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In vitro and In vivo experimental study of Aqueous and Ethanolic Extracts of Selected Medicinal Plants for Free Radical Scavenging and Hypoglycemic Activity

Rishiram Baral
Kyungpook National University
Prakash Rawal
Pokhara University
Sushil Panta
sushilmax@hotmail.com

Pokhara University

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Abstract

Background

Diabetes mellitus (DM) is a condition affecting metabolism characterized by a high level of glucose in the bloodstream, either due to failure of the Islets of Langerhans of the pancreatic beta cell to produce enough insulin (Type I diabetes) or when there is insulin resistance in body cells (Type II diabetes). Oxidative stress caused by the free radicals produced during the oxidation of glucose, protein glycation by non-enzymatic pathway, and the gradual breakdown of glycated protein by oxidation, is a significant factor in the pathogenesis of diabetic complications. This study was designed for the qualitative and quantitative phytoconstituent determination, free radical lowering activity, and in-vitro and in-vivo glucose-lowering effect of selected parts of five medicinal plants of Nepal using two solvents, i.e., methanol and water of different polarity. The selected plant species are *Artocarpus heterophyllus* Lam., *Dendrocalamus hamiltoni*, *Fagopyrum megacarpum*H. Hara., *Somallanthus sonchifolius* (poepp)H. Rob., *Urtica parviflora*Roxb.

Results

The result from the study revealed that among the tested samples, *A. heterophyllus, F. megacarpum,* and *S. sonchifolius*have higher Total Phenolic Content (TPC) and Total Flavonoid Content (TFC), conclusively showing significant free radical scavenging properties. The *in-vitro*study for the hypoglycemic activity of plant samples showed that ethanolic and water extracts of *S. sonchifolius* have potent alpha-amylase inhibitory effect showing the half-maximal inhibitory concentration as 0.136 mg/ml and 0.168 mg/ml respectively. *In vivo* hypoglycemic activity study of *S. sonchifolius* depicted the blood glucose lowering effect in a dose-dependent manner having a comparative release profile to standard Metformin. *S. sonchifolius* with the dose of 500 mg/kg showed the optimal decrement in blood glucose level in normal mice by 17.99%, while the same dose of *S. sonchifolius* diminished the glucose level in blood by 15.74% in glucose-loaded hyperglycemic mice in 120 minutes.

Conclusion

The results from the study outlined that the positive qualitative and quantitative phytoconstituent determination and the ability to eliminate free radicals align with the *in vitro* and *in vivo*hypoglycemic activity test.

Introduction

Diabetes mellitus (DM) is a longstanding disease with impaired glucose metabolism characterized by a rise in blood glucose levels and carbohydrate, fat, and protein metabolism disturbance. DM is associated with an absolute or relative deficiency in insulin secretion and action [1]. Type I diabetes is characterized by insulin deficiency due to severe defects in Islets of Langerhans in the pancreas's beta cell (β-cell). In contrast, resistance to the action of insulin and insufficiency in insulin secretion are common

characteristics of Type II diabetes. The complications associated with DM are diabetic retinopathy, diabetic nephropathy, diabetic neuropathy, and cardiovascular diseases such as coronary heart disease, myocardial infarction, heart stroke, peripheral arterial disease, and aortic disease [2]. The global existence of diabetes is rising each year and a study by WHO revealed that the number of diabetic patients worldwide has reached 200 million and is expected to exceed 366 million by 2030[3].

Oxidative stress plays a vital role in the occurrence of diabetes complications. Oxidative stress alters the enzymatic systems, depletes lipid peroxidation, impairs glutathione metabolism, and decreases Vitamin C levels, damaging enzymes and cellular machinery and increasing insulin resistance[4]. Several studies showed that in diabetic patients, oxygen free radicals (OFR) could be formed by the reaction between the molecular form of the oxygen with glycated proteins resulting in complications such as perforating ulcers, microangiopathy, retinopathy, and polyneuritis. The WHO recommended natural antioxidants to minimize the diabetic complications associated with oxidative stress: ascorbic acid, α-tocopherol, β-carotene, glutathione-flavonoids, uric acid, cysteine, vitamin-K, serum, albumin, bilirubin and trace elements such as zinc and seleniuman[5].

Pancreatic alpha-amylase (α-amylase) is a crucial enzyme in carbohydrate digestion. It mediates the beginning step in the breakdown of starch to a mixture of smaller oligosaccharides consisting of maltose, maltotriose, and several α-(1–6) and α-(1–4)-oligo glucans. These oligosaccharides are further breakdown into the absorbable form of glucose by alpha-glucosidases[6]. The action of human pancreatic α-amylase directly correlates to a rise in postprandial glucose levels, and control of this is essential in treating Type II diabetes. Furthermore, inhibitors of pancreatic α-amylase delay carbohydrate digestion, reduce the rate of glucose absorption and lower the postprandial serum glucose levels[7]. Synthetic small molecule inhibitors targeting α-amylase and α-glucosidase are acarbose, imiglitol, and volgibose. Their non-specificity in targeting different glucosidases crowned these agents with several side effects such as bloating, abdominal discomfort, diarrhea, flatulence, low blood sugar level, skin rash and itching, kidney complications, tiredness, dizziness, etc. [8]. Therefore, researchers are interested in finding safer, more effective, and specific antidiabetic medicines, a critical investigation area. The natural plant sources and valuable extracts obtained from them are highlighted for their significant potential for discovering new hypoglycemic agents[9, 10].

Medicinal plants play a significant role in managing DM, attributed to several phytoconstituents such as phenolics, flavonoids, tannins, terpenoids, saponins, alkaloids, glycosides, etc. [11]. These compounds are synthesized by a secondary metabolic pathway proving themselves as potent phytoconstituents to mitigate several bodily ailments[9, 12]. The phytochemicals act in several metabolic pathways involving glucose or its derivatives as substrate or product. They affect glucose metabolism by acting in the glycolysis pathway and Kreb's cycle, alter pentose phosphate pathways, glycogenesis, gluconeogenesis, glycogenolysis, glucose absorption via the alimentary canal, and increase insulin release production and its efficacy[13]. Phytoconstituents modulate blood sugar levels through these different roles in glucose metabolism through various mechanisms[14]. Variations in the plant parts, extraction method, and solvent may yield different active ingredients showing different glucose-lowering effects [15]. In this

study, five different medicinal plants were collected from various areas of the Kaski district of Nepal. The selected medicinal plants are *Artocarpus heterophyllus* Lam., *Dendrocalamus hamiltoni*, *Fagopyrum megacarpum* H. Hara., *Somallanthus sonchifolius* (poepp)H. Rob., *Urtica parviflora* Roxb.

A. heterophyllus, also known as Jackfruit, belongs to the family Moraceae and is a large evergreen tree found in the tropical, equatorial, and subtropical region where the warm and humid climate is prevalent[16]. The fruit, leaf, and root contain different phytoconstituents such as flavonoids, phenolic acids, organic acids, carotenoids, stilbenes, triterpenes, and sterols[17]. Different parts of these plants are commonly used in ethnomedicine as antioxidant, anti-inflammatory, antidiabetic, anti-aging, antihyperglycemic, and antihyperlipidemic agents[18].

D. hamiltoni, also known as bamboo belonging to the family Poaceae is an evergreen clump-forming tree with a height of 15–18 meters and is widely distributed in tropical and subtropical regions around the globe. This plant's young shoots are edible and rich in tannins, steroids, phenols, glycosides, flavonoids, carbohydrates, and protein[19]. With the availability of these phytoconstituents, it is known for their antibacterial, antimicrobial, and antioxidant activities[20].

F. megacarpum, locally known as Bhote Khair, belongs to the family Polygonaceae distributed in tropical and sub-tropical climatic regions. The *F. megacarpum* is the least studied plant species without any publications to the date specified in this species. While several studies in other species, such as *F. esculantum* shows that hulls and flour have significant hydrogen peroxide and superoxide radical scavenging activity. The study depicted that the strong peroxyl radical-scavenging activity is due to the presence of antioxidant compounds catechin and rutin[21].

