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Qualitative and Quantitative Phytoconstituent Determination, DPPH Free Radical Lowering Effect and *in vitro* Hypoglycemic Activity Study by Alpha-amylase Enzyme Assay along with Membrane Diffusion Technique

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Abstract

Diabetes mellitus, a physiological disorder is characterized by low secretion of insulin due to the attack in insulin producing beta cell (Type I) or the body cell become insulin resistance (Type II). This study was designed to evaluate the DPPH lowering effect, in-vitro alpha amylase and glucose diffusion inhibition of the selected medicinal plants. Five different plant sample Amomum subulatum, Choerospondias axillaris, Musa sp, Myrica esculenta and Nephrolepis cordifolia were taken for the study. From the result it was revealed that the methanol extracts of Myrica esculenta stem bark and small branches showed potent DPPH free radical scavenging activity with the IC50 value of 4.23 µg/ml and 3.14 µg/ml respectively which is almost comparable to standard Ascorbic acid taken. Meanwhile, alpha-amylase inhibitory study showed that Myrica esculenta stem bark showed potent subsidiary effect on methanol extracts with IC50 value of 0.96 mg/ml which is comparable to standard volgibose taken. Lastly, membrane diffusion study with glucose and plant sample showed that Amomum subulatum seed and Choerospondias axillaris fruit have potent glucose diffusion inhibition with highest GDRI %. From the result, it could be correlated that the free radical scavenging activity and glucose lowering effect of these plant extracts is due to the presence of phytoconstituents like phenolics, flavonoids, alkaloids, terpenoids, glycosides saponins etc. as well as several other uncompetitive modes of inhibition.

Keywords: Diabetes mellitus, free radical scavenging, alpha amylase, glucose diffusion, phytochemicals

1. Introduction

Diabetes mellitus is a group of physiological disorder which is characterized by low secretion of insulin due to irreversible damage of insulin producing β-cell of pancreas due to of autoimmune disorder (Type I). Likewise, target cell of insulin become insulin resistance (Type II) or excessive glucagon secretion which results in elevation of blood glucose level (Blair, 2016). This increment in the level of blood glucose leads to several other life threatening complications such as damage to small and large blood vessels, cardiovascular disease (CVD), neuropathy, retinopathy and nephropathy (Zarkogianni et al., 2015). According to IDF diabetes atlas, the global prevalence of diabetes in adult is 8.8% and it is predicted to rise up to 10.4% until 2040 with around 16.2% live birth associate with hyperglycemia during pregnancy (Whiting et al., 2011). Similarly, vascular complications during diabetes are particularly caused by oxidative stress. Oxidative stress is particularly elevated in diabetic patient due to inability of endogenous antioxidant system such as catalase, superoxide dismutase and glutathione peroxidase enzyme to neutralize the increased production of Reactive Oxygen Species (ROS). Therefore, targeting ROS induced oxidative stress is also an effective strategy for the treatment of Diabetes Mellitus (Asmat et al., 2016).

Among, various enzymes present in human body as a part of digestive system, pancreatic alpha amylase is one of the calcium metalloenzymes which cannot function in the absence of calcium (Agarwal et al., 2016). Alpha-amylase catalyzes hydrolysis of the α-1, 4 glycosidic linkages in starch, amylopectin, amylose, glycogen and numerous maltodextrins in polysaccharides (Nair et al., 2013). Hydrolysis of such complex polysaccharides leads to the formation of smaller monosaccharides which are absorbable by different modes of passive and active transport through the intestinal barrier and reach the blood circulation (Ernest et al., 2012). This leads towards the rapid increment in the level of blood glucose causing Post Prandial Hyperglycemia (PPH) which is the indicator of Type II diabetes mellitus. Therefore, for controlling the Type II diabetes mellitus two major enzyme alpha-amylase and alpha glucosidase are the effective targets to inhibit their catalytic activity in the breakdown of complex polysaccharides to smaller monosaccharides (Dastjerdi et al., 2015).

Meanwhile, the diffusion of glucose across the intestinal membrane to the blood circulation can be inhibited with the use of complex polysaccharides and various dietary fibers. With the inhibition of such glucose diffusion from the intestinal lumen to the blood leads to the decrement of postprandial blood glucose level (Shadhan et al., 2017).

Plants have always been an exemplary sources of drugs and many of the currently available drugs have been derived directly or indirectly from them (Arumugam et al., 2013). Meanwhile, for the reduction of blood glucose level various medical plants are being used either as a dietary adjuvant or in the form of crude extract. Some plant secondary metabolites like alkaloids, carbohydrates, glycosides, flavonoids, steroids, terpenoids, peptides, phenolics have shown blood glucose lowering effect revealing their anti-diabetic potential. The antidiabetic effect shown by herbal medicine is either due to the insulin like activity or affecting insulin secreting beta cell or by modifying glucose utilization (Gulati et al., 2012a).

Despite widespread use of herbal medicine in Nepal for treatment of various bodily ailments, scientific study to prove the safety, efficacy, quality control and other aspects are limited (Kunwar et al., 2010). In this study five different medicinal plants which are being used as herbal remedies by different indigenous group of Nepal for treatment of diabetes complication were

evaluated. The plants are Greater Cardamom or Large Cardamom (*Amomum subulatum* Roxb.), Lapsi (*Choerospondias axillaris* Roxb.), Banana (*Musa* sp.), Box berry or Kafal (*Myrica esculenta* Buch. - Ham.ex D Don), Pani Amala (*Nephrolepis cordifolia* (L). C. Presl).

Greater cardamom or large cardamom (*Amomum subulatum* Roxb.) member of Gingeberaceae family is a well-known flavoring spice, used to treat various ailments in different medicinal system all over (Verma et al., 2012). *Amomum subulatum* is used traditionally for analgesic properties, antimalarial properties, antimicrobial properties, antioxidants, anti-diabetic and anthelmintic activities (Verma et al., 2012). Lapsi (*Choerospondias axillaris* Roxb.) belonging to family Anacardiaceae is a popular fruit tree of Nepal and many other Asian countries. In a study by Puja et al. 2018, it was reported that *Choerospondias axillaris* fruit juice is being used traditionally for the glucose lowering effect by various indigenous group of Nepal (Shrestha et al., 2018).

