 10% $CHCL_3$ at one time and eluted one liter for one combination up to 100% $CHCL_3$. In order to further raise the polarity of the eluent, ethyl acetate was added 10% at each step. Thin layer chromatography (TLC) was continuously carried out after every 10% rise in polarity. Fractions showing same Rf values were combined and at the end (1-6), (11-16) and (19-25) fractions were obtained. On the basis of TLC, fraction 3-5 were mixed and loaded onto the smaller column. No, single spot compound was identified. The mixed sample was subjected to HPLC analysis. Similarly, TLC of fraction 12-15 showed two UV active spots, which were also combined and loaded again onto the Sephadex LH-20 column for purification purpose. Ethyl acetate was used as an eluent whose polarity was subsequently increased by mixing 20% CH_3OH at each step. At combination of 10% ethyl acetate and 90% methanol, TLC showed single UV active spot and then 2 liter of eluent was passed from the column to purify the isolated compound. The purity of isolated compound was checked by TLC at 2 different ratios of mobile phase: ethyl acetate, $CHCl_3$, methanol and water (8:3:3:1) and (8:5:4:1) to obtain single spot of the isolated compound. The melting range of the purified secondary metabolite was noted and characterized by modern spectroscopic techniques like IR and NMR.

2.8. Assessment of alpha-amylase enzyme inhibition activity

The alpha-amylase enzyme inhibition study was carried out by using reported protocol after slight modifications [13]. Briefly, stirred 1.0 g of potato starch in 0.02 M sodium phosphate buffer (pH = 6.9) to yield 1.0 percent w/v starch solution. Added 50 mg of α-amylase enzyme in 100 mL of 0.02 M sodium phosphate buffer (pH =6.9) to make the enzyme solution (0.5 mg/mL). Colorimetric reagent was prepared by mixing sodium potassium tartrate and 3, 5 di-nitro-salicylic acid (96 mM) solution. Different concentrations of RBFCE ranging from 20, 50, 100, 250, 500, and 750 µg/mL were prepared from stock solution. Mixed the 250 µL each of extract and enzyme solutions and pre-incubated at 25℃ for 10 min followed by addition of 250 µL of 1.0% starch solution. The reaction mixture was incubated at 25℃ for 10 min. To stop the reaction, added 1 mL of the colorimetric reagent, and the mixture was incubated in a boiling water bath for 5 min. The mixture was cooled to room temperature and 10 mL of purified water was added to dilute the reaction mixture. Absorbance of the solution was measured at 540 nm with acarbose as a positive control. A control solution (blank) was prepared in a similar way by replacing plant extract with DMSO and representing 100% enzyme activity [14]. The experiment was repeated five times and results were reported as ± S.E.M and IC_{50} values were calculated.

2.9. Assessment of alpha-glucosidase enzyme inhibition activity

The inhibition potential of RBFCE against of alpha-glucosidase enzyme was calculated using the slightly modified method [15, 16]. The reaction mixture contained 2.9 mM pNPG, different concentrations (100, 250, 500, 750, and 1000 µg/mL) of RBFCE individually and 1.0 U / ml of α-glucosidase enzyme in sodium phosphate buffer with pH 6.9. Control tubes contained only DMSO, enzyme, and substrate. While acarbose replaced the sample solution in the positive control. Mixture without enzyme, sample solution and acarbose used as blank. Acarbose, a standard α-glucosidase enzyme inhibitor served as the positive control. The reaction mixtures were incubated for 5 min at 25°C, followed by boiling for 2 min to stop the reaction. The absorbance of the resulting pNP solution was noted at 405 nm using a UV-Vis spectrophotometer. The experiment was repeated five times and results were reported as ± S.E.M and IC_{50} values were calculated.

2.10. In-vivo antidiabetic activity

Animals were divided into six groups (n = 6) and injected intravenously with 120 mg/kg of ALXN to induce diabetes. After 48-72 h of ALXN injection, the animals with a fasting blood glucose level of more than 240 mg/dL were considered diabetic and included in the study [17]. Animals from the normal control and diabetic (negative) control **received a** standard pelleted diet and water. Diabetic animals of the positive control group received GBM (10 mg/kg) orally for 21 days [18]. However, diabetic animals from low, medium, and high dose groups were orally given 500, 750, and 1000 mg/kg of RBFCE, respectively for 21 days.

2.11. Physical monitoring and blood collection

The general condition of rats, such as locomotor activity, food intake, water consumption, and urine output, was observed throughout the study period. Blood samples were drawn from the overnight fasted rats by rupturing the tail vein to check glucose level on days 1, 7, 14, and 21. The glucose level in the blood samples checked using one-touch glucometer and commercially available glucose test strips. Animal weight variation was calculated by subtracting the weight on the first day from the weight at the end of the study period [19]. On the 21st day, all animals were anesthetized with an overdose of 1% chloralose (5ml/kg) in 25% urethane (w/v) and sacrificed to collect samples of kidney and pancreas. Tissues were immediately excised, perfused with ice-cold saline and kept in 10% buffered formalin for histological processing.

2.12. Histopathological investigations

Histopathological examinations were performed on all of the frozen kidney and pancreas tissue samples. Sections of tissue (5 m thick) were stained with hematoxylin and eosin (HE) dye. A skilled pathologist using a deca-head microscope identified pathological alterations and scored for histopathology.

2.13. Statistical analysis

The significance of differences between groups was determined using a one-way analysis of variance (ANOVA), and data were presented as the mean SEM. Means of *in-vitro* bioactivities were compared across groups using the post hoc Tuckey's multiple comparison test. The cutoff for significance was a p value of less than 0.05. Data analysis was performed using GraphPad Prism® (GraphPad Software, Version 6.01 for Windows).

3. RESULTS

3.1. Characterization and structure elucidation of isolated compound

Characterization of the isolated compound was carried out by spectral studies including IR, NMR and mass spectroscopy and melting range. The published data was examined and matched with reported compound catechin. The observations regarding compound under study are as follows (**Table 1**).