S. sonchifolius, also known as Yacon, belonging to the family Urticaceae is a perennial, monoecious herb native to South America at an altitude up to 3200m. The tuberous roots of the plants have a much sweeter taste and mainly contain carbohydrates, lipids, and proteins. The root and leaves are being used by people who have diabetes or are suffering from digestive and renal disorders. Studies depicted that *S. sonchifolius* has antibacterial, anti-inflammatory, antifungal, and antiparasitic properties[22]. There is growing interest in studying other pharmacological properties, such as hypoglycemic properties and efficacy against leukemia and pancreatic cancer cells[23].

U. parviflora, also known as Chinese elm or lacebark elm belonging to the family Urticaceae, is a perennial, monoecious herb distributed primarily in the tropical region at the height of 1700 to 2800 meters from the sea level[24]. The leaves and stem of the plant contain trichomes which could be utilized for treating burning and itching sensations due to the presence of histamine and 5-hydroxytryptamine. The plant's leaves and roots are used in rheumatism, hemorrhage, diuretic, inflammation, and hyperglycemic condition. Furthermore, *U. parviflora* and a few other *Urtica* species are rich in phytoconstituents such as hydroxycinnamic acid derivatives and flavonoids, exhibiting antibacterial, antioxidant, anti-inflammatory, antimicrobial, and mast cell stabilizing properties[25].

Although the comprehensive utilization of these medicinal plants by numerous ethnic communities and indigenous societies by incorporating them into different traditional and alternative systems of medicine, minimal scientific studies are being made to prove the efficacy, extrapolate the mechanism of action, and highlight their effectiveness[26]. With these reference basis of their utilization in different systems of traditional and alternative medicine, this study was designed to prove the effectiveness of these medicinal plants experimentally and scientifically.

Materials and Methods

Chemicals and Reagents

1,1 Diphenyl-2 picryl hydrazyl radical (DPPH) was procured from Tokyo Chemical Industry, Japan. α -amylase (1,4-α-D -Glucan-glucano hydrolase), Ascorbic acid, quercetin, gelatin powder and α -napthol was purchased from Himedia laboratories, India. Starch, Chloroform, methanol, ethyl acetate, mercury, Sulfuric acid, Sodium hydroxide, Sodium carbonate, Sodium citrate, Copper sulphate, Ferric chloride, Sodium nitrate, and Folin reagent was bought from Thermo Fisher Scientific, India. Metformin was provided by Sumy Pharmaceuticals, Nepal, and Voglibose was offered by Quest Pharmaceuticals, Nepal, as a bounteous gift. All chemicals and reagents utilized were of a quality suitable for analytical testing.

Collection and Identification of Plant Samples

Plant species selected for the study, as listed in Table 1, were collected from various locations in the Kaski district, Nepal. Preparation and identification of herbaria were performed by the registered taxonomist in National Herbarium and Plant Laboratory, Godawari, Kathmandu, Nepal. The voucher number was assigned for each herbarium and stored in the Department of Pharmaceutical Sciences crude drug museum, School of Health and Allied Sciences, Pokhara University, Nepal. The collected parts of the samples were washed quickly to remove dust and waste, cut into small pieces, and shaded dried. Followingly, the samples were stored in the incubator at 40˚C for complete moisture removal, which was later verified by the weight variation test. The selected medicinal plant samples are shown in Fig. 1.

Table 1
List of selected medicinal plants

Scientific Name	Family	Local Name	Part Used	Crude Drug Voucher No.	Sample No.
Artocarpus heterophyllus Lam.	Moraceae	Jack fruits	Fruit	PUH-2020-15	S4
Dendrocalamus hamiltonii	Poaceae	Dhunge Baans	Shoot	PUH-2020-12	S2
Fagopyrum megacarpum H. Hara	Polygonaceae	Bhote Khair	Tuber	PUH-2019-01	S5
Smallanthus sonchifolius (Poepp.) H. Rob.	Asteraceae	Yacon	Leaf	PUH-2020-14	S3
Urtica parviflora Roxb.	Urticaceae	Sisnu	Leaf	PUH-2020-13	S1

Sample Extraction

When the sample was dried entirely, a double maceration extraction technique was used to extract active phytoconstituents from the crude drugs. The solvents used for the extraction are ethanol and water. 50g of each plant sample was dissolved in 500 ml of each solvent, implying a ratio of 1:10. Then, the sample was left for 24 hours for maceration in a conical flask with occasional shaking. To acquire purified extracts, Whatmann-1 filter paper was used to filter the samples, followed by the double maceration of the residue obtained for the next 24 hours. The extract was mixed and evaporated using a rotary vacuum evaporator, followed by storage in a vacuum desiccator for drying. The formula below was employed to calculate the percentage yield of the unrefined extracts;

$$\%Yield = \frac{\mathrm{Weightofdriedextract}}{\mathrm{Weightofplantssample}} * 100$$

Phytochemical Screening

The method outlined by Hossain et al. 2013 and Benariba et al. 2013 was followed to conduct the phytochemical analysis to determine the presence or absence of phytoconstituents such as alkaloids, carbohydrates, glycosides, saponins, tannins, terpenoids, phenols and flavonoids[27, 28].

Total phenolic content (TPC)

Total phenolic content (TPC) was determined using the Folin-Ciocalteu method, as Gao et al., 2019[29] mentioned. Gallic acid was taken as a standard phenolic compound. The different concentration of gallic acid ranging from 0.01mg/mL to 0.1 mg/mL was prepared in ethanol while a working solution of 1 mg/mL of each plant extract was prepared. 1 mL of each plant extract was added to 1 mL of 2N Folin-Ciocalteu reagent and 5 mL of distilled water. This was followed by adding 1 mL of 10% Sodium carbonate, with subsequent incubation at room temperature for 1 hour. Finally, the UV spectrophotometer

at λ = 725 nm setup read the absorbance. Each experiment was performed thrice (n = 3), and the results were depicted as mean ± SD. The unit measurement of TPC was quantified and reported as µg of Gallic acid equivalent per mg (GAE/mg) of the extract.

Total flavonoid content (TFC)

Total flavonoid content (TFC) was determined using the aluminum chloride method mentioned by Pekal et al., 2019[29]. Quercetin was taken as a standard flavonoid compound. The different concentrations of quercetin ranging from 0.05 mg/mL to 0.5 mg/mL were prepared in ethanol, while a working solution of 1 mg/mL of the plant was prepared. 1 mL of 1 mg/mL of each plant extract was mixed with 4 mL of distilled water and 0.3 mL of 5% Sodium nitrite. Five minutes of incubation was followed by adding 10% aluminum chloride and 1 mL of 1M Sodium hydroxide. Finally, after 30 minutes of incubation, a UV spectrophotometer at λ = 510 nm read the absorbance. Experiments were performed thrice (n = 3), and the data were presented as mean ± SD. The TFC was expressed as µg of the plant extract's quercetin equivalent per mg (QE/mg).

In vitro activity analysis

DPPH free-radical scavenging activity

The DPPH free radical assay was carried out per the method mentioned by Musa et al., 2016[30]. Plant extracts of different concentrations ranging from 0.1 µg/mL to 100 µg/mL of five samples were mixed with a 60 µM solution of DPPH prepared in ethanol. The reaction was completed by incubating the reacting solution in the dark for 30 minutes. Finally, the optical density of the sample was measured with a UV spectrophotometer at λ = 517 nm. The antioxidant property of each sample was studied using the following equation. Ascorbic acid was utilized as a positive control in the experiment.

Radical scavenging activity (%) = $[(A_0 - A_S)/ A_0] \times 100$

Where;

A_0 = absorbance of control

A_S = absorbance of the sample

Half maximal inhibitory concentration (IC_{50}) was calculated by plotting optical density against the sample concentration.