Musa sp. (Musaceae) called Banana in English, are one of the interesting tropical plants which have been consumed since centuries by human and animals as a nutritious food. In a study on *Musa sapientum*, mainly used in Indian folk medicine for the treatment of diabetes mellitus, oral administration of chloroform extract of the banana flowers in alloxan induced diabetic rats for 30 days resulted in a significant reduction in blood glucose, glycosylated hemoglobin and an increased in total hemoglobin (Orhan, 2001a). *Myrica esculenta* (Myricaceae) commonly known as box berry or Kafal is an important Asian medicinal plant. It is found in foothill track of eastern Himalayas Meghalayas, Nepal, China, and Pakistan. Stem bark of *M. esculenta* is reported for various pharmacological activities like radical scavenging, antioxidant, anti-diabetic, anxiolytic, anti-bacterial, anti-helminthic, allergic, anti-inflammatory, anti-microbial, mass cell stabilizing and anti-asthamatic (Srivastava et al., 2016).

Nephrolepis cordifolia is a member belonging to the Nephrolepidaceae family. It grows on hillside, riverside etc. It is adaptable and abundant in resources. It is traditional folk medicine. Modern medical experiment shows that the water extracts from the tuber of *N. cordifolia* possess significant blood glucose lowering activity. It opens up broad prospects for this kind of medicine for having anti-diabetic activity (Chai et al., 2015). These plants were collected from the Kaski district of Nepal. This study is focused for qualitative and quantitative determination of phytoconstituents, DPPH radical scavenging effect, digestive enzyme inhibitory study along with membrane permeability study of glucose in presence of plant extracts.

2. Materials and Methods

Chemicals and Reagents: Positive control inhibitor Volgibose IP was provided from Asian Pharmaceuticals Pvt. Ltd, Bhairahawa, Nepal as bounteous gift. Purified anhydrous Dextrose, Potassium sodium tartrate tetra hydrate, Di-sodium hydrogen phosphate, Benzene, Copper sulphate pentahydrate, Sodium anhydrous, 1-napthol were bought from Merck specialties, India. Central drug house, India provided 3,5-Dinitrosalicyclic acid while 1,1 Diphenyl-2 picryl hydrazyl radical (DPPH) was procured from Tokyo Chemical Industry, Japan. Dimethyl sulfoxide (DMSO), Benedict's reagent, Hydrochloric acid, Mercuric chloride, Sodium hydroxide pellets and starch were purchased from Thermo Fischer Scientific, India. Himedia Laboratories, India delivered the dialysis membrane and L-ascorbic acid on placement of order. All chemicals and reagents used were of analytical reagent grade.

Collection and identification of plant samples: Medicinal plants listed on Table 1

were gathered from numerous locations of western Nepal. Botanist of National Herbarium and Plant Laboratories, Godawari, Kathmandu, Nepal helped in the plant identification after observing the prepared herbaria. Crude drug museum of School of Health and Allied Sciences, Pokhara University in housed the voucher specimen of all collected medicinal plants. Plants samples were cleaned, chopped into small pieces, and left in shed for drying. Moisture was removed from the samples by placing the samples in hot air oven at 40˚C. Weight variation test determined the complete moisture removal and fine powdering was done with the help of grinder.

Table 1. List of plants

Scientific Name	Family	Local Name	Parts Used	Crude Drug Voucher No.	Sample No.
Amomum subulatum Roxb.	Zingiberaceae	Alaichi	Seed	PUCD-2019-12	S1
			Outer cover	PUCD-2019-13	S2
Choerospondias axillaries (Roxb.) B.L. Burrt	Anacardiaceae	Lapsi	Fruits	PUCD-2019-14	S3
Musa sp.	Musaceae	Kera	Leave	PUCD-2019-15	S4
			Leaves vein	PUCD-2019-15	S5
Myrica esculenta Buch. - Ham.ex D Don	Myricaceae	Kafal	Stem bark	PUCD-2019-16	S6
			Small branches	PUCD-2019-17	S7
Nephrolepis cordifolia (L). C. Presl	Nephrolepidaceae	Pani amala	Fruits	PUCD-2019-18	S8
			Leaves	PUCD-2019-19	S9

Sample Extraction: Single maceration for 24 hours was done for the extraction of crude drugs. The sample was macerated with hexane and methanol extracts separately and ratio of sample and solvents is 1:5 (w/v). Subsequently, filtration of the extract was followed by the Soxhlet extraction to obtain concentrated extracts after removing the residual solvent.

Qualitative Phytochemical Screening: Phytochemical screening was done as described by Anthony et al. 2013 to detect presence/ absence of secondary metabolite such as Alkaloid, Carbohydrate, Glycoside, Saponins, Phenolics, Flavonoids, Tannins, Terpenoids in the plants extract (Adegor et al., 2013).

Quantitative Phytochemical screening

Total phenolic content (TPC): TPC was determined by the Folin-Ciocalteu method as reported earlier by Juan Carlos et al. 2013, (Abreu-Villela et al.). The experiment employed the mixing of 5 ml of distilled water in test tubes containing 1 ml of sample along with 1 ml of Folin reagent. The tubes were incubated for 5 minutes and 1 ml of 10% 10% Na_2Co_3 was dropped. This was further followed by the incubation for another 1 hour in the dark at room temperature. Absorbance was measured at wavelength 725 nm. Experiments were performed in triplicate (n=3) and mean along with the standard deviation values were considered during the calculation. µg of Gallic acid equivalent per mg (GAE/mg) of extract expressed the TPC.

Total flavonoid content (TFC): Aluminum chloride method was employed to determine TFC as given by Mooza et al. 2014. 0.2 ml of 5% sodium nitrite solution was mixed with 4 ml of distilled water

which was subsequently added to the test tube containing 1 ml of plant extract. The tubes were incubated in dark for 5 minutes. Then, 0.2 ml of 10% of aluminum chloride and 2 ml of 1 M sodium hydroxide was added. Followingly, the absorbance was measured with UV-Vis spectrophotometer at λ = 510 nm. Experiments were performed in triplicate (n=3) and mean along with the standard deviation values were considered during the calculation. µg of quercetin equivalent per mg (QE/mg) of the plant extract expressed the TFC.

