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PHYTOCHEMICAL EVALUATION AND ANTIOXIDANT ACTIVITIES OF *SPILANTHES CALVA* DC.

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Abstract

Many active chemical substances are synthesized from plants especially medicinal plants; some substances of them produce important pharmacological effects on various physiological systems of organisms. In this study, ethanolic extract of whole part of the plant ***Spilanthes calva*** (Family-Asteraceae) was taken to explore phytochemicals and antioxidant activities. The phytochemical research of the plant extract exhibited the existence of glycosides, tannins, carbohydrates, flavonoids, alkaloids gums, phenolic compounds, steroids, proteins & amino acids, acidic compounds etc. The percentage yield of extract was found to be **1.54% w/w**. In DPPH Free Radical Scavenging Assay, the IC_{50} value of extract was **32.42 µg/ml** where the IC50 value of ascorbic acid (standard antioxidant) was **12.90 µg/ml.** Ethanol extract showed considerable total phenolic content(TPC) of **459.89 mg GAE/gm** of dry extract by using Gallic acid calibration curve, total flavonoid content(TFC) of **123.59 mg QE/gm** of dry extract by using Quercetin calibration curve and total tannin content (TTC) of **22.83 mg GAE /gm** of dry extract by using Gallic acid calibration curve. The above study suggests that the plant may be an important source of natural products of health benefits.

Keywords: Spilanthes calva, Antioxidant, Antibacterial, ascorbic acid , DPPH, Gallic acid, Quercetin

Introduction

The plant named *Spilanthes calva* DC is also well-known as marhatitiga in Bangladesh which is a member of the family Asteraceae. This plant is a herb which grows annually [1].

People eat this herb mainly by using fresh leaves in salad and now-a-days the people of other countries, are trying marhatitiga in as food, in search of new kind of taste. So, it is not cultivated for its culinary purposes that much yet rather than for medicinal uses. It is well known to the folklore medicine as a toothache plant. The name is given due to its analgesic properties against toothache. Let along this main property, marhatitiga exhibits lots other activities that might be useful in discovering new drugs with low risk.

Materials & Methods

1.1. Chemicals, reagents and equipments

Mayer's Reagent, Fehling's Solution A ,Fehling's Solution B, Benedicts Reagent ,Molish's Reagent, Hager's Reagent, Wagner's Reagent , Legal's Reagent, Keller-Kiliani Reagent and Salkowski's Reagent were used for the different chemical group test[2].

Ethanol, Methanol, Concentrated H_2SO_4 (98 %) , Folin-Ciocalteu reagent, Quercetin, Gallic acid and DPPH, Ascorbic were used to find out antioxidant activities of the extract. Shimadzu UV spectrophotometer was used to find the absorbance of the ethanolic extract solution. All reagents and chemicals were used of analytical grade[3].

1.2. Plant material identification and preparation of the extract

In Bangladesh, *S. calva* has many different local names such as Marhatitiga, Nakful, etc. This plant is known in the tribal region as Ozonshak (Chakma), Hamfoi (Marma). The fresh plant parts (*Spilanthes calva* aerial part) were collected in January 2018 from Singair, Manikganj, Dhaka and identified by the experts of Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh.

265 gm of *S. calva* (aerial part) powder were taken in clean, glass containers and soaked in 90% ethanol and kept for 15 days accompanying shaking and stirring. Then it was filtered by using filter paper.

The filtrate obtained was evaporated and finally it became gummy concentrate which was designated as crude extract of Ethanol and yield of plant extract was **1.54%**[4].

1.3. Phytochemical screening

The ethanol extract of ***Spilanthes calva*** was subjected to a series of phytochemical group test to identify the presence of major phytochemical groups. To recognize the specific chemical constituents of ethanolic plant extract, some standard procedures were followed[5,6]. By using the specific reagents and chemicals, the crude extract solution was used to test quantitatively to identify the specific chemical constituents which were present in the sample solution.

1.4. DPPH free radical scavenging assay

1.4. 1 Qualitative Antioxidant Assay based on TLC

A diluted crude ethanolic stock solution of the plant extract were spotted on TLC plates then the plates were run in polar solvent, medium polar solvent and non-polar solvent systems to separate polar and non-polar components of the extract. The TLC plates were kept to dry at normal temperature and spraying was performed with a chemical named 0.02% DPPH in ethanol. After spraying DPPH on the TLC plate, the plates were seen for 10 minutes to observe resolved bands and the color of the plate changes usually yellow on the purple background were noted which specified the presence of antioxidant components in the plant extract[7].