In vitro α -amylase inhibitory activity

In vitro α -amylase inhibition study was performed by the starch iodine method, as mentioned by Sun et al., 2020 and Taha et al., 2017[31, 32]. Plant extracts of five different concentrations ranging from 0.125 mg/mL to 2 mg/mL were prepared for each plant sample. 1 mL of α -amylase solution prepared in a phosphate buffer solution of pH 7 was mixed with 1 mL of each plant extract, followed by incubation of

the sample at 37 ˚C for 10 minutes. Incubation was followed by adding 1% starch solution and further incubation for 1 hrs at 37 ˚C. The reaction was terminated by adding 50 µL of 1M HCL and 100 µL of Iodine solution. Finally, 2 mL of RO water was added to dilute all the test tubes equally. A blank solution was prepared by replacing the α-amylase enzyme with phosphate buffer to deduct the background signals. Voglibose was taken as the reference standard. Finally, a UV spectrophotometer measured the optical density for each sample at λ = 565 nm.

Inhibition of enzyme activity was measured by using the equation below:

Percentage Inhibition (%) = $[(A_0\text{-}A_1/A_0) \times 100\%]$

Where;

A_1 = absorbance of control

A_0 = absorbance of the sample

In vivo hypoglycemic activity study in Normal mice

The glucose-lowering effect of given plant extracts was studied in adult Swiss albino mice of both sex weighing about 32 to 34 grams as per the method mentioned by Taya et al., 2020 and Mechchate et al., 2021[33, 34]. The animals used in the experiment were housed in a controlled environment with a temperature range of 25 ± 2 ˚C and relative humidity (RH) of 55 ± 5% for one week before the study began to allow them to adapt to the environment. The animals were fasted overnight before the experiment while still having access to water. The plant extract of *S. sonchifolius* doses of 100 mg/kg, 250mg/kg, and 500 mg/kg was administered to different treatment groups at different concentrations. Blood sampling was done from each mouse's tail tip at the defined time interval, and blood glucose level was determined using Clinical Glucometer based on the Glucose oxidase method. The alteration in the concentration of glucose was determined by applying the equation given below;

% glycemic Change (%) = $[(G_0\text{-}G_t)/G_0] *100\%$,

Where;

G_0 is the initial blood glucose level, and

G_t is blood glucose level at 30, 60, and 120 minutes.

Five groups (I-V) were prepared by dividing the adult Swiss albino rate. Each group contains five mice:

Group I: Control group without treatment receiving 0.5 mL of distilled water.

Group II: Treatment group receiving 100 mg/kg of *S. sonchifolius.*

Group III: Treatment group receiving 250 mg/kg of *S. sonchifolius*.

Group IV: Treatment group receiving 500 mg/kg of *S. sonchifolius.*

Group V: Treatment group receiving 100mg/kg of Metformin.

At the beginning of the experiment, fasting blood glucose level was measured. The administration of test samples was followed by the blood glucose level measurement at 30, 60, and 120 minutes.

Oral Glucose Tolerance Test (OGTT)

The OGTT was measured as per the method mentioned by Jagannathan et al., 2020. Mice were fasted for 16 hrs only with the excess of water. The mice were divided into five categories containing five mice in each group (n = 5).

Group I: Control group without treatment receiving 0.5 mL of distilled water and 2g/kg of glucose.

Group II: Treatment group receiving 100 mg/kg of *S. sonchifolius* and 2g/kg of glucose.

Group III: Treatment group receiving 250 mg/kg of *S. sonchifolius* and 2g/kg of glucose.

Group IV: Treatment group receiving 500 mg/kg of *S. sonchifolius* and 2g/kg of glucose.

Group V: Treatment group receiving 100mg/kg of Metformin and 2g/kg of glucose.

Blood sampling was done from the tail vein of mice just before the administration of glucose and at 30, 60 and 120 minutes after loading glucose to measure the blood glucose level.

Results

Qualitative and quantitative phytochemical screening

Different standard test methods were employed to determine the presence or absence of different active phytoconstituents and the positive study test towards several secondary metabolites such as flavonoids, terpenoids, carbohydrates, phenols, tannins, saponins, and alkaloids. In contrast, the study revealed the absence of glycoside and gelatin in extracts of both solvents. **Table 2** shows the qualitative data of phytochemical screening**.**

Table 2: Phytochemical Screening of Aqueous and Ethanolic Extracts of Plant Samples

Phytochemical Constituents	Specific Test	Aqueous extract					Ethanolic extract				
		S1	S2	S3	S4	S5	S1	S2	S3	S4	S5
Alkaloid	Mayer	-	-	-	-	-	-	-	-	-	-
	Wagner	+	+	+	+	-	+	+	+	+	-
Carbohydrate	Molish	+	+	+	+	+	+	+	+	+	+
	Benedict	+	+	+	+	+	+	+	+	+	-
Glycoside	Modified Borntrager	-	-	-	-	-	-	+	-	-	-
Saponin	Foam	+	+	+	+	+	+	+	-	+	+
Phenol	Ferric Chloride	+	+	+	+	+	-	-	+	+	+
Flavonoid	Alkaline Reagent	+	+	+	+	+	+	+	+	+	+
Tannin	Gelatin	-	-	-	-	+	-	-	-	-	+
	Ferric Chloride	-	-	+	-	+	-	-	+	+	+
Terpenoid	Salkowaski	+	+	+	+	+	+	+	-	+	+
	Copper acetate	+	+	-	+	-	+	+	+	+	-

Where; (+) represent the presence of phytoconstituent, and (-) represents the absence of phytoconstituent.

Total phenolic content (TPC)

The quantitative determination of TPC of the extracts was determined by the Folin Ciocalteu method in terms of gallic acid equivalent (GAE). Phenol oxidation in an alkaline solution by yellow molybdotungstophosphoric heteropolyanion reagent and measurement of the resultant molybdotungstophosphate blue by colorimetric technique is the principle reaction step. The maximum absorption of blue pigment was observed based on the quantitative composition of a phenol mixture besides the solutions' pH. The gallic acid standard curve was prepared for calibrating the experimental procedure and condition. The TPC of each sample based on the standard regression line for gallic acid was determined, and the result is shown in **Table 3,** which is expressed as µg gallic acid equivalent (GAE) per mg of extracts. The result showed that both water and ethanol extract of **S5** possesses high TPC of 341.75 ± 1.56 µg GAE/mg of extract and 273.44 ± 1.29 µg GAE/mg of extract, respectively.

Total flavonoid content (TFC)

Aluminum chloride colorimetric assay determined the TFC in various plant extracts regarding quercetin equivalent (QE) as standard. Flavonoids give a dark yellow fluorescence signal when mixed with aluminum chloride, and optical density was measured with a UV spectrophotometer at λ=510 nm. The calibration curve of quercetin as a standard was prepared for calibrating the experimental procedure and

condition. The TFC of each sample based on the standard regression line for quercetin was determined, and the result is shown in **Table 3,** which is expressed as quercetin equivalent (QE) per mg of extracts. The result showed that both water and ethanol extract of **S5** possesses high TFC with the quantitative value of 1153.59 ± 7.05 µg QE/mg of extract and 1110.50 ± 3.46 µg QE/mg of extract, respectively.