Antioxidant Activity Study

DPPH Free Radical Scavenging Activity: The method described Marinova et al. 2011 was taken as reference protocol to study the DPPH free radical scavenging activity (Marinova et al., 2011). To describe it in brief, 2ml plant sample of different concentration ranging from 1 µg/ml to 100 µg/ml was mixed with 2 ml of DPPH solution of concentration 60 µM. Reaction was completed after the incubation of the mixture for 30 minutes in dark. At the end, the absorbance obtained at 517 nm was taken to calculate the relative free radical scavenging effect at each sample concentration. Finally, following formula converted the relative scavenging effect into the %DPPH Scavenging activity:

$$\%\ DPPH\ Scavenging\ activity = \frac{Abs_{\ control} - Abs_{sample}}{Abs_{\ control}} \times 100\%$$

Where,

$Abs_{control}$ = Absorbance of control

Abs_{sample} = Absorbance of sample

Alpha- Amylase Inhibition Assay: The protocol defiend by Nyambe-Silavwe et al. 2015 was taken as reference for Alpha-inhibition assay by starch iodine method (Nyambe-Silavwe et al., 2015). Briefly describing, plant samples of concentration ranging from 0.25 mg/ml upto 2 mg/ml was mixed with alpha amylase solution of concentration 0.025 mg/ml. With the 10 minute incubation at 37°C, 1 % starch solution was added and the sample was incubated again for 1 hour in dark. Addition of 1% iodine solution was followed with the dilution of test tube with 5 ml of distilled water. Absorbance at 565 nm was converted into the relative alpha amylase enzyme inhibition effect of the plant extract. Volgibose IP was taken as reference standard. Test tubes containing buffer instead of enzyme was used as a background signal. Test tubes containing buffer instead of starch but having enzyme were taken as tracer control. Following formula converted the absorbance into % inhibition of enzyme activity.

$$Inhibition\ of\ enzyme\ activity(\%) = \frac{(A - C)}{(B - C)} * 100\%$$

Where,

A= Absorbance of sample

B= Absorbance of blank (no alpha amylase)

C= Absorbance of control (no starch)

Glucose Diffusion Inhibition Assay

Measurement of Glucose Diffusion Inhibition Activity: Few modifications on the protocol of Archit et al. 2013 was done to determine the glucose diffusion inhibitory activity of the plant samples (Archit et al., 2013). Dialysis membrane (12000 MW cutoff) of 5 cm was taken for the study purpose after the removal of the sulphate content of the membrane by boiling in water for 25-30 minutes. The membrane was then loaded with dextrose solution 5 % containing 0.15 M NaCl and different concentration of plant extracts. Both ends of the membrane were tied by the nylon thread. RO water replaces the plant extracts in negative control. 250 ml conical flasks containing 40 ml of 0.15 M NaCl, and 10 ml of RO water were prepared for each sample inside the membrane. NaCl aided in the equalization of the internal and external strength. Water bath shaker was

maintained at temperature of 37°C and 100 rpm and prepared conical flasks with samples were placed over it. Sample was withdrawn from each conical flask on regular time interval and glucose concentration was determined on every half an hour time interval. The process was repeated thrice to determine the reproducibility of the experiment.

3,5 Dinitro salicylic Acid (DNSA) Method to determine the reducing sugar from sample withdrawn 500 µl of samples were mixed with 1 ml of DNSA reagent and 2 ml of deionized water. To initiate the reaction between the glucose present in sample and DNSA, the tubes were placed on 95 °C for 5 minutes. Color change from yellow to reddish is the indicator of the reaction process. This was followed by cooling the sample thoroughly with the addition of RO water of 3.5 ml to each tube again. Absorbance measured at 540 nm gave the glucose content in the solution after subtracting the background signal. The natural glucose present on the plant sample might cause the experimental outcome to be errorful. Therefore, plant sample and DNSA was mixed outside the membrane and the resulted absorbance was converted to determine the glucose content on the plant samples. The glucose concentration determined was converted into Glucose Diffusion Retardation Index by using following formula:

$$GDRI\% = 100 - \frac{glucose\ content\ with\ addition\ of\ sample}{glucose\ content\ of\ control} * 100\%$$

Standard calibration curve was plotted with different concentration of dextrose in the range from 0.1% to 10%. This curve was taken as reference standard to determine the concentration of glucose as dextrose equivalent from the obtained graph.

3. Results

Qualitative Phytochemical Screening: Secondary metabolite (phytoconstituents) present in Hexane and methanol plant samples were determined by qualitative method and the result obtained are listed in **Table 2** and **Table 3.** From, the result it is seen that phenol, saponin and flavonoids are the secondary metabolites present in Hexane extract. The result shows that **S8** and **S9** possesses most of the phytoconstituents and leave extract of **S9** contains alkaloid in it. The result shows that Methanol extracts of plant samples contain almost all the phytoconstituents. Mayer and Wagner test revealed that all plant samples except **S4** and **S5** have alkaloid in them. Similarly, most of the methanolic plant extracts possesses glycoside, saponin, phenol, flavonoid, tannin and terpenoid. *Myrica esculenta* (bark and small branches), *Amomum. subulatum* and *Musa sp.* have almost all tested phytoconstituents.