Source	*Rosa brunonii* L. fruit
State	Yellowish powder
Yield	23 mg
Molecular Weight	290.10
Molecular Formula	$C_{15}H_{14}O_6$
Melting Point	176 -179 ℃

Proton and C-13 NMR of the isolated compound was carried out using Bruker instrument as shown in table 2. IR spectrum indicated peaks at wave numbers 3000 cm-1 indicates C-H bond vibrations. Similarly, the mass spectrum showed maximum at 290.3 and minimum at 122.4. The other fragments were seen at 138.4, 146.4, 164.3, 180.3, 206.2, 248.1 and 272.2. The molecular mass corresponding to 290 g/mol was observed, which also confirmed the isolated compound as catechin (**Figure 1**). Melting range of the isolated compound was found to be 176 - 179° C which confirmed that compound under study was catechin as it has melting point 175°C.

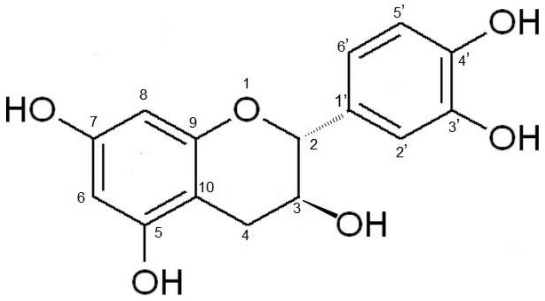

Figure 1: Structure of catechin.

Table 2: C_{13} and H1 NMR data of isolated compound.

C.No	Multiplicity	^{13}C-NMR (δ)		^{1}H-NMR (δ)	
	O	**Experimental**	**Reported** [20]	**Experimental**	**Reported** [20]
C-2	CH	66.09	66.3	4.83, d [*J* = 8.0 Hz]	4.56, d [*J* = 7.8 Hz]
C-3	CH	78.48	80.90	4.19, m	3.97, m
C-4	CH2	27.88	27.7	α = 2.74, dd [*J* = 4.5, 16.5 Hz] β = 2.88, dd [*J* = 3.0, 16.5 Hz]	α = 2.49, dd [*J*=8.6, 16.0 Hz] β = 2.82, dd [*J*=1.6, 16.0 Hz]
C-5	C	156.61	156.1		
C-6	CH	94.51	93.9	5.96, d [*J* = 2.5 Hz]	5.86, d [*J* = 2.1 Hz]
C-7	C	156.26	156.4		
C-8	CH	95.01	95.1	5.94, d [*J* = 2.5 Hz]	5.92, d [*J* = 2.1 Hz]
C-9	C	155.98	155.3		
C-10	C	130.90	130.6		
C-1’	C	118.02	118.4		
C-2’	CH	98.69	99.1	6.99, d [*J* = 2.0 Hz]	6.83, d [*J* = 1.8 Hz]
C-3’	C	144.54	144.6		
C-4’	C	144.38	144.8		
C-5’	CH	113.93	115.1	6.78, d [= 8.0 Hz]	6.75, d [*J* = 8.1 Hz]
C-6’	CH	114.52	114.5	6.82, dd [*J* = 2.0, 8.0 Hz]	6.70, dd [*J* = 8.1, 1.8 Hz]

3.2. Alpha-amylase enzyme inhibition activity

Table 3 demonstrates the percentage inhibitory action of RBFCE against alpha-amylase in comparison to the acarbose (positive control) at different concentrations ranging from 20, 50, 100, 250, 500, and 750 µg/mL. The standard drug, acarbose showed the highest alpha-amylase inhibition (91.84±1.74%) followed by RBFCE (64.85±1.92%) at the highest concentration tested (750 µg/mL).

Table 3: Alpha-amylase inhibition activity of RBFCE

Sr. No	Concentration (µg/mL)	Inhibition potential of acarbose	Inhibition potential of RBFCE
1	20	32.95±4.50	11.30±2.57
2	50	47.25±1.50	22.20±1.33
3	100	61.50±1.90	31.70±3.26
4	250	77.54±2.52	44.10±3.90
5	500	83.84±2.38	59.10±2.92
6	750	91.84±1.74	64.85±1.92
7	IC_{50}	64.64±3.70	322.06±17.40

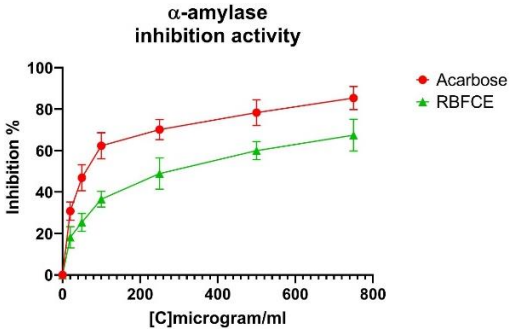

Figure 2: Alpha-amylase inhibition potential of RBFCE.

A dose-dependent inhibitory potential of RBFCE was observed against alpha-amylase enzyme with an IC50 value of 322.64± 17.40 µg/mL, respectively. The low IC50 value demonstrated by RBFCE indicated a higher alpha-amylase enzyme inhibition potential. Acarbose showed an IC50 value of 64.64±3.70 µg/mL, as demonstrated in **Figure 2**.

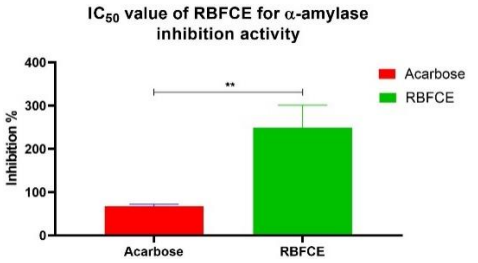

Figure 3: IC_{50} calculations of acarbose and RBFCE

3.3. Alpha-glucosidase enzyme inhibition

Table 3 shows the percent inhibitory potential of RBFCE against alpha-glucosidase enzyme at various concentrations in comparison to the acarbose (standard drug).

Table 3: Alpha-glucosidase inhibition activity of RBFCE

Sr. No	Concentration (µg/mL)	Acarbose (%) inhibition	RBFCE (%) inhibition
1	20	30.82±1.98	18.20±2.30
2	50	46.90±2.80	25.40±1.94
3	100	62.31±2.83	36.57±1.70
4	250	70.13±2.16	48.92±3.37
5	500	78.34±2.80	59.98±1.96
6	750	85.40±2.50	67.44±2.43
	IC_{50}	67.60±4.20	248.93±1.62

The RBFCE revealed a significant inhibitory activity of alpha-glucosidase enzyme at concentration of 750 µg/mL (67.44±2.43%) in comparison to the positive control (85.40±2.50%) at the same concentration.