1.4. 2 Quantitative DPPH Free Radical Scavenging Assay

DPPH free radical scavenging assay of the plant extract was calculated according to a validated method[8]. Stock solution of ascorbic acid and the crude extract solution were prepared separately. The stock solution was prepared in ethanol having

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the concentration 1024 µg/ml. Then serial dilution was carried out to obtain the concentrations of 512, 256, 128, 64, 32, 16, 8, 4, 2 and 1 µg/ml for ascorbic acid and extract solution. Add 2ml extract/standard solution of different Concentrations in 6ml DPPH solution. The contents were stirred vigorously for 15 seconds. Then the solution was kept at dark place for 30 min at room temperature. After 30 min, absorbance was taken against a control at 517 nm with UV spectrophotometer. The percentage of DPPH radical-scavenging activity of plant extract and ascorbic acid was measured by using the following formula:

Percent scavenging activity=(1–Absorbance of sample or standard/Absorbance of control) ×100

To determine the concentration of extract and ascorbic acid which are necessary to decrease DPPH radical-scavenging by 50% (called IC_{50}) the percent DPPH radical-scavenging activity was diagramed against the plant extract and ascorbic acid concentration (µg/ml) separately.

1.5 Total Phenolic Content Assay

The total phenolic content(TPC) of the ethanol extract of plants was determined by the Folin-Ciocalteu method[9] . At first 0.5 ml of methanol solution of extract (I mg/ml) was mixed with 5 ml 10% (v/v) Folin-Ciocalteu reagent in where Folin-Ciocalteu (FC) reagent was dissolved in distilled water. Then 4 ml 7.5% w/v aqueous Sodium carbonate solution was added to the mixture. Blank was concomitantly prepared, containing 0.5 ml methanol, 5 ml 10% (v/v) Folin-Ciocalteu's reagent dissolved in distilled water and 4 ml 7.5% w/v aqueous Sodium carbonate solution. Then final solution is diluted by 10 times. The mixture was vortexed for 15 seconds and allowed to incubated in a thermostat at 40° C for 30 minutes. The absorbance was measured at λ max=765 nm against the blank by using spectrophotometer**.**

1.6 Total Flavonoid Content Assay

The test for total flavonoid content of ethanol extracts was done according to Aluminum trichloride colorimetric method[10]. At first, 1. 1 ml methanol solution of ethanol extract (1 mg/ml) was taken with 4 ml distilled water and 0.3 ml 5% (w/v) sodium nitrate solution. Five minutes later, 0.3 ml 10% (w/v) aluminum chloride was also added to the mixture. After one minute 2 ml of 1 M sodium hydroxide solution was mixed to the mixture. The volume of the mixture was finally adjusted to 10 ml with distilled water. Blank was also prepared. The mixture was mixed vigorously for atleast 15 seconds and the solution was kept to stand for 30 min for reaction at room temperature. The absorbance was calculated at λ max = 510 nm against the blank by using spectrophotometer.

1.7 Total Tannin Content Assay

The test for total tannin content assay was done using the Folin-Ciocalteu phenol reagents as reported method[11]. Briefly 0 .1 ml of the sample of plant extract was mixed with 7.5 ml of distilled water and then mixed with 0.5 ml of Folin-Ciocalteu phenol reagent, 1 ml of 35% sodium carbonate solution and diluted to l0 ml with distilled water. The mixture was mixed well and kept to stand for 30 minutes at room temperature and absorbance was taken at 725 nm with a double beam UV/ Visible spectrophotometer.

Results

2.1 Phytochemical screening

In Table 1.1, the experimental findings from the study showed that the extract of *S. calva* has organic compounds like Carbohydrate, Alkaloid, Phenolic compounds, Flavonoids Glycoside, Tannins, Steroids, Protein & Amino acids, Gum and Acidic compounds.

2.2 Antioxidant activity evaluation

2.2.1 Qualitative Antioxidant Assay based on TLC

The observed plates were viewed under UV detector both in short (254 nm) and long (365 nm) wavelength. When the plates were viewed under UV detector a lot of colored and fluorescent positive components were found in short and long wavelengths respectively which indicated the presence of UV positive materials in the plant extract and they were marked. Materials viewed in shorter

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wavelength are marked by the sign ( ) and materials viewed in longer wavelength are marked by [ ].

In figure 1.1, yellow color on purple background after applying DPPH on the TLC plate was observed which indicated the presence of antioxidant components in the extract of plants.