Table 2. Phytochemical Analysis of Hexane Extracts of Plants Samples

Phytochemical Constituents	Specific Tests	S1	S2`	S3	S4	S5	S6	S7	S8	S9
Alkaloid	Mayer	-	-	-	-	-	-	-	-	+
	Wagner	+	+	-	-	-	-	-	+	+
Carbohydrate	Molish	-	-	-	+	-	-	-	+	-
	Benedict	-	-	-	-	-	-	-	-	-
Glycoside	Modified Borntrager	-	-	-	-	-	-	-	-	-
Saponin	Foam	+	+	+	+	+	+	+	+	+
Phenol	Ferric Chloride	+	+	-	+	+	+	-	+	-
Flavonoid	Alkaline Reagent	-	+	+	+	-	+	+	+	-
Tannin	Gelatin	-	-	-	-	-	-	-	-	-
Terpenoid	Salkowaski	+	+	+	-	+	-	-	-	-

Table 3. Phytochemical Analysis of Methanol Extracts of Plants Samples

Phytochemical Constituents	Specific Tests	S1	S2	S3	S4	S5	S6	S7	S8	S9
Alkaloid	Mayer	+	+	+	+	-	+	+	+	+
	Wagner	+	+	+	+	-	+	+	+	+
Carbohydrate	Molish	+	-	-	+	+	+	+	-	-
	Benedict	-	-	-	-	-	-	-	+	+
Glycoside	Modified Borntrager	+	+	-	+	+	+	+	+	-
Saponin	Foam	+	+	+	+	+	+	+	+	+
Phenol	Ferric Chloride	-	-	+	+	+	+	+	+	-
Flavonoid	Alkaline Reagent	+	+	-	+	+	+	+	-	-
Tannin	Gelatin	+	+	+	+	+	+	+	+	+
Terpenoid	Salkowaski	+	+	+	+	+	+	+	+	+

Quantitative phytochemical screening

Total Phenolic content (TPC)

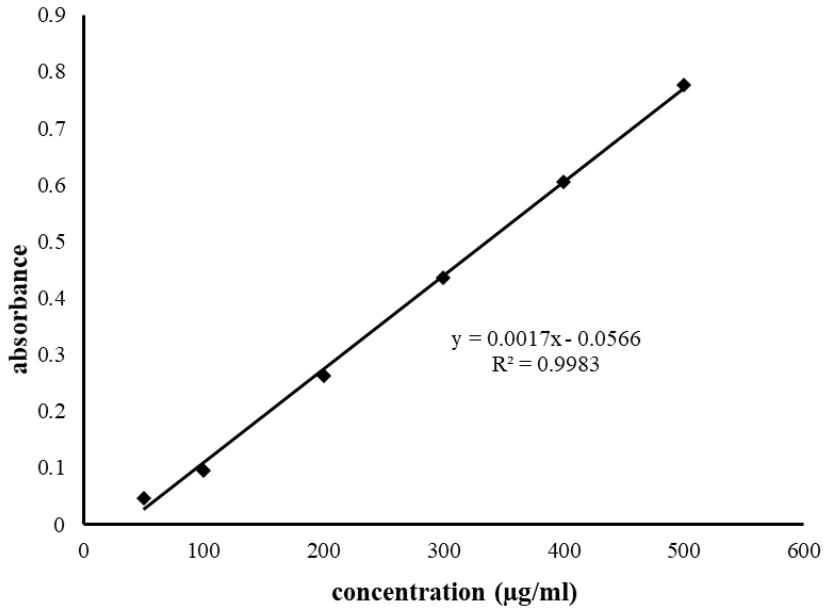

Figure 1. Calibration curve of gallic acid for total phenolic content.

Table 4. Total phenolic expressed as µg GAE/mg extracts

Sample	Total phenolic content (µg GAE/ mg ± SD) of plant extracts	
	Hexane	Methanol
S1	23.02±0.12	56.31±1.89
S2	12.56±0.46	101.25±2.41
S3	27.19±0.78	160.32±1.63
S4	63.41±0.29	168.58±1.57
S5	45.21±1.29	178.42±1.48
S6	72.09±0.95	180.21±0.79
S7	18.73±1.59	154.21±0.83
S8	24.10±0.72	174.61±0.79
S9	14.21±0.87	89.21±2.01

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

Results of TPC contents are expressed as µg gallic acid equivalent per mg of extract. Among the selected plant samples the methanol extracts of **S6** showed the highest TPC of 180.42±1.48 µg GAE/mg of extracts which is followed by the 178.58±1.57 µg GAE/mg of extracts of **S5**. The calibration curve of different concentration of gallic acid is shown in **Figure 1** and the phenolic content of each plant samples is given in **Table 4**.

Total flavonoid content: Results of TFC are expressed as µg quercetin equivalent per mg of extract. Among the selected plant sample the sample **S6** showed the maximum content of total flavonoid given as 301.51±1.89 µg QE/mg of extracts which is followed by 296.42±2.58 µg QE/mg of extracts in **S5**. The calibration curve of different concentration of gallic acid is shown in **Figure 2** and the flavonoid content of each plant samples is given in **Table 5**.

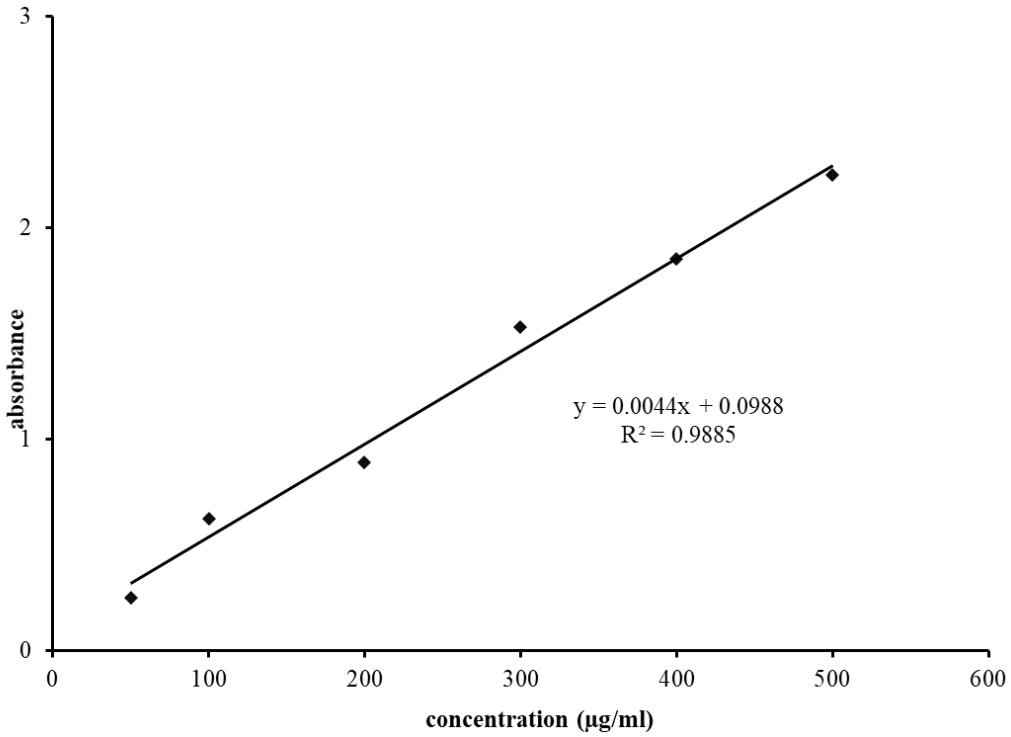

Figure 2. Calibration curve of quercetin for total flavonoid content.