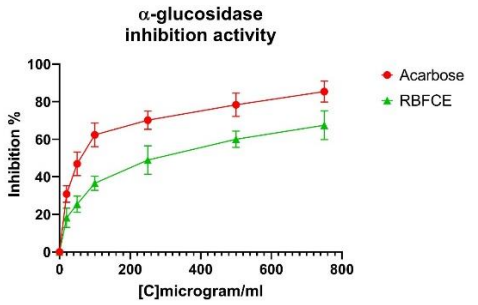

Figure 4: Alpha-glucosidase inhibition activity of RBFCE

RBFCE exhibited IC_{50} values of 248.93±1.62 µg/mL as shown in Figure 4. There was significant difference in the IC_{50} values of acarbose in comparison to RBFCE ($p < 0.05$). Lower IC50 value indicates that small dose is required to inhibit the alpha-glucosidase enzyme at the given concentration. Moreover, better alpha-glucosidase inhibition potential of RBFCE also supports its folk use in treatment of DM.

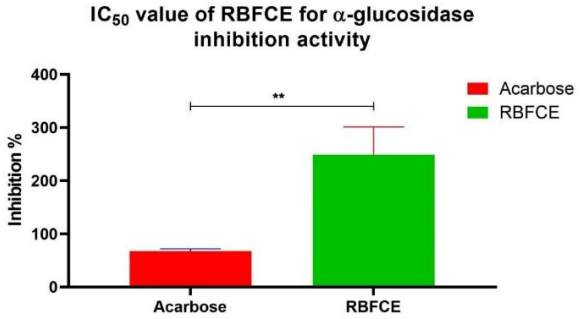

Figure 5: IC_{50} calculations of RBFCE

3.4. In-vivo antidiabetic activity

The current study revealed the regulation of blood glucose level at the low, medium, and high doses of RBFCE in dose-dependent manner. The parameters focused for antidiabetic potential were alteration in body weight and blood glucose level in all the groups. No significant difference in body weights of control and RBFCE treated groups were noted. Similarly, no psychological and physical changes were observed in the rats of high dose RBFCE treated group compared to normal control. Whereas, the rats of low, medium and diabetic control group showed the signs of diabetes such as a change in their behavior, excessive water consumption, high urination, less food intake, wet body fur, and reduced body weight. Except the normal control, animals of all groups showed signs of diabetes during the 1st week of the study period and tend to become normal with the passage of time. Overall, no significant change was observed during the 2nd and 3rd week of the study period except that one animal died in each group; i.e., in diabetic control group and 500 mg/kg RBFCE treated group on 06th and 09th day of study respectively. Blood glucose level was found to be increased by 248% (Mean = 266, $p < 0.001$) in negative (diabetic) control group compared to the normal group (Mean = 107) on 21st day of study. Negative value indicates reduction in glucose level. All extract treated groups were compared to the negative control. Alterations in glucose level with 500 mg/kg RBFCE, 750 mg/kg and 1000 mg/kg treated rats were found to be – 19% (Mean = 246, $p > 0.05$), – 21.43% (Mean = 231, $p > 0.05$) and – 27.21% (Mean = 206, $p < 0.05$) respectively as shown in **Table 3**.

Table 4. Assessment of antidiabetic potential of RBFCE in 21-day study period.

Group	Dose	Glucose regulation on 1st day	Glucose regulation on 7th day	Glucose regulation on 14th day	Glucose regulation on 21st day	% glucose alteration on 21st day
Normal control	2 ml water	116±5.1	111±5.6	123±3.8	107±5.9	----
Positive control	Diabetic + GBM	296±16.1	140±12.7	146±13.6	113±7.5	-61.83%***
Negative control	60 mg/kg STZ (Single dose)	343±17.5	312±18.9	296±16.6	266±18.4	248.5%†††
500 mg/kg RBFCE	Diabetic + 500 mg/kg	307±13.6	291±11.9	271±19.3	246±15.5	-19.1%
750 mg/kg RBFCE	Diabetic + 750 mg/kg	294±9.2	266±19.2	250±22.6	231±12.1	-21.43%
1000 mg/kg RBFCE	Diabetic + 1000 mg/kg	283±15.0	270±13.3	241±10.4	206±23.6	-27.21%*

† indicates comparison with normal control group. * indicates comparison with diabetic control group. * shows $p < 0.05$, ** represents $p < 0.01$ and ***, ††† represents $p < 0.001$.

Table 4 shows the alteration in blood biochemical parameters of Wistar albino rats among normal, negative, and positive controls, low, medium and high dose RBFCE groups in 21-day study period.

Table 5. Sub acute study showing effect of low, medium, and high dose of RBFCE on biochemical parameters of diabetic rats. Mean values are expressed with standard error of means (±S.E.M.)

Group	Dose	Glucose regulation on 1st day	Glucose regulation on 7th day	Glucose regulation on 14th day	Glucose regulation on 21st day	% glucose alteration on 21st day
Normal control	2 ml water	116±5.1	111±5.6	123±3.8	107±5.9	----
Positive control	Diabetic + GBM	296±16.1	140±12.7	146±13.6	113±7.5	-61.83%***
Negative control	60 mg/kg STZ (Single dose)	343±17.5	312±18.9	296±16.6	266±18.4	248.5%†††
500 mg/kg RBFCE	Diabetic + 500 mg/kg	307±13.6	291±11.9	271±19.3	246±15.5	-19.1%
750 mg/kg RBFCE	Diabetic + 750 mg/kg	294±9.2	266±19.2	250±22.6	231±12.1	-21.43%
1000 mg/kg RBFCE	Diabetic + 1000 mg/kg	283±15.0	270±13.3	241±10.4	206±23.6	-27.21%*

3.5. Histopathological investigations

Histopathological observations of the pancreas showed lobulated architecture composed of acini and few ducts in normal tissue. Diabetic pancreatic tissue showed destructed acinar structures with a reduced number and size of islets of Langerhans. Low dose RBFCE has recovered the damaged acinar structures to some extent in pancreatic tissue. Serous acinar structures containing moderate eosinophilic cytoplasm and basal nuclei have been observed in medium and high dose groups (**Figure 6**).