2.2.2 Quantitative DPPH Free Radical Scavenging Assay

In figure 1.2, there is a comparison of DPPH scavenging activity of *S. calva* and Ascorbic acid. After calculation of IC_{50} values of extracts with ascorbic acid in DPPH assay are given below.

Plant Extracts	IC50 (ug/ml)
Ascorbic acid	~13
S. calva	~32

2.3 Total Phenolic Content Assay

In figure 1.3, the calibration curve of Gallic acid is plotted to determine total phenolic content. In Table 1.2, there is a data to determine Total Phenolic Content of *S. calva* extract and the result showed that total phenolic contents of *S. calva* extract was found **459.89** mg GAE/gm of dry extract respectively.

2.4 Total Flavonoid Content Assay

In figure 1.4, the calibration curve of Quercetin is graphed determine total flavonoids content. In table 1.3, there is a data to determine Total Flavonoids Content of *S. calva* extract. The result showed that total flavonoid contents of *S. calva* extract was found **123.59** mg QE/ gm of dry extract respectively.

2.5 Total Tannin Content Assay

In figure 1.5, the calibration curve of Gallic acid is plotted to determine total tannin content. In table 1.4, there is a data to determine Total Tannin Content of *S. calva* extract. The result showed that total tannin contents of *S. calva* extract was found **22.83** mg GAE/gm of dry extract respectively.

Discussion

The freshly prepared extract of *Spilanthes calva* was subjected to preliminary phytochemical screening for various constituents. The percentage yield of ethanol extract was 1.54% w/w. The qualitative Phytochemical investigation of ethanol extract of *S. calva* revealed the presence of tannins, flavonoids, steroids, glycosides, alkaloids, carbohydrates, Phenolic compounds, gums, proteins and amino acids, acidic compounds, etc. which may lead to the development of drug formulation after investigation of further pharmacological and other activity.

Derivatization of the plates with various detecting reagent used did not give good resolution and number of the spots were also less compared, latter was seen under UV light, so UV light detection at 254 nm and 366 nm was used [12]. Antioxidants combine with DPPH, a stable free radical which is reduced to the DPPH-H and as a result, absorbance is decreased to the DPPH-H form from the DPPH radical. The extent of discoloration indicates the scavenging activity of the antioxidant compounds or extracts in terms of hydrogen donating ability. DPPH radical acts as an oxidizing radical to be reduced by the antioxidant[13].

Phenolic compounds are secondary metabolites that are derivatives of the shikimate, pentose phosphate, and phenylpropanoid pathways in plants [14]. Phenolic compounds in plants are vital part of the human diet, and are of considerable interest due to their antioxidant properties. These compounds have an aromatic ring bearing one or more hydroxyl groups and these hydroxyl groups are responsible for their free radical scavenging ability. So, the phenolic compounds act as natural antioxidants [15]. Probably *S. calva* contains many types of phenolic compounds and these phenolic compounds act as free radical scavenger and heavy metal chelators. The total phenolic content of the ethanol extract of *S. calva* was determined by the modified Folin-Ciocalteu method [16]. The total Phenolic content of extract was 459.89 mg GAE/gm of dry extract by using Gallic acid calibration curve ($R2 = 0.991$). Some

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important factors such as sample treatment and extraction condition will affect the phenolic content of marhatitiga. Since the ethanol extract was found to contain phenolic compounds, *S. calva* will protect different organs of the body from free radical damage and free radical causing diseases.

Flavonoids are usually polyphenolic compounds which can be classified according to their chemical structure into flavanones, flavonols, flavones, catechin isoflavones, chalcones and anthocyanidins. These flavonoids have potential beneficial effects on our human health. They have been reported to have anti-inflammatory, antiviral, anti-allergic, anti-tumor, antiplatelet, and antioxidant activities[17]. Flavonoids help us to provide protection against these diseases as well as antioxidant defense system of the human body. Epidemiological studies showed that flavonoid ingestion is inversely linked to death from the coronary heart disease[18]. Flavonoids are identified for their anti- oxidant activities and the positions of the substituent's also have an effect on the physiological system of different flavonoids. In view of the fact that, antioxidants are able of thwarting oxidative damage so that the extensive use of natural product antioxidants as a substitution of conventional antioxidants in plants ,food and other food supplements has been known that natural products have been considered to be promising and safe source of drug. The total flavonoid content of ethanol extract of *S. calva* was determined according to Aluminum trichloride colorimetric method. Total flavonoid content of ethanol extract was 123.59 mg QE/gm dry extract by quercetin calibration curve (R2 = 0.9906) .