Table 5. Total flavonoid content expressed as µg QE/mg extracts

Sample	Total flavonoid content (µg QE/ mg ± SD) of plant extracts	
	Hexane	Methanol
S1	30.21±1.47	264.53±1.28
S2	42.19±7.28	278.21±4.51
S3	51.28±1.49	156.31±2.81
S4	64.78±1.75	289.51±1.89
S5	54.72±0.47	296.42±2.58
S6	81.24±5.26	301.72±1.01
S7	78.21±2.15	294.58±2.51
S8	57.96±1.24	142.21±1.21
S9	37.41±0.78	124.63±1.15

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

DPPH Free Radical Scavenging Activity: DPPH free radical scavenging method evaluated the electron contributing ability or the free hydrogen atom. The scavenging effects of plant extracts at different concentration are shown in **Table 6** and **Table 7**. Hexane extracts showed very poor DPPH free radical scavenging activity. **S3** fruits showed maximum inhibition of DPPH free radical which is 62.23±2.03 at 100µg/ml with IC_{50}value of 80.06 µg/ml.

Table 6. DPPH Free Radical Scavenging Activity and IC_{50} of Hexane Extracts of Selected Plants

Plants	Concentration			IC_{50}
	1 µg/ml	10 µg/ml	100 µg/ml	
S1	1.25±0.76	4.31±0.5	12.76±0.25	>100
S2	2.69±1.27	5.03±0.5	9.89±1.27	>100
S3	3.23±2.03	7.01±1.27	62.23±2.03	80.06
S4	0.36±0.5	2.87±2.54	9.53±3.3	>100
S5	4.49±0.76	9.17±1.78	12.05±0.25	>100
S6	4.86±0.25	10.25±2.79	51.61±0.76	96.48
S7	1.07±0.5	2.33±1.78	12.41±1	>100
S8	1.8±0.5	3.78±1.78	2.48±2.7	>100
S9	0.71±0.5	7.73±1.27	9.1±5.95	>100
Ascorbic acid	23.96±4.26	96.34±0.22	97.62±0.22	>4.23

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

Table 7. DPPH Free Radical Scavenging Activity and IC_{50} of Methanol Extracts of Selected Plants

Plants	Concentration			IC_{50}
	1 µg/ml	10 µg/ml	100 µg/ml	
S1	20.48±4.71	26.34±0.89	40.15±5.61	>100
S2	16.98±2.02	19.52±0.67	39.04±2.69	>100
S3	21.11±2.46	40.95±1.34	89.52±0.44	26.77
S4	26.34±3.59	43.33±1.12	84.92±2.91	24.43
S5	16.5±0.44	18.41±1.34	25.87±1.57	>100
S6	24.92±1.12	94.76±1.57	96.03±0.22	4.23
S7	36.35±5.19	93.8±0.22	94.44±0.22	3.13
S8	26.98±2.69	34.29±0.44	95.07±0.22	33.26
S9	18.09±0	26.98±2.69	73.68±4.04	54.36
Ascorbic acid	23.96±4.26	96.34±0.22	97.62±0.22	4.23

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

The result revealed that most of the methanolic plant extracts are potent DPPH free radical scavenger. Methanol extract of S6 and S7 showed IC50 value of 4.23 µg/ml and 3.14 µg/ml respectively which is comparable to ascorbic acid standard. S8, S3 and S4 also showed potent inhibitory activity with IC_{50} value of 33.26 µg/ml, 26.76 µg/ml, 24.43 µg/ml, respectively.

In vitro Alpha-amylase Inhibition Activity: *α*- amylase inhibition assay was performed using spectrophotometric assay at 540nm. The inhibitory effects of hexane and methanol extracts at different concentration and solvents are shown in **Table 8** and **Table 9**. Hexane extracts showed minimum alpha-amylase inhibition at the concentration up to 2mg/ml**.**

Table 8. Alpha-amylase Inhibitory Activity and IC_{50} Value of Hexane Extracts of Selected Plant Samples

Plants	Concentration				IC_{50}
	0.25 mg/ml	0.5 mg/ml	1 mg/ml	2 mg/ml	
S1	16.85±0.23	20.83±0.92	35.28±1.96	61.61±3.17	1.55
S2	2.71±1.11	8.52±1.19	12.9±0.83	21.7±1.26	>2
S3	18.92±0.36	28.55±1.46	42.13±1.19	57.07±1.26	1.52
S4	14.97±0.77	26.32±1.14	35.68±2.22	55.79±2.11	1.71
S5	4.03±0.59	12.11±1.03	22.26±0.65	28.63±0.73	>2
S6	15.77±1.09	21.62±1.68	37.19±0.88	57.46±1.09	1.68
S7	5.97±2.12	8.72±0.97	12.31±0.83	34.33±2.05	>2
S8	2.27±1.26	7.37±1.82	9.96±0.81	19.39±0.92	>2
S9	12.86±0.92	19.43±0.65	33.930.47	51.41±2.86	1.91
Volgibose	48.71±1.08	69.16±4.14	82.2±4.15	94.31±2.45	0.26

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

Table 9. Alpha-amylase Inhibitory Activity and IC_{50} Value of Methanol Extracts of Selected Plant Samples