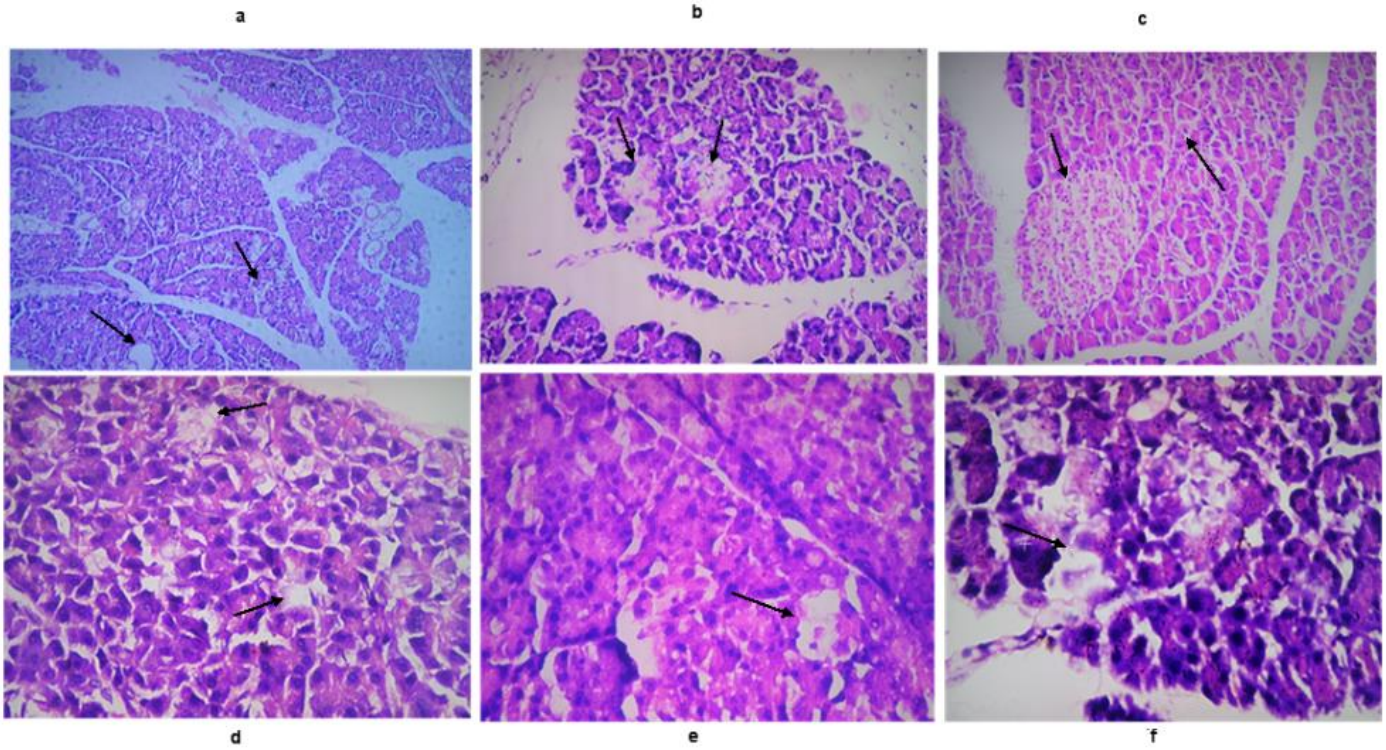

Figure 6: Effect of RBFCE on histopathology of pancreas. Photomicrographs of stained sections of (**a**) Normal pancreas showing lobulated architecture composed of acini with ducts (**b**) Diabetic (negative control) pancreas showing focal destruction of acinar structure (**c**) GBM treated (positive control) showing lobulated architecture with recovered islets (**d**)Low dose RBFCE treated group showing focal destruction of lobulated architecture (**e**) Medium dose RBFCE group showing focal tissue destruction and rest tissue with normal acini (**f**) High dose RBFCE group showing serous acinar structures containing moderate eosinophilic cytoplasm and basal nuclei.

On the other hand, focal tubular atrophy and oedema can be appreciated in the kidney tissue of the low dose RBFCE treated group. However, a medium dose treated group displayed normal glomeruli with no signs of inflammation, proliferation, and necrosis. Normal tubules and glomeruli with no signs of inflammation, cipher of thyroidization and fibrosis can be seen in high dose treated group (**Figure 7**).