Tannin is a very complex group of plant secondary metabolites and is identified from other polyphenolic compounds by their ability to precipitate proteins. These phenolic compounds interact with FCR (Folin-Ciocalteu Reagent) under basic conditions. The phenolic proton is dissociated and guides to a phenolate anion, which are capable of reducing FCR. This helps to find that the reaction occurred through electron transfer mechanism. The blue compounds produced between phenolate and FCR which are independent of the structure of phenolic

compounds, therefore ruling out the possibility of coordination complexes formed between the metal center and the phenolic compounds. The total tannin content of *S. calva* was determined using the Folin-Ciocalteu phenol reagents as reported method [19]. Total tannin content of extract was 22.83 mg GAE/gm of dry extract by using Gallic acid calibration curve (R2 = 0.9983). Tannins have been reported to possess anti carcinogenic and antimutagenic potentials as well as antimicrobial properties.

Conclusion

It can be concluded that the ethanol extract of *S. calva* contributes in national health care sector through identification of potential source of antioxidant and relative antioxidant which justify the basis of using this plant's extract as folkloric medicine.

Conflict of interest

The authors have no conflict of interest to declare.

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Table 1.1: Different chemical group tests for plant extract

Phytochemical Group	***S. calva***
Carbohydrate	+
Alkaloid	+
Phenolic compounds	+
Flavonoids	+
Tannins	+
Glycoside	+
Steroid	+
Protein & Amino acids	+
Saponin	-
Gum	+
Acidic compounds	+

(+) indicates the presence

&

(-) indicates the presence

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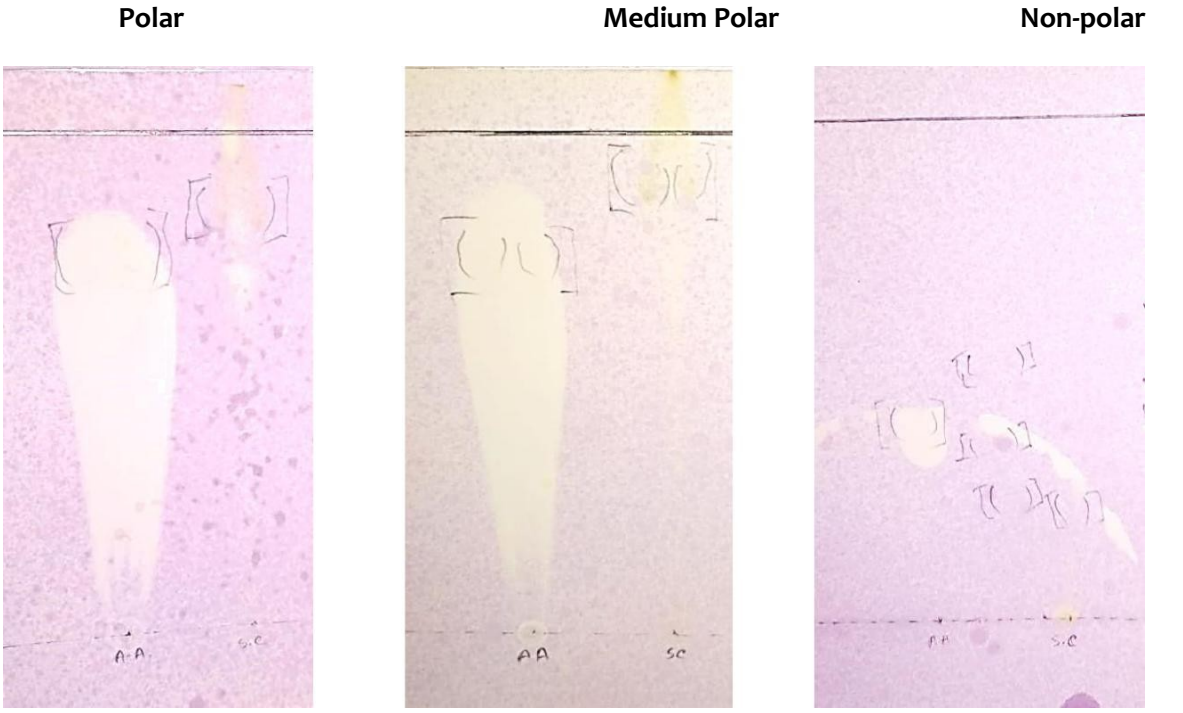
Figure 1.1: Comparison of TLC plate for *Spilanthes calva* with Standard (Ascorbic acid) after applying DPPH.



In figure 1.2, Comparison of DPPH scavenging activity of *S. calva* and Ascorbic acid

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