Plants	Concentration				IC_{50}
	0.25 mg/ml	**0.5 mg/ml**	**1 mg/ml**	**2 mg/ml**	
S1	11.95±0.9	35.05±0.73	45.28±0.66	66.03±0.84	1.22
S2	3.5±0.54	12.18±1.09	18.08±0.01	22.93±1.31	>2
S3	11.38±0.9	28.04±0.73	36.16±0.54	60.09±1.46	1.57
S4	14.69±1.31	15.53±0.66	34.01±0.3	50.77±1.13	1.95
S5	0.43±1.01	7.08±0.53	8.44±0.6	25.24±0.95	>2
S6	15.69±0.93	32.41±0.26	51.17±1.06	55.43±0.97	0.96
S7	2.11±0.61	9.04±0.65	10.51±0.63	22.98±0.65	>2
S8	1.39±0.36	5.49±0.43	7.13±0.48	20.35±0.48	>2
S9	14.29±0.6	15.49±1.19	31.1±0.84	50.37±1.04	1.98
Volgibose	48.71±1.08	69.16±4.14	82.2±4.15	94.31±2.45	0.26

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

Among all the hexane extracts only **S3** showed considerable inhibition of alpha amylase with IC50 value of 1.52 mg/ml. Methanol extract of **S6** showed potent alpha-amylase inhibition with IC_{50} value of 0.96 mg/ml which is comparable to volgibose. *Nephrolepis cordifolia* leaves and *Musa* sp. fruit even shows potent alpha-amylase inhibition with IC50 below 2 mg/ml.

Glucose Diffusion Inhibition Study

Studies have revealed that, mainly glucose diffusion inhibition could be achieved due to the presence of fibrous compound in the samples. Hexane is a non-polar solvent in which resinous or fibrous compounds are extracted in least quantity. Therefore, glucose diffusion inhibition study was performed only with methanol extracts where fibrous and resinous compounds are expected to get extracted in substantial amount. **Fig 3** represented the standard calibration curve. Based on this curve as referencing sample, the amount of glucose lied exterior of the dialysis membrane was calculated.

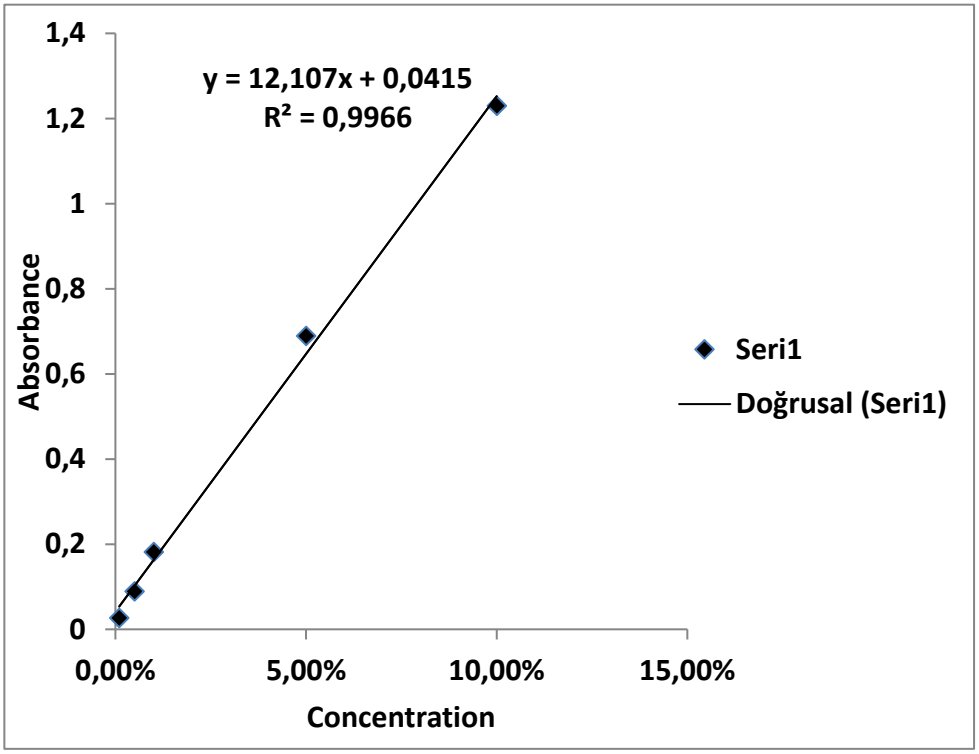

Figure 3. Calibration curve of dextrose exterior to the dialysis membrane

From the result, 20 mg/ml methanol extract of **S2** showed potent GDRI% from 120-180 minutes, with maximum GDRI% of 22.5 at 150 minutes. Followingly, the GDRI index of **S5** is 20% within first 90 minute. Meanwhile, **S7** also revealed potent GDRI% at 180 minutes.

When the concentration of sample was increased by two times with methanol extract, the GDRI% of **S1** was found maximum i.e., 49% as shown in **Fig 5**. This proved that **S1** was found highly potent at 40 mg/ml. **S3** was proved potent in all time intervals with 31% GDRI in 150-minute time.