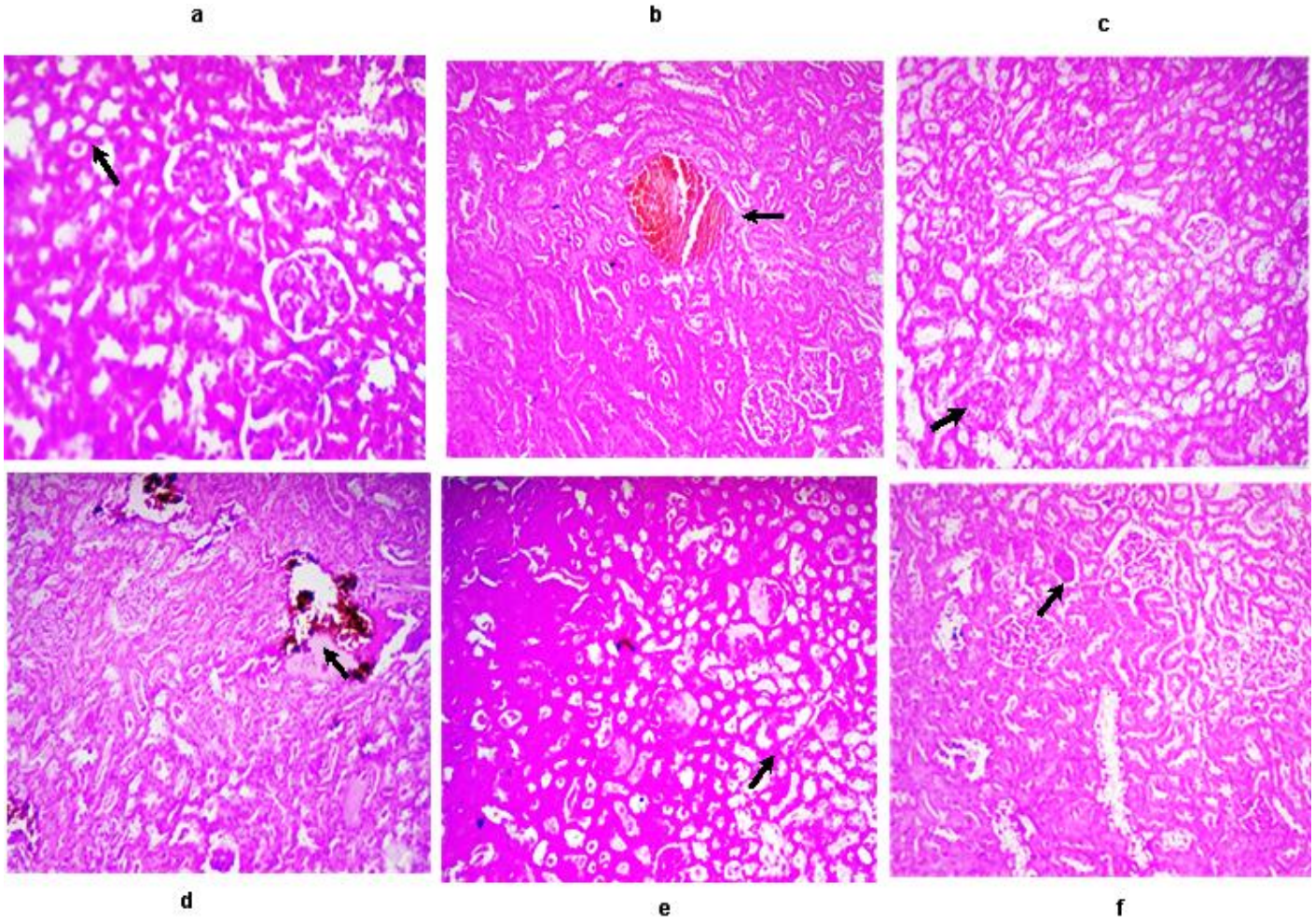

Figure 7: Effect of RBFCE on histopathology of kidneys. Photomicrographs of stained sections of (**a**) Normal kidneys with ordinary glomerular structure (**b**) Diabetic (negative control) kidneys showing destructed glomerular tubules with secretions in Bowman space (**c**) GBM treated (positive control) showing protected glomerular tubules **(d)** Low dose RBFCE treated group showing distorted trabecular structure (**e**) Medium dose RBFCE group showing normal tubules with focal thyroidization (**f**) High dose RBFCE group showing normal glomerular architecture with no inflammation and malignancy.

DISCUSSION

DM is an eminent metabolic disease marked by high blood glucose levels. The decrease in insulin production by the pancreas, or the cells' reduced responsiveness to the generated insulin, is the cause of this disorder [21]. T2DM is a chronic metabolic disorder that stimulates production of ROS because of autoxidation of sugars. Production of ROS damages the cellular DNA, proteins and lipids, leading to the progression of late complications of T2DM [22]. It is estimated that approximately 90 to 95% of all the diabetes diagnosed cases are of T2DM. It is the major source of mortality and morbidity described by defective carbohydrate, lipoproteins and lipid metabolism leading to hyperglycemia [4-6]. Diabetic patients prefer to rely on complementary and alternative medicine systems as they are considered to be devoid of any side effects, economic and cheaper than the allopathic drugs [23]. Different surveys have shown that up to 71% of diabetic individuals use herbal remedies around the globe [24-27]. Plant materials (fruits, herbs, spices, vegetables) considered a rich source of antioxidants [28] which play vital role in regulation of T2DM [29].

Plants have been used as an alternative treatment for various physiopathologic disorders, including *DM*, in many developed, and developing countries for a long time. To date, alpha-amylase inhibition by a number of plants from family *Rosaceae including Sarcopotarium spinosum* L., *Rubus caesius* L., and *Eriobotrya japonica* were reported [30]. Researchers are currently focusing their efforts on the medicinal plants because they are known to be beneficial, readily available, and have fewer adverse effects. Till date, there are no literature reports showing alpha-amylase inhibition potential of *Rosa brunonii* L. fruit. In the present research work, inhibitory potential of RBFCE against alpha-amylase, alpha-glucosidase enzymes by *in-vitro* study followed by *in-vivo* antidiabetic potential of RBFCE was investigated parallel to isolation and structure elucidation of secondary metabolite.

In the current research work, proton NMR data of the isolated compound showing nine protons out of which 5 are in aromatic region. The coupling pattern shows that two of the protons appeared with meta coupling while the three other separately displaying 1,4,5 coupling pattern i.e. one proton with meta coupling constants, other one showing ortho coupling constants and the third one appeared as double doublet with two coupling constants. The other four protons distinctly matched with flavan-3-ol. One downfield proton around 4.8 appeared due to H-2 proton, H-3 appeared at 4.19 as multiplet as it is coupling with one H-2 and two H-4 protons, while the two protons at H-4 appeared as double doublets because of axial and equatorial coupling constants. The C-13 NMR data also matched exactly with the reported structure of catechin. Previously, catechins have been reported from tea [31], *Acacia catechu* [32], *Arbutus unedo* [33], green tea [34], *Roscoea purpurea* [35] and Bengal catechu [36] etc. they are highly antioxidant polyphenolic compounds also abundantly found in black grapes, tea leaves, peaches, strawberries, apricots, and beans. They prevent cell damage and diminish free radical formation in the body [37-39]. Catechins inhibit the production of metalloproteinase enzymes and activate collagen synthesis, thereby protecting skin from damage [40]. They significantly improve photo-stability, shield the skin from harmful UV rays, regulate cellular death and angiogenesis.