Table 3: TPC and TFC expressed as µg GAE/mg of extract and µg QE/mg of extract, respectively

Plant Sample	Total Phenol Content (mg GAE/g dry extract weight)		Total Flavonoid Content (mg QE/g dry extract weight)	
	Water	Ethanol	Water	Ethanol
S1	39.63±4.63	49.40±5.80	70.15±2.38	26.13±2.38
S2	51.98±4.36	66.81±3.27	85.74±2.10	41.26±4.12
S3	88.46±2.13	70.71±4.22	424.12±2.86	224.66±2.86
S4	49.61±1.36	57.55±1.88	213.66±3.46	190.28±4.20
S5	341.75±1.56	273.44±1.29	1153.59±7.05	1110.50±3.46

Data are expressed as mean ± standard deviation (n=3)

DPPH free radical scavenging activity

During the DPPH experiment, the change of the violet color of the methanolic solution of DPPH depicts the ability of plant extract to donate either hydrogen atoms or electrons. Bleaching of violet color to yellow results in the decrement of absorbance when measured using a UV spectrophotometer at λ=517 nm, where the decrement in absorbance was inversely correlated with the antioxidant property of the sample. Our study revealed that aqueous and ethanolic extracts of **S5** showed potent DPPH free radical scavenging activity depicting IC_{50} values of 3.52 µg/mL and 3.65 µg/mL, respectively, compared to standard ascorbic acid. The aqueous and ethanolic extract of **S4** showed IC_{50} of 47.69 µg/mL and 49.13 µg/mL, respectively, followed by **S3,** which showed IC_{50} of 47.64 µg/mL and 58.86 µg/mL, respectively, in water and ethanolic extract. The detailed result of the antioxidant activity study is shown in **Table 4** and **Table 5.**

Table 4: DPPH Free-Radical Scavenging Activity of Aqueous Extracts.

Plant	% DPPH Scavenging Activity				IC_{50} µg/mL
Sample	0.1 µg/mL	1 µg/mL	10 µg/mL	100 µg/mL	
S1	3.41±0.56	7.79±0.56	12.90±0.56	26.28±0.37	>100
S2	6.69±0.92	11.56±0.42	20.56±0.42	48.42±0.56	>100
S3	10.34±1.29	16.67±0.21	40.15±1.67	82.12±0.73	47.64
S4	13.26±0.56	16.18±1.88	36.49±0.97	82.73±0.76	47.69
S5	18.76±0.92	42.21±0.21	92.58±0.21	92.94±0.56	3.52
Ascorbic acid	13.38±0.76	44.28±0.55	95.38±0.21	96.48±0.21	3.56

Data are expressed as mean ± standard deviation (n=3)

Table 5: DPPH Free-Radical Scavenging Activity of Ethanolic extracts.

Plant	% DPPH Scavenging Activity				IC_{50} µg/mL
Sample	0.1 µg/mL	1 µg/mL	10 µg/mL	100 µg/mL	
S1	2.07±0.92	7.79±0.56	10.71±0.56	17.40±1.73	>100
S2	9.37±1.28	12.41±0.73	15.08±0.21	44.40±0.21	>100
S3	5.84±0.56	10.95±0.73	26.64±0.97	75.91±0.0.73	58.86
S4	4.62±0.76	14.84±1.12	35.77±1.09	84.56±0.56	49.13
S5	22.87±0.21	36.50±0.97	91.97±0.97	92.21±0.56	3.65
Ascorbic acid	13.38±0.76	44.28±0.55	95.38±0.21	96.48±0.21	3.56

Data are expressed as mean ± standard deviation (n=3)

In vitro α -amylase inhibitory activity

In vitro hypoglycemic activity of given plant samples was determined by α -amylase enzyme inhibition technique. The inhibitory activity of different aqueous and ethanolic extracts of plant samples towards α -amylase enzyme with their IC_{50} values are shown in **Table 6** and **Table 7,** respectively. Among the various plant samples, aqueous and ethanolic extracts of **S3** showed potent α -amylase inhibitory activity with the IC_{50} value of 0.168 mg/mL and 0.136 mg/mL, respectively, followed by aqueous and ethanolic extracts of **S5** showing half-maximal inhibitory concentration of 0.248 mg/mL and 0.196 mg/mL respectively. The dose-dependent inhibition was shown by y all plant samples towards α -amylase enzyme.

Table 6: *In vitro* α -amylase inhibitory activity of aqueous extract of plant sample.

Plant Sample	Concentration (mg/mL)					IC_{50} mg/mL
	0.125	0.25	0.5	1	2	
S1	9.80±5.17	24.19±0.82	41.33±3.77	54.02±0.53	67.27±1.46	0.841
S2	8.73±3.86	10.32±2.77	18.13±2.67	23.71±2.83	35.38±1.99	2.968
S3	41.03±5.28	56.01±3.72	74.65±0.22	83.31±0.91	91.80±0.31	0.168
S4	19.56±4.55	37.77±1.65	48.53±0.99	64.03±0.49	68.91±1.56	0.873
S5	37.73±4.77	55.17±1.16	66.84±0.74	76.03±0.98	83.11±1.07	0.248
Voglibose	44.58±6.31	70.51±1.20	85.09±0.81	91.06±0.74	94.68±0.31	0.126

Data are expressed as mean ± standard deviation (n=3)

Table 7: *In vitro* α -amylase inhibitory activity of ethanolic extract of plant sample.

Plants Samples	Concentration (mg/mL)					IC_{50} mg/mL
	0.125	0.25	0.5	1	2	
S1	24.45±4.04	47.69±1.31	60.61±3.38	76.23±0.91	87.31±0.41	0.426
S2	8.22±1.95	23.41±3.47	42.27±1.36	53.66±1.02	58.70±1.51	1.318
S3	48.51±2.83	69.47±1.57	83.33±0.14	89.70±0.25	93.62±0.07	0.136
S4	41.10±2.55	59.11±1.82	66.52±1.44	79.20±0.28	88.11±0.42	0.198
S5	42.58±4.72	56.63±3.64	75.17±0.42	80.42±0.32	86.01±0.43	0.196
Voglibose	44.58±6.31	70.51±1.20	85.09±0.81	91.06±0.74	94.68±0.31	0.126

Data are expressed as mean ± standard deviation (n=3)

In Vivo Hypoglycemic activity study

Acute hypoglycemic effects on normal mice

Among the studied plant samples extracted in aqueous and ethanolic solvent media, the ethanolic extract of **S3** showed potent α-amylase enzyme inhibitory activity. Therefore, for the study of *in vivo* glucose-lowering activity study, the ethanolic extract of **S3** was selected. The mice were fasted for 16 hours, and the hypoglycemic effect of different doses of ethanolic extract of **S3** was assessed at different time intervals. The result outlined the dose-dependent decrement in the blood glucose level at different time intervals. The optimal dose of **S3** taken, which is 500 mg/kg, showed 4.16%, 10.20%, and 17.99% at 30 minutes, 60 minutes, and 120 minutes, respectively.

Table 8: Acute hypoglycemic effect of ethanolic extract of S3 at different concentrations.

Group (n=5)	Average Blood Glucose Level (mg/dl) at various Time Intervals (Percentage Change in Blood Glucose Level Concerning Zero Time)			
	0 min	30 min	60 min	120 min
Control	149.20±5.76	155.60±3.44 (4.29%)	149.60±3.36 (0.27%)	144.60±6.88 (-3.08%)
S3 100 mg/kg	162.80±8.32	174.00±13.55 (6.88%)	167.80±11.58 (3.07%)	157.00±10.51 (-3.56%)
S3 250 mg/kg	183.20±8.01	193.60±4.34 (5.68%)	174.60±13.13 (-4.69%)	171.80±12.66 (-6.22%)
S3 500 mg/kg	149.00±12.37	142.80±10.11 (-4.16%)	133.80±12.24 (-10.20%)	122.20±10.28 (-17.99%)
Metformin 100 mg/kg	171.40±6.77	149.80±5.26 (-12.60%)	126.60±6.15 (-26.14%)	116.80±7.26 (-31.86%)

Data are expressed as mean ± standard deviation (n=5). Values in parentheses represent the % change in blood glucose level concerning the blood glucose level at 0 minutes, and the negative sign indicates the decrease in blood glucose level.