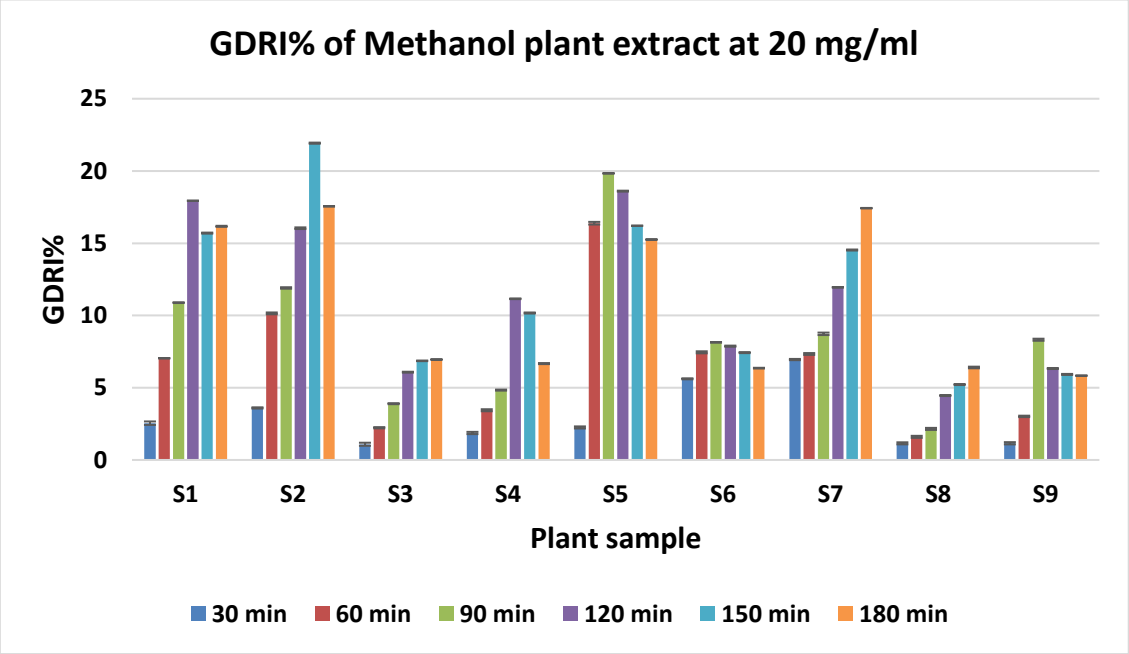

Figure 4. GDRI% of Methanol Extracts at 20 mg/ml

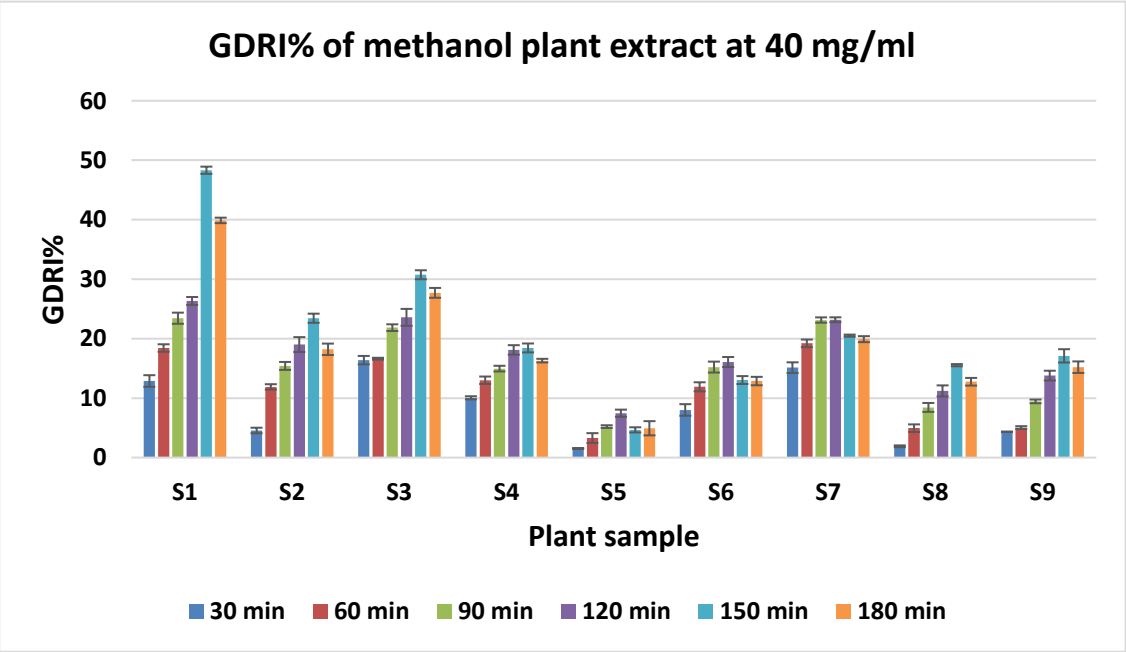

Figure 5. GDRI% of Methanol Extracts at 40 mg/ml

4. Discussion

Based on the local and ethnomedicinal utilization as hypoglycemic agents, different parts of five different medicinal plants were selected. From the phytochemical result obtained, it was determined that hexane extracts have very low phytoconstituents or secondary metabolites present on it. But in contrast, methanol extracts are found to be rich in phytoconstituents like glycosides, saponins, flavonoids, tannins and terpenoids which can be further correlated

with the positive in-vitro alpha amylase and glucose diffusion inhibition activity.

Phenol and polyphenolic compounds are proved to be potent free radical scavenger acknowledged to protect cell against oxidative stress (Rasouli et al., 2017). In a study by Daniil et al. 2014, it was shown that phenolic phyto substances were proved as the perspective natural compounds which exhibited antidiabetic activity as well (Olennikov et al., 2014). Common phenolic compounds present in plants which shows pharmacological activity are gallic acid, caffeic acid, ferulic acid, protocatechuic acid, coumaric acid, L-DOPA, ellagic acid, resveratrol, quercetin, rosmarinic acid etc. (Lin et al., 2016). According to Sandeep et al., 2011, *Myrica esculenta* revealed anti-oxidant properties due to the phenolic compound present on it. More specifically, gallic acid which is efficiently absorbed in the body contributed more to scavenge the free radicals present in them. Oxidative injuries in epithelial layer of various smooth muscles could be overcome by the phenolic constituents like chlorogenic acid, catechin and P-coumaric acid(Rawat et al., 2011).

On the other hand, the DPPH free radical scavenging effect of the *Choerospondias axillaries* fruit is correlated with the high presence of Vitamin C on plant extracts (Labh et al., 2015). In accordance with previous study, it has been reported that *Musa sp.* can protect itself from oxidative stress producing large amount of antioxidants thus is considered as good source of natural antioxidant due to the high presence of poly phenolic compounds (Orhan, 2001b). Not only phenolic compounds but also the presence of alkaloids, flavonoids, tannins, terpenoids, saponin and glycoside are also linked towards the scavenging properties of free radical. The results from our study revealed that the methanolic extracts of S6 and S7 showed potent DPPH free radical scavenging effect with the IC50 of 4.23 µg/ml and 3.13 µg/ml respectively. The result could be correlated with the presence of maximum amount of TPC and TFC in those plant extracts.