Literature revealed the anticancer activities of catechins in various organs including mammary gland, bladder, lungs, stomach, prostrate, liver, small intestine, and pancreas etc. [41-43]. Catechins also possess antihyperlipidemic, antioxidative, antiproliferative, antihypertensive, and anti-inflammatory

properties. They act as pro-oxidants, metal ion chelators and scavenge free radicles [44]. Diabetes, neurodegenerative diseases, cancer, and cardiovascular diseases are linked to free radicals. Due to their promising antioxidant potential, catechins help in preventing oxidative stress-induced diseases. Catechins suppress stress-induced inflammation pathways by modulating ligand-receptor interactions [45]. They regulate lipid profile, endothelin-1, prostaglandins, prostacyclin, muscle cell proliferation, platelet aggregation and vascular inflammation [46, 47]. Results of the present study revealed that RBFCE showed small inhibitory activity against alpha-amylase and alpha-glucosidase enzyme and demonstrated a significant difference ($p < 0.001$) in inhibitory potential at all concentrations compared to activity of acarbose (positive control). The carbohydrates in food are rapidly absorbed in the intestines by the alpha-amylase enzyme. It breaks complex carbohydrates (polysaccharides, oligosaccharides) into simple absorbable sugars (monosaccharides), causing a sudden rise in blood glucose levels. Oral hypoglycemic agents that inhibit alpha-amylase enzymes are effective in treating hyperglycemia, particularly in individuals with T2DM. Alpha-amylase inhibitors extend carbohydrate digestion time, leading to a reduced rate of glucose absorption and thereby lower blood glucose levels [48].

The alpha-glucosidase inhibitors slow down the degradation of complex carbohydrates into glucose, and lower the pace of glucose absorption from the gut, resulting in reduced blood glucose levels. The alpha-glucosidase inhibition method was declared as the first-line of treatment by The Third Asia-Pacific Region Diabetes Treatment Guidelines for decreasing postprandial hyperglycemia. Due to the side effects of antihyperglycemic medications, people increasingly prefer to use herbal medications to treat DM and its complications around the world. In the present study, inhibitory activity of RBFCE on yeast alpha-glucosidase enzyme was investigated. The alpha-glucosidase inhibition potential of acarbose has been linked to the excessive suppression of pancreatic amylase. Use of acarbose may cause serious gastrointestinal adverse side effects including bloating, flatulence, and diarrhea [49, 50]. These adverse side effects occur due to excessive alpha-amylase inhibition, which causes abnormal bacterial fermentation of undigested carbohydrates in the large intestine. RBFCE displayed the alpha-amylase and alpha-glucosidase inhibition potential in a dose-dependent manner. This observation also suggested that the bioactive compounds inhibiting alpha-amylase and alpha-glucosidase activity are present in RBFCE which are still to be explored. Further studies are required to isolate the bioactive metabolites with significant alpha-amylase and alpha-glucosidase inhibition potential.

Results of *in-vivo* antidiabetic activity indicates that RBFCE showed normalization of biochemical parameters in a dose-dependent manner and overcome the effects of hyperglycemia. ALXN induces diabetes by damaging insulin producing *islet of Langerhans* DNA, mitochondria, lysosomes and plasma membrane which leads to swelling and degeneration of insulin-secreting beta cells [51]. Treatment with low dose of RBFCE did not showed any significant normalization of biochemical parameters and protective effect on the histology of the pancreas as well as biochemical parameters. Whereas, medium dose of RBFCE normalized the biochemical parameters and displayed recovery of beta cells to secrete insulin and acinar structures to perform normal pancreatic functions. Besides this, high dose of RBFCE showed prominent effect on regulation of biochemical parameters and normalized the lobulated architecture of the pancreas composed of acini. It also showed considerably regenerated *islets of Langerhans* which might increase the secretion of insulin. Similarly, kidneys of low dose RBFCE treated animals showed focal tubular atrophy and oedema. Whereas, normal tubules and glomeruli with no signs of inflammation, cipher of thyroidization, fibrosis, proliferation, and necrosis were observed in medium and high dose administered groups.

The plants of the family *Rosaceae* are known for their antioxidant and antidiabetic potential. Other members of family *Rosaceae* including *Rosa damascena* and *Rosa alba* also possess antidiabetic activity [52]. *Rosa canina* and *Sorbus decora* are too widely used for regulation of blood glucose level around the globe [53, 54]. One of the key factors in the development of T2DM is the oxidative stress [7]. Deficiencies in antioxidant protection, lipid peroxidation and over production of free radicles showed involvement in the development of T2DM [8, 9]. Patients with T2DM have reduced levels of antioxidant capacity and increased levels of DNA damage due to increased production of ROS. Members of family *Rosacea* are rich source of phenolic and flavonoid compounds [55, 56]. Flavonoids possesses strong antioxidant potential as well as beneficial health effects in diabetes, obesity and other metabolic disorders [57]. The presence of flavonoid constituents, like tiliroside, quercetin-3-O-rhamnoside, and astragalin from *Rosa brunonii* L. fruit can be considered as the major contributing factor towards the glucose regulation due to their antioxidant potential [58, 60]. Similarly, rutin, kaempferol, and apigenin also possess potential antioxidant activity and are abundant in plants of family *Rosaceae* including *Rosa brunonii* L. [58]. The presence of highly antioxidant phenolic and flavonoid metabolites in RBFCE might contributed in regulation of blood glucose levels in a dose-dependent manner.

CONCLUSIONS

From the results of current *in-vitro* and *in-vivo* studies, it can be concluded that RBFCE possesses moderate inhibitory potential of alpha-amylase and alpha-glucosidase enzyme and thereby regulates blood glucose level with minimal side effects. More research is needed to isolate the potential bioactive compounds from the plant and to perform the *in-vitro* alpha-amylase and alpha-glucosidase inhibition and *in-vivo* pharmacological activities of the already isolated compounds followed by clinical trials.

AUTHOR CONTRIBUTIONS

Conceptualization, E.A, M.J, Z.M.A, N.I.B and T.A.; methodology, E.A, M.J, Z.M.A, N.I.B and T.A; software, T.A; validation, A.A.S; formal analysis, T.A.; investigation, E.A, M.J, Z.M.A, N.I.B and T.A; resources, M.A and A.A.S.; data curation, T.A.; writing—original draft preparation, T.A and E.A; writing—review and editing, T.A and A.F.A; visualization, A.A.S; supervision, T.A and B.I.; project administration, A.A.S and M.A ; funding acquisition, T.A

FUNDING

This research work received no external funding.

ACKNOWLEDGMENTS

The authors greatly acknowledge and express their gratitude to the Researchers Supporting Project number (RSP2023R462), King Saud University, Riyadh, Saudi Arabia

DATA AVAILABILITY STATEMENT

We have presented all our data in the form of figs. and tables. The datasets supporting the conclusions of this article are presented in the paper. The Rosa brunonii L. fruits were collected from Murree, Punjab, Pakistan and identified by an expert taxonomist at Department of Botany, GC University, Lahore, Pakistan. A voucher specimen (GC.Herb.Bot.3315) was deposited in the Herbarium of GC University, Lahore, Pakistan for future reference.