Oral glucose tolerance test (OGTT)

Glucose-induced hyperglycemic test was performed by OGTT. The ability of ethanolic extract of S3 to reduce blood glucose levels in mice that had been loaded with glucose was tested. Throughout the experiment, the blood glucose levels were measured at various time intervals to track changes over time. With the administration of 2g/kg of oral glucose, blood glucose levels increased dramatically in all study groups, which decreased subsequently with the administration of different doses of the **S3**. The decrease in blood glucose level by ethanolic extract of **S3** at a concentration of 500 mg/kg was 5.16% and 15.74% after 60 minutes and 120 minutes of administration, respectively. The details of the hypoglycemic effect of ethanolic extract of S3 at different doses up to 120 minutes are shown in **Table 9 and Figure 2.**

Table 9: Acute hypoglycemic effect in glucose-induced hyperglycemic mice of ethanolic extract of S3 at different concentrations.

Group (n=5)	Average Blood Glucose Level (mg/dl) at various Time Intervals (Percentage Change in Blood Glucose Level Concerning Zero Time)			
	0 min	30 min	60 min	120 min
Control	144.44±8.23	220.40±11.93 (52.63%)	191.00±6.60 (32.27%)	172.80±8.26 (19.67%)
S3 100 mg/kg	158.40±7.77	206.60±6.43 (30.43%)	188.60±8.65 (19.07%)	179.80±5.72 (13.51%)
S3 250 mg/kg	155.60±7.30	195.80±7.63 (25.84%)	171.00±8.51 (9.90%)	159.00±7.18 (2.19%)
S3 500 mg/kg	158.80±5.89	195.80±9.31 (23.30%)	150.60±4.28 (-5.16%)	133.80±8.90 (-15.74%)
Metformin 100 mg/kg	159.40±7.09	179.00±6.44 (12.30%)	132.20±13.44 (-17.06%)	115.40±5.08 (-27.60%)

Data are expressed as mean ± standard deviation (n=5). The values in parentheses indicate the percentage change in blood glucose levels compared to the initial measurement at zero time. A negative sign preceding the values indicates a reduction in blood glucose levels.

Discussion

Plants have long been used as a complementary and alternative source for treating several acute and chronic human diseases. Natural products derived from botanical sources continue to be one of the most important sources of lead compounds for different traditional medicinal systems around the globe and modern allopathic and homeopathic treatment procedures [36]. The use of herbal remedies to lower blood glucose levels in hyperglycemic patients has also been pronounced highly [37]. The primary criteria for the selection of plant source of medicine in diabetes include the stage of progression of the disease, the type of comorbidities the patient is suffering from which is associated with diabetes, local availability of the herbal remedies along with the safety and efficacy profile shown by the utilized parts of the medicinal plants[38]. Based on these criteria, local availability and utility by different ethnic groups, local practitioners, and less scientific exploration, five different medicinal plants were selected in this study. Qualitative and quantitative phytoconstituent determination, free radical scavenging activity study, and *in vitro* and *in vivo* hypoglycemic activity study were performed with few exciting results.

Plant secondary metabolites exhibit different pharmacological and therapeutic efficacy series, which highly depend on their chemical structure and functional group. The therapeutic efficacy of most secondary metabolites is broad and shows direct interaction with the receptors, cell membranes, and

nucleic acids[39]. Among the numerous secondary metabolites, our study qualitatively detected the presence of alkaloids, carbohydrates, saponin, phenols, flavonoids, terpenoids, and tannin, while the study showed a negative glycoside test in most of the extracts. These phytoconstituents each have a unique mechanism for showing a glucose-lowering effect. The highly pronounced mechanism of these phytoconstituents towards glucose lowering property is the reduction of oxidative stress by lowering the free radical concentration[40, 41]. Type I diabetes occurs in animals through the action of free radicals on the pancreatic beta cells, leading to cell death. Though there is the presence of copper and zinc-containing superoxide dismutase (Cu-ZnSOD), manganese-containing superoxide dismutase (MnSOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase to act against free radicals in pancreatic cells, the total antioxidant enzyme activity of the beta cell is low[42]. This shows that the pancreatic β-cells and endothelial cells are highly prone and sensitive to free radicals. Furthermore, an imbalance in the generation of free radicals and their scavenging of those molecules even upsurges oxidative stress[43]. The correlation between the high presence of TPC and TFC, high free radical scavenging property, significant *in vitro* α -amylase inhibitory property, and *in vivo* hypoglycemic activity of ethanolic extracts of **S3** could be correlated with the noticeable free radical scavenging property of the phytoconstituents present in plant sample.

The study depicted that aqueous and ethanolic extract of **S5** showed optimal TPC of 341.75 ± 1.56 and 273.44 ± 1.29 µg GAE/mg of extract, respectively, which is followed by **S3** with the TPC content of 88.46 ± 2.13 and 70.71 ± 4.22 µg GAE/mg of extract respectively. The study of TFC showed that both aqueous and ethanolic extracts of S5 showed maximum flavonoid content digitizing the value of 1153.59 ± 7.05 and 1110.50 ± 3.46 µg QE/mg of extract, respectively, which S3 follows with the TFC of 424.12 ± 2.86 and 224.66 ± 2.86 µg QE/mg of extract respectively. The high TPC and TFC content could correlate to the positive free radical scavenging property. Both aqueous and ethanolic extracts of **S5** showed potent free radical scavenging properties with IC_{50} values of 3.52 µg/mL and 3.65 µg/mL, which is subsequently followed by **S3** with IC_{50} values of 47.64 µg/mL and 58.86 µg/mL respectively. [44]. An ideal free radical scavenger should have one of the following properties as defined by Bors; i) 3 and 5-hydroxyl groups with a 4-oxo function, ii) hydroxy group in the 3, 4 positions on the B ring, which provides stability to the radicals formed and iii) 2,3-positions on the B ring with a double bond which eases in the higher conjugation with other double bonded molecules [45]. Therefore, the phenolic and flavonoid compounds in **S5** and **S3** are predicted to have one of these properties, showing significant antioxidant activity. The aromatic ring bearing one or more hydroxyl constituents scavenges free radicals and lowers oxidative stress[46]. The free radicals scavenging ability of polyphenols is based primarily on their hydrogen or electron donating ability, the stabilization and delocalization of unpaired electrons with polyphenols derived radicals. This is known as the chain-breaking function and their chelation potential towards metal ions. Flavonoids are hydroxylated phenolic moieties with a benzo-γ-pyrone ring in their structure and are widely present in plants[47]. The hydroxyl groups chelate metal ions to scavenge free radicals and show antioxidant activity. Metal ion chelation is crucial in preventing radical generation and damaging target biomolecules[48].

This significant free radical scavenging property of **S5** and **S3** is correlated with the maximum α -amylase enzyme inhibitory activity. The result revealed that aqueous and ethanolic extract of **S5** showed potent α -amylase enzyme inhibition with IC_{50} values of 0.248 mg/mL and 0.196 mg/mL, respectively. Here the aqueous and ethanolic extract of S3 showed more potent α -amylase enzyme inhibition than **S5** depicting the IC_{50} value of 0.168 mg/mL and 0.136 mg/mL, respectively. Though the TPC and TFC of **S5** are higher than that of **S3**, the result outlines the potency of S3 over S5. Studies showed that *S. sonchifolius* is rich in several phytoconstituents, as shown in Fig. 5, known for hypoglycemic and glucose-lowering ability. Caffeic acid, Chlorogenic acid, 3,4-dicaffeoylquinic acid, 3,5-dicaffeolyquinic acid, 4,5-dicaffeoylquinic acid, gallic acid, rosmarinic acid, ferulic acid, P-coumaric acid, protocatechuic acid and quercetin are widely present in *S. sonchifolius* which are widely known for their role towards inhibition of α -amylase enzyme[35]. These phytoconstituents in S. sonchifolius are predicted to subside starch hydrolysis and break them into smaller oligosaccharides like maltose, maltotriose by α -amylase. α-glucosidase further acts on these smaller oligosaccharides, converting to the absorbable form of glucose, which could be transported to the bloodstream by the hepatic portal vein. Significant depletion in the blood glucose level will be the possible outcome when the α-amylase mediated starch hydrolysis is depleted[49]. Therefore, the present study aimed to determine the natural phytoconstituent moieties that down-surge starch hydrolysis in the presence of α -amylase. Our study even proved that the inhibition effect of phytoconstituents on a pancreatic digestive enzyme such as α -amylase depends on the presence of TPC and TFC and in the presence of other phytochemicals such as alkaloids, steroids, terpenoids, saponins, etc. Oleanane, ursane, lupeol, and lupane group of terpenoids represent efficient sources as potentially active natural compounds with α -amylase inhibitory activity[50].