Meanwhile, natural products from plant sources are also gaining interest nowadays for inhibition of digestive enzymes such as alpha-amylase and alpha-glucosidase to control the blood glucose level. This is mainly due to the minimal side effect and therapies based on natural compounds compared to other hypoglycemic agent currently available (Gulati et al., 2012a). In a study by Nguyen et al. 2018, it was seen that isolated flavonoids showed the most active alpha amylase and alpha glucosidase inhibitory activity with IC50 value of 112.8 ± 15.1 and 785.9 ± 12.7 µg/mL respectively. This results showed the correlation between flavonoids and alpha-amylase inhibitory activity (Thao et al., 2018). Glucose lowering ability of epigallocatecin gallate, epicatechin and flavonoids are highly pronounced. Study from Uddin et al., 2014, revealed that alpha-amylase inhibitory activity of medicinal plants is more credited for the role of terpenoids, steroids and saponins to inhibit the respective enzyme. The study even reported that the hydrolysis is the major step for the breakdown of carbohydrate into glucose which is catalyzed by both alpha-amylase and alpha glucosidase enzyme. This step could be prevented even with the natural polyphenols(Uddin et al., 2014). In our experiment, S6 showed potent alpha-amylase inhibition on methanol extracts with IC_{50} value of 0.96 mg/ml which can be correlated with the presence of natural flavonoids, saponins, polyphenols and terpenoids. The quantitative phytochemical data revealed that the TPC in S6 is 180.21±0.79 µg GAE/mg of extracts and TFC in S6 is 301.72±1.01 µg QE/mg of extracts. This high amount of phenolic and flavonoid content could be correlated with the potent alpha-amylase inhibitory activity of the S6.

Likewise, in a study by Roberta et al 2011, spices and herbs commonly used in Italian cuisine were studied thoroughly for their combine anti-oxidant and alpha amylase

inhibitory activity and results revealed that these herbs and spices can improve the condition of non-insulin-dependent diabetes patients for their anti-oxidant activity (Cazzola et al., 2011). Phenolic acids such as hydroxycinnamic acid and gallic acid are subsequently found in plants which have both glucose lowering as well as free radical scavenging properties (Gulati et al., 2012b). Reactive oxygen species (ROS) formed with the oxidation of glucose and denaturation of glycated proteins consecutively results on oxidative stress. This is also a major cause for diabetic complication in human. So, in our study as well we could predict that the alpha amylase inhibitory activity of S6, S3, S9 and S4 is due to the potent free radical scavenging effect. Aside from the competitive binding study of plant extract with alpha amylase enzyme, uncompetitive mode of inhibition was also reported. The study suggested that binding strength of the enzyme for the substrate given by Km and rate of reaction given by Vm could also be decreased by the plant extracts (Picot et al., 2014b). Therefore, the alpha amylase inhibitory activity of our methanolic plant extracts can be correlated with uncompetitive mode of inhibition as well.

Complex polysaccharides such as oats, gum, psyllium husk and guar are revealed for their unique ability to lower the blood glucose level. Diffusion of glucose from the biological membrane in-vivo is predicted due to the physical boundary created by insoluble fiber particles. The fibrous network is supposed to entrap glucose moieties with in the networking residue preventing the upsurge of postprandial glucose level. Downsurge in glucose uptake in blood after crossing the biological semi-permeable membrane as well as the prolongation of gastric emptying time are the major outcome of viscous matrix. The accessibility of glucose molecule to the epithelial layer of intestine got decreased due to viscous gel which minimize the rise in postprandial glucose peaks (Gallagher et al., 2003). GDRI is a useful in-vitro index to predict the effect of fiber to decrease delay in glucose diffusion in the intestinal tract. In our study, we used a normal dialysis membrane to test the glucose diffusion inhibition by the plant sample at two different concentration 20 mg/ml and 40 mg/ml. We prepared glucose in NaCl solution as Glucose need carrier molecule to diffuse across cells. Our result showed that 20 mg/ml methanol extract of S1 showed potent GDRI which can be matched with the presence of insoluble fibers and complex polysaccharides in the plant extracts. S3, S6 and S7 even showed time dependent increment in GDRI% . The resinous network of the insoluble fiber particles is the cause glucose molecule to be entrapped resulting in the downfall of glucose diffusion to exterior of membrane (Bhutkar et al., 2017). Furthermore, several studies even highlighted that decrease in alpha-amylase enzyme activity is also directly correlated to the glucose diffusion retardation through semi permeable membrane. Subsequent decrement in the release of glucose from starch is the final output. On the other hand, the encapsulation of starch and enzyme within the resinous matrix as well as the action of the inhibitors might be the other cause for the inhibition of alpha amylase(Picot et al., 2014a).

5. Conclusion

The result of present study indicates that out of the five plants taken Methanol extract of stem bark and small branches of *Myrica esculenta, Nephrolepis cordifolia* fruit, *Choerospondias axillaris* fruit and *Musa sp.* leaves showed potent DPPH free radical scavenging effect. Similarly, the maximum alpha-amylase inhibition was shown by *Myrica esculenta* stem bark followed by *Choerospondias axillaris* fruits, *Nephrolepis cordifolia* leaves and *Musa sp.* leaves.
Similarly, among the plants taken *Amomum subulatum* and *Choerospondias axillaries* fruit showed maximum glucose diffusion inhibition. Above all, the local and traditional utilization of the selected medicinal plants to lower the blood glucose

level was substantially supported by the outcomes of this study. Conclusively, isolation of the active phytocompounds, investigation on the molecular level to determine the receptor-ligand binding affinity could be further encouraged with the outcome of the study.

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Authors Contribution

Conceptualization, Rishiram BARAL, Nirmala JAMMARKATTEL; methodology, Rishiram BARAL, Laxman SUBEDI, Sabita OJHA; investigation, Rishiram BARAL, Laxman SUBEDI, Sabita OJHA, Monika GURUNG, Basanta SHRESTHA; writing-original draft preparation, Rishiram BARAL, writing-review and editing, Rishiram BARAL, Nirmala JAMMARKATTEL; supervision and project administration, Nirmala JAMMARKATTEL.

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Conflict of Interest

The author declares no conflict of interest.

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