COMPLIANCE WITH ETHICAL STANDARDS

All the animal studies were carried out according to internationally accepted protocols, which were approved by the institutional animal ethical committee College of Pharmacy, University of Punjab (AEC/PUCP/1077) dated 03–05–2018.

CONFLICTS OF INTEREST

"The authors declare no conflict of interest."

REFERENCES

1. Saeedi, P., et al., *WITHDRAWN: Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: results from the International Diabetes Federation Diabetes Atlas.* 2019: p. 107843.
2. Meo, S.A., et al., *Type 2 diabetes mellitus in Pakistan: Current prevalence and future forecast.* 2016. **66**(12): p. 1637-1642.
3. Marathe, P.H., H.X. Gao, and K.L.J.J.o.d. Close, *American D iabetes A ssociation S tandards of M edical C are in D iabetes 2017.* 2017. **9**(4): p. 320-324.
4. Organization, W.H., *Diabetes programme*. 2012.
5. David, J.A., et al., *The Nrf2/Keap1/ARE pathway and oxidative stress as a therapeutic target in type II diabetes mellitus.* 2017. **2017**.
6. Prasad, N., et al., *To study the anti-hyperglycemic activity of porridge (A fibre rich dietary supplement) in management of type II diabetic subjects.* 2018.
7. Mancini, F.R., et al., *Dietary antioxidant capacity and risk of type 2 diabetes in the large prospective E3N-EPIC cohort.* Diabetologia, 2018. **61**(2): p. 308-316.