Since the ethanolic extract of **S3** showed potent α-amylase enzyme inhibition, different concentration of ethanolic extract of **S3** was taken for *in vivo* hypoglycemic test. The *in vivo* experiment was performed with normal mice and glucose-loaded hyperglycemic mice. The result showed that ethanolic extract of **S3** showed 17.99% depletion in glucose level at 500 mg/kg concentration in normal mice at 120 minutes of administration. When the same dose of ethanolic extract of **S3** was administered to glucose-induced hyperglycemic mice, the down surge of blood glucose concentration was 15.74% at 120 minutes of administration. As soon as the administration of a different dose of **S3**, there is an upsurge in the blood sugar level, possibly occurring in the presence of the reducing sugar content in the plant sample or as a physiological phenomenon. The binding of active hit and lead molecules in the plant sample to different glucose uptaking receptors at physiological conditions prevents the excessive glucose release in blood showing hypoglycemic activity. Few *in silico* and molecular docking studies showed that the antidiabetic macromolecules targets of several phytoconstituents are dipeptidyl peptidase-4 (DPP4), protein tyrosine phosphatase-1B (PTP 1B), glucokinase, as well as α -glucosidase.

Similarly, phytoconstituents such as caffeic acid produce decrement in the glucose level markedly in diabetic rats, while chlorogenic acid upsurges glucose tolerance and decreases insulin resistance in obese rats. Few studies reported that the phytoconstituents increase the glucokinase and glucose-6-phosphate dehydrogenase activities and decrease glucose-6-phosphatase activity in diabetic rats, playing

a significant role in the homeostasis of glucose. Therefore, among several mechanisms of action, decreasing gluconeogenesis and oxidative stress are two highly pronounced mechanisms by which active phytoconstituents show a glucose-lowering effect[51]. Furthermore, in *in vivo* physiological conditions, phytoconstituents show hypoglycemic activity by subsiding transformation polysaccharides and oligosaccharides to glucose in hepatic cells, uprising insulin production, enhancing uptake of glucose by adipose tissue as well as skeletal muscle, and working as a barrier to transport glucose across intestine[40]. Few studies were performed at the molecular level, which involves pancreatic and duodenal homeobox 1 (PDX-1), Insulin 1(Ins-1), neurogenin-3 (ngn-3), Glucose transporter type-4 (GLUT-4) and Insulin receptor substrate 1 (IRS-1) in insulin signaling pathway and Tumor necrosis factor receptor-associated factor (Traf-4), Tumor necrosis factor receptor-associated factor (Traf-6) and Mitogen-activated protein kinase-8 (MAPK) downstream Jun N-terminal kinase (JNK) cascade. The results from the study revealed that oxidative stress in pancreatic β cells was reduced by rejuvenating antioxidant property of the phytoconstituent, reducing the mRNA expression of (MAPK-8), Traf-4, and Traf-6, and increasing the Pdx-1, Ins-1, ngn-3, GLUT-4, and IRS-1 expression ensuing regeneration of β cells and subsequent insulin release from the pancreas[52]. Furthermore, the retardation of blood glucose levels *in vivo* is even attributed to the physical barrier and obstacle property of the phytoconstituent entrapping glucose molecules inside the networked fiber, preventing a rise in the postprandial glucose level[53].

Though our study was not carried out at the molecular level to determine the actual mechanism of action, we could predict that one or more of the mentioned pathways were utilized by the plant sample to show the hypoglycemic property. Followingly, though the treatment of diabetes mellitus is associated chiefly with managing hyperglycemia, the approach towards reducing oxidative stress associated with reactive oxygen species (ROS) and reactive nitrogen species (RNS) support essentially to defends β-cells of the pancreas against oxidative stress and decreases diabetic complications. Therefore, this study indicated that powerful free radical scavengers could also serve as hypoglycemic agents. Our study also highlighted the potent α -amylase enzyme inhibition activity of **S5** and **S3**, and the significant in vivo hypoglycemic property of **S3** is attributed to free radical scavenging properties and some other mechanistic pathways mentioned above.

Conclusion

Hyperglycemia and hence hyperglycemic complications are profound medical threats, demanding the need to find specific and effective treatment strategies from natural sources. Exploring the chemical compounds from plant species to find a mechanistic pathway to highlight their hypoglycemic role and efficacy to mitigate diabetic complications will support further the development of an effective treatment regimen. This study studied five plant species to highlight their *in vitro* and *in vivo* anti-diabetic effects. The study showed that *F. megacarpum* and *S. sonchifolius* showed potent in vitro α-amylase enzyme inhibition, and *S. sonchifolius* depicted significant hypoglycemic activity during *in vivo* animal study. Further study at the molecular level to isolate active hit and lead molecules from these plant species and determine the isolated chemical compounds' binding sites and regions by *in silico* study and docking strategy are encouraged. Determining the actual mechanistic pathway showing antidiabetic activity for

each compound isolated from specific plant sample help in finding potent hit and lead molecules that could be later utilized in the pharmaceutical setting.

"Medicinal plants heal more than the physical body. They help open our hearts, reconnect us with spirit, and guide us in developing respectful and joyful relationships with the natural world."

-Deb Soule

Abbreviations

α -amylase: Alpha-amylase ((1,4-α-D -Glucan-glucano hydrolase)

DPPH: 1,1 Diphenyl-2 picryl hydrazyl radical

α -napthol: Alpha-napthol

DM: Diabetes mellitus

OFR: Oxygen free radicals

TPC: Total phenolic content

TFC: Total flavonoid content

λ: wavelength

mg/mL: milligram per milliliter

µg/mL: microgram per milliliter

UV: Ultraviolet

IC_{50}: Half-maximal inhibitory concentration

RO: Reverse Osmosis

RH: Relative Humidity

OGTT: Oral Glucose Tolerance Test

GAE: Gallic acid equivalent

OGTT: Oral glucose tolerance test

ROS: Reactive oxygen species

RNS: Reactive nitrogen species

PDX-1: Pancreatic and duodenal Homeobox 1

DPP4: Dipeptidyl peptidase-4

PTP 1B: Protein tyrosine phosphatase-1B

Ins-1: Insulin-1

Ngn-3: Neurogenin-3

GLUT-4: Glucose transporter type-4

IRS-1: Insulin receptor substrate 1

Traf-4: Tumor necrosis factor receptor-associated factor-4

Traf-6: Tumor necrosis factor receptor-associated factor-6

MAPK-8: Mitogen-activated protein kinase-8

JNK: Jun N-terminal kinase

Declarations

Ethics approval and consent to participate

The ethical approval to carry out *in vivo* animal experiment was obtained from Pokhara University Research Center and Institutional Review Committee (IRC). Identification of herbaria were performed by the registered taxonomist in National Herbarium and Plant Laboratory, Godawari, Kathmandu Nepal.

Consent for Publication

Availability of data and material

The datasets generated and/or analyzed during the study are available from the corresponding author on reasonable request.

Competing interests

The author declare that they have no competing interests.

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Authors' Contributions

PR, SP: Conceptualization. PR, RB, SP: Methodology. PR, RB: Investigation. RB, PR: Writing-original draft preparation. PR, RB, SP: Writing-review and editing. SP: Supervision and project administration. All authors have read and approved the final manuscript.