8. Opara, E.J.T.j.o.t.R.S.f.t.P.o.H., *Oxidative stress, micronutrients, diabetes mellitus and its complications.* 2002. **122**(1): p. 28-34.
9. Singh, R., et al., *Role of free radical in atherosclerosis, diabetes and dyslipidaemia: larger-than-life.* 2015. **31**(2): p. 113-126.
10. Montonen, J., et al., *Dietary antioxidant intake and risk of type 2 diabetes.* 2004. **27**(2): p. 362-366.
11. Ishaque, M., et al., *Fruits of Rosa brunonii–A Source of Antioxidant Phenolic Compounds.* Natural Product Communications, 2017. **12**(11): p. 1934578X1701201106.
12. Vincent, H.K., et al., *Effects of antioxidant supplementation on insulin sensitivity, endothelial adhesion molecules, and oxidative stress in normal-weight and overweight young adults.* Metabolism, 2009. **58**(2): p. 254-262.
13. McCue, P.P. and K. Shetty, *Inhibitory effects of rosmarinic acid extracts on porcine pancreatic amylase in vitro.* Asia Pacific Journal of Clinical Nutrition, 2004. **13**(1).
14. Pandithurai, M., et al., *In vitro α-amylase and α-glucosidase inhibition activity of methanolic extract of marine brown alga Spatoglossum asperum.* Int J Adv Pharm, 2015. **4**(5): p. 83-87.
15. Bräunlich, M., et al., *Extracts, anthocyanins and procyanidins from Aronia melanocarpa as radical scavengers and enzyme inhibitors.* Nutrients, 2013. **5**(3): p. 663-678.
16. Matsui, T., et al., *In vitro survey of α-glucosidase inhibitory food components.* Bioscience, biotechnology, and biochemistry, 1996. **60**(12): p. 2019-2022.
17. OECD, O., *Guidance Document on Acute Oral Toxicity Testing*. 2000, OECD, Paris, France.
18. Sreejesh, P., B.H. Thampi, and E.J.I.J.o.P.S. Sreekumaran, *Hypoglycaemic effect of glibenclamide: a critical study on the basis of creatinine and lipid peroxidation status of streptozotocin-induced diabetic rat.* 2017. **79**(5): p. 768-777.
19. Nagappa, A., et al., *Antidiabetic activity of Terminalia catappa Linn fruits.* Journal of ethnopharmacology, 2003. **88**(1): p. 45-50.
20. Hye, M., et al., *Isolation of (+)-catechin from Acacia catechu (Cutch Tree) by a convenient method.* Journal of Scientific Research, 2009. **1**(2): p. 300-305.
21. Khavandi, K., et al., *Strategies for preventing type 2 diabetes: an update for clinicians.* Therapeutic advances in chronic disease, 2013. **4**(5): p. 242-261.
22. Lazalde-Ramos, B.P., et al., *DNA and oxidative damages decrease after ingestion of folic acid in patients with type 2 diabetes.* 2012. **43**(6): p. 476-481.
23. Moravej, H., et al., *Complementary and alternative medicine to treat diabetes mellitus type 1 in Southern Iran*. 2016. **41**(1): p. 71-72.
24. Azizi-Fini, I., M. Adib, and Z.J.E.J.I.M. Gharehboghlou, *Herbal medicine use among patients with type 2 diabetes in Kashan, Iran.* 2016. **22**: p. 449-54.
25. Ali-Shtayeh, M.S., R.M. Jamous, and R.M. Jamous, *Complementary and alternative medicine use amongst Palestinian diabetic patients.* Complementary Therapies in Clinical Practice, 2012. **18**(1): p. 16-21.
26. Naveed et al., Characterization and Evaluation of the Antioxidant, Antidiabetic, Anti-Inflammatory, and Cytotoxic Activities of Silver Nanoparticles Synthesized Using Brachychiton populneus Leaf Extract. Processes **2022**, 10, 1521.
27. Balamurugan, E. and K. Ganesh, *Prevalence and pattern of self medication use in coastal regions of South India.* Br J Med Pract, 2011. **4**(3): p. a428.
28. Lourenço, S.C., M. Moldão-Martins, and V.D.J.M. Alves, *Antioxidants of natural plant origins: From sources to food industry applications.* 2019. **24**(22): p. 4132.
29. Penckofer, S., D. Schwertz, and K. Florczak, *Oxidative stress and cardiovascular disease in type 2 diabetes: the role of antioxidants and pro-oxidants.* Journal of Cardiovascular Nursing, 2002. **16**(2): p. 68-85.
30. Hamdan, I.I. and F.U. Afifi, *Screening of Jordanian flora for α-amylase inhibitory activity*. Pharmaceutical Biology, 2008. **46**(10-11): p. 746-750.
31. Vuong, Q.V., et al., *Extraction and isolation of catechins from tea.* Journal of Separation Science, 2010. **33**(21): p. 3415-3428.
32. Acharya, P.P., G.R. Genwali, and M. Rajbhandari, *Isolation of catechin from acacia catechu willdenow estimation of total flavonoid content in camellia sinensis kuntze and camellia sinensis kuntze var. assamica collected from different geographical region and their antioxidant activities.* Scientific World, 2013. **11**(11): p. 32-36.
33. Mrabti, H.N., et al., *Separation, identification, and antidiabetic activity of catechin isolated from Arbutus unedo L. Plant roots.* Plants, 2018. **7**(2): p. 31.
34. Dong, J.-J., et al., *Isolation of antioxidant catechins from green tea and its decaffeination.* Food and Bioproducts Processing, 2011. **89**(1): p. 62-66.
35. Kaur, G., et al., *Isolation of Catechins from Roscoea purpurea*. Journal of Young Pharmacists, 2020. **12**(4): p. 389.
36. Azad, A.K., K. Ogiyama, and T. Sassa, *Isolation of (+)-catechin and a new polyphenolic compound in Bengal catechu.* Journal of Wood Science, 2001. **47**(5): p. 406-409.
37. Kwik-Uribe, C. and R.M. Bektash, *Cocoa flavanols: measurement, bioavailability and bioactivity.* Asia Pacific Journal of Clinical Nutrition, 2008. **17**.
38. Barickman, T.C., D.A. Kopsell, and C.E. Sams, *Selenium influences glucosinolate and isothiocyanates and increases sulfur uptake in Arabidopsis thaliana and rapid-cycling Brassica oleracea*. Journal of agricultural and food chemistry, 2013. **61**(1): p. 202-209.
39. Cheng, G.W. and C.H. Crisosto, *Browning potential, phenolic composition, and polyphenoloxidase activity of buffer extracts of peach and nectarine skin tissue*. Journal of the American Society for Horticultural Science, 1995. **120**(5): p. 835-838.
40. Gul R et al**.,** Phytochemical, antimicrobial, radical scavenging and in-vitro biological activities of Teucrium stock, sianum leaves. J.Chil Chem Soc 2023, **68** (1), 5748–5754.
41. Lambert, J.D., et al., *Inhibition of carcinogenesis by polyphenols: evidence from laboratory investigations.* The American Journal of Clinical Nutrition, 2005. **81**(1): p. 284S-291S.
42. Yang, C.S., P. Maliakal, and X. Meng, *Inhibition of carcinogenesis by tea.* Annual review of pharmacology and toxicology, 2002. **42**(1): p. 25-54.
43. Xiao, H., et al., *Green tea polyphenols inhibit colorectal aberrant crypt foci (ACF) formation and prevent oncogenic changes in dysplastic ACF in azoxymethane-treated F344 rats.* Carcinogenesis, 2008. **29**(1): p. 113-119.
44. Fraga, C.G., et al., *Basic biochemical mechanisms behind the health benefits of polyphenols.* Molecular Aspects of Medicine, 2010. **31**(6): p. 435-445.
45. Fan, F.-Y., L.-X. Sang, and M. Jiang, *Catechins and their therapeutic benefits to inflammatory bowel disease.* Molecules, 2017. **22**(3): p. 484.
46. Rauf B, Evaluating the influence of Aloe barbadensis extracts on edema induced changes in C-reactive protein and interleukin-6 in albino rats through in vivo and in silico approaches. Acta Biochim Pol. 2023, 17;70(2):425-433.
47. Galley, H.F. and N.R. Webster, *Physiology of the endothelium.* British Journal of Anaesthesia, 2004. **93**(1): p. 105-113.
48. Wild, S., et al., *Global prevalence of diabetes: estimates for the year 2000 and projections for 2030.* Diabetes Care, 2004. **27**(5): p. 1047-1053.
49. McIver, L.A. and J. Tripp, *Acarbose*. 2018.
50. Rosak, C. and G. Mertes, *Critical evaluation of the role of acarbose in the treatment of diabetes: patient considerations.* Diabetes, metabolic syndrome and obesity: targets and therapy, 2012. **5**: p. 357.
51. Sen, S., et al., *Hypoglycemic and hypolipidemic effect of Meyna spinosa leaves in high fat diet-alloxan induced type 2 diabetic rats.* 2013. **8**(2): p. 181-185.
52. Ahmad, M., et al., *Traditional herbal remedies used for the treatment of diabetes from district Attock (Pakistan).* 2009. **41**(6): p. 2777-82.
53. Yeşilada, E., et al., *Traditional medicine in Turkey IX:: Folk medicine in north-west Anatolia.* 1999. **64**(3): p. 195-210.
54. Vianna, R., et al., *In vivo anti-diabetic activity of the ethanolic crude extract of Sorbus decora CK Schneid.(Rosacea): a medicinal plant used by Canadian James Bay Cree nations to treat symptoms related to diabetes.* 2011. **2011**.
55. Abdel-Hameed, E.-S.S., S.A. Bazaid, and M.S. Salman, *Characterization of the phytochemical constituents of Taif rose and its antioxidant and anticancer activities.* BioMed Research International, 2013. **2013**.
56. CierniaK, M.K.-D.a.J.J.o.F. and N. Research, *Antioxidant and genoprotective properties of extracts from edible flowers.* 2019. **58**(1): p. 42-50.
57. Middleton, E., C. Kandaswami, and T.C. Theoharides, *The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer.* Pharmacological reviews, 2000. **52**(4): p. 673-751.
58. Ishaque, M., et al., *Fruits of Rosa brunonii–A Source of Antioxidant Phenolic Compounds.* 2017. **12**(11): p. 1934578X1701201106.
59. **Saleem A.,** et al., HPLC, FTIR and GC-MS Analyses of Thymus vulgaris Phytochemicals Executing In Vitro and In Vivo Biological Activities and Effects on COX-1, COX-2 and Gastric Cancer Genes Computationally. Molecules 2022, 27, 8512.
60. Ahmad B et al., Curative Effects of Dianthus orientalis against Paracetamol Triggered Oxidative Stress, Hepatic and Renal Injuries in Rabbit as an Experimental Model. Separations 2023, 10, 182