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Figures



Figure 1

Medicinal plants selected for the study. *The entire figure represents selected medicinal plants in their ready-to-extraction form. Each sample was rinsed quickly after being collected, cut into small fragments, and shaded dried for complete moisture removal before extraction.*

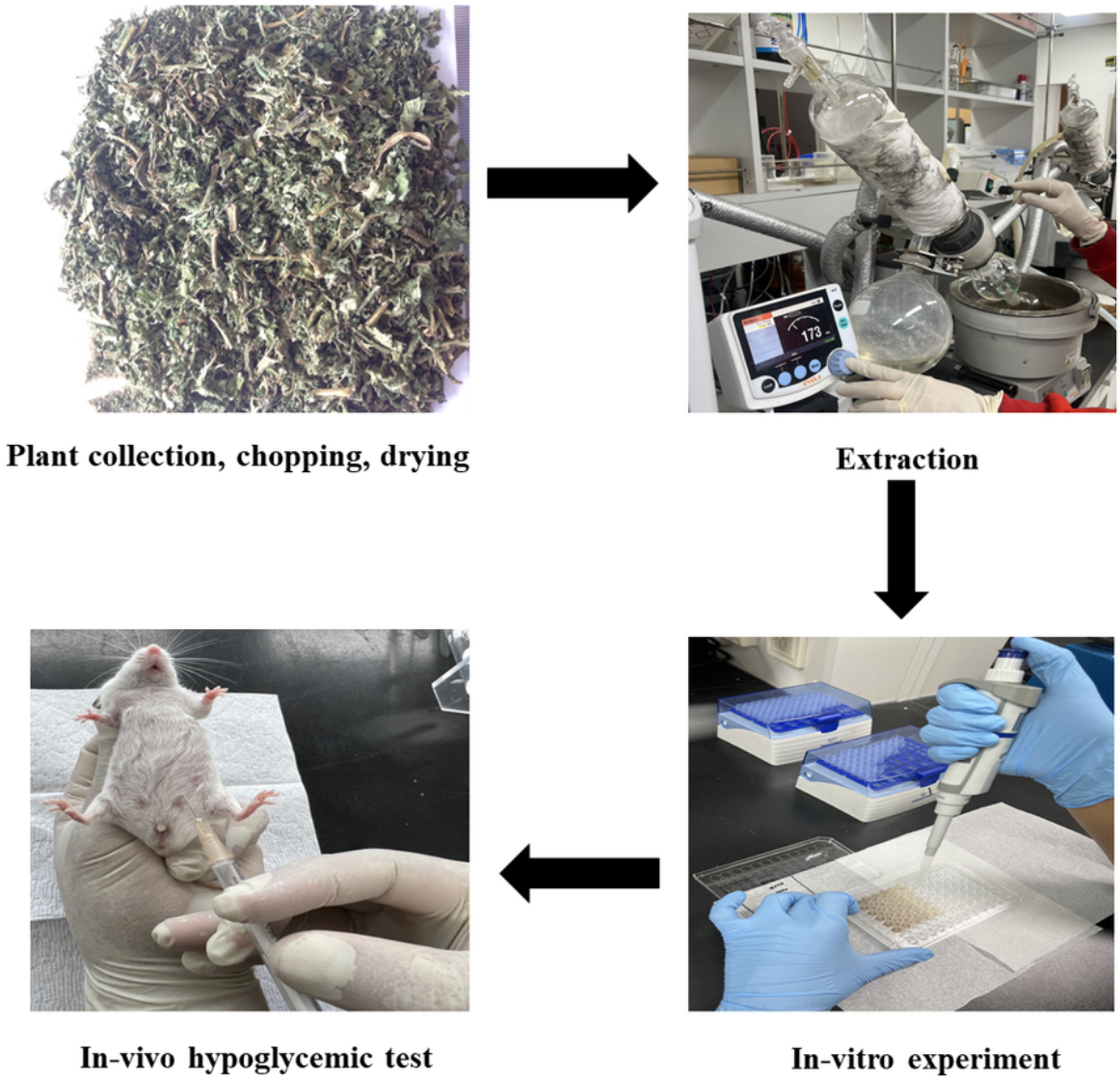


Figure 2

Schematic diagram showing the general framework of the experiments carried out during the study. *Standard protocols and ethical guidelines were followed correctly during the experiments. Appropriate validation and calibration of equipment were performed, and standard aseptic and sterile conditions were maintained whenever required. Reference standards were taken in each experimental condition to verify the experiment performed.*

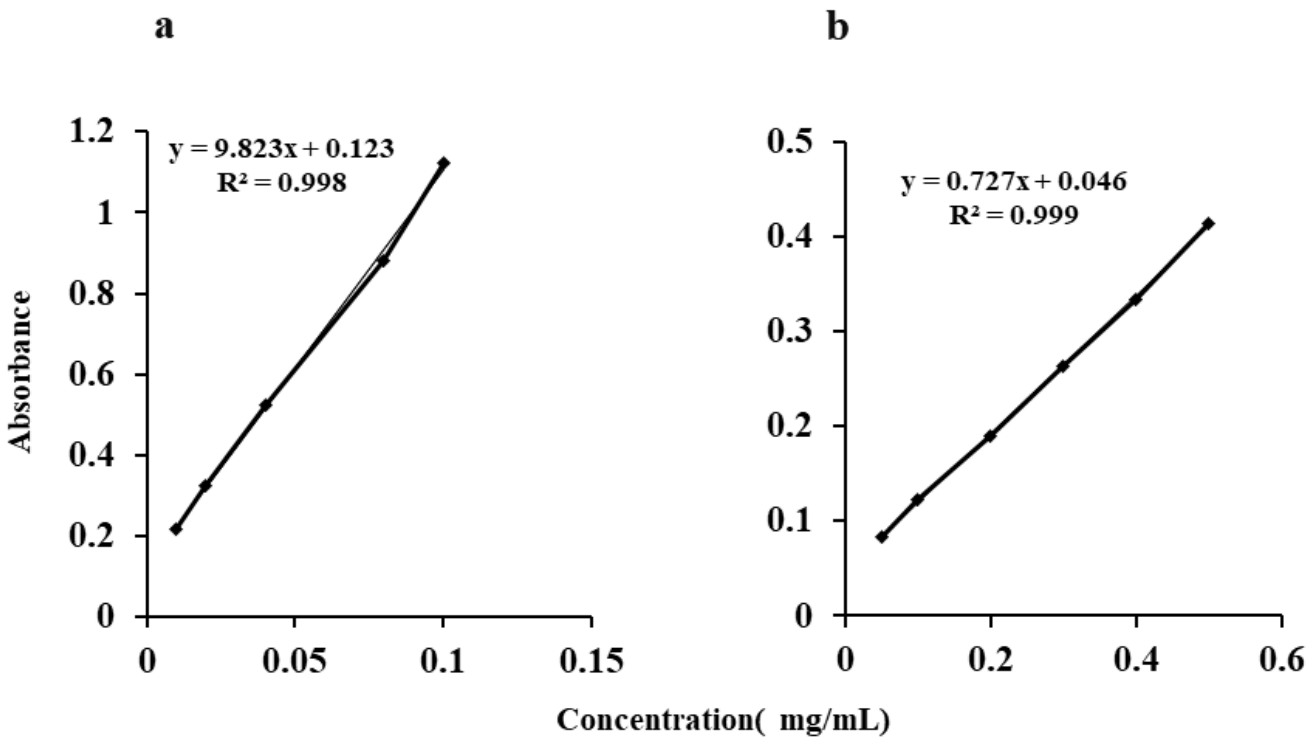


Figure 3

Calibration curve for Gallic acid and quercetin. A) *The figure represents the calibration curve of gallic acid for TFC; the concentration range of gallic acid taken is 0.01 mg/mL to 0.1 mg/mL. The standard regression equation obtained was used for the determination of TFC.* ***B)*** *The figure represents the calibration curve of quercetin for TPC, and the concentration range of quercetin taken is 0.05 mg/mL to 0.5 mg/mL. The standard regression equation obtained was used for the determination of TPC.*

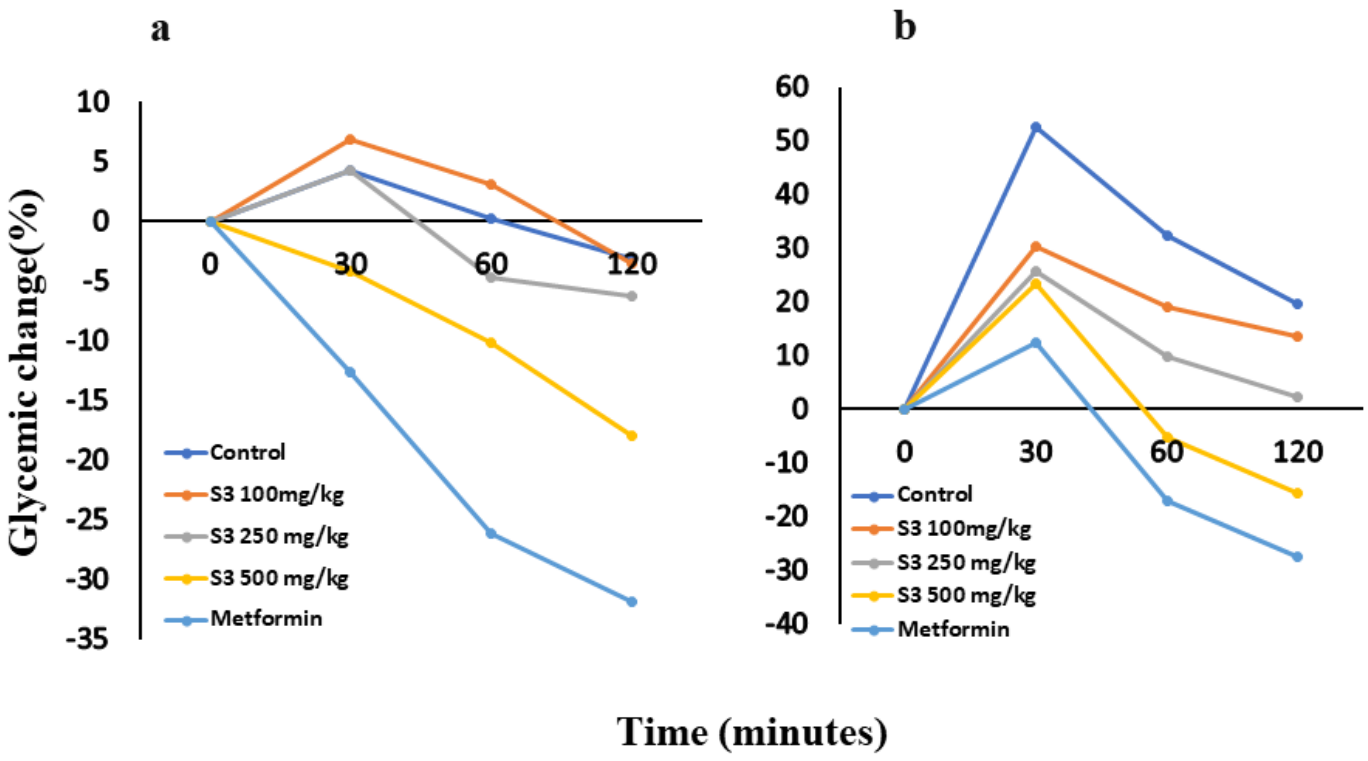


Figure 4

Percentage of Glycemic change after administration of a different dose of ethanolic S3 at a different time interval. A) *The figure represents a change in the blood glucose concentration of normal mice after administering different doses of S3 at different time intervals of up to 120 minutes.* ***B)*** *The figure represents an alteration in the blood glucose concentration of glucose-induced hyperglycemic mice after administering different doses of S3 at different time intervals of up to 120 minutes. Metformin was taken as a reference standard in both in vivo experimental conditions.*

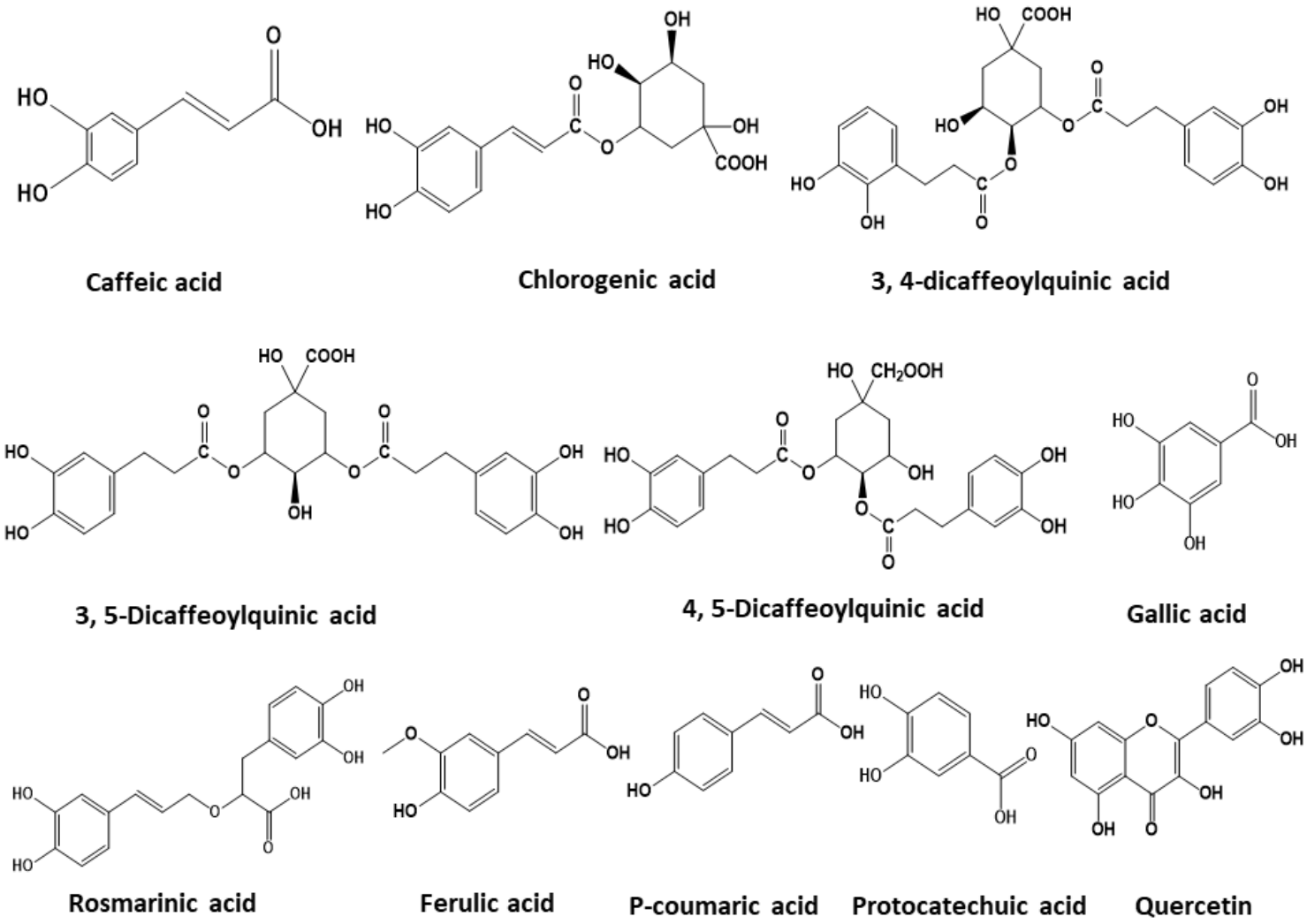


Figure 5

Chemical structures of active phytoconstituents present in *S. sonchifolius* showing hypoglycemic activity[35]. *The structures are drawn with ChemDraw (version chemdraw ultra 12.0). The hypoglycemic activity of most of these chemical compounds is associated with their free radial scavenging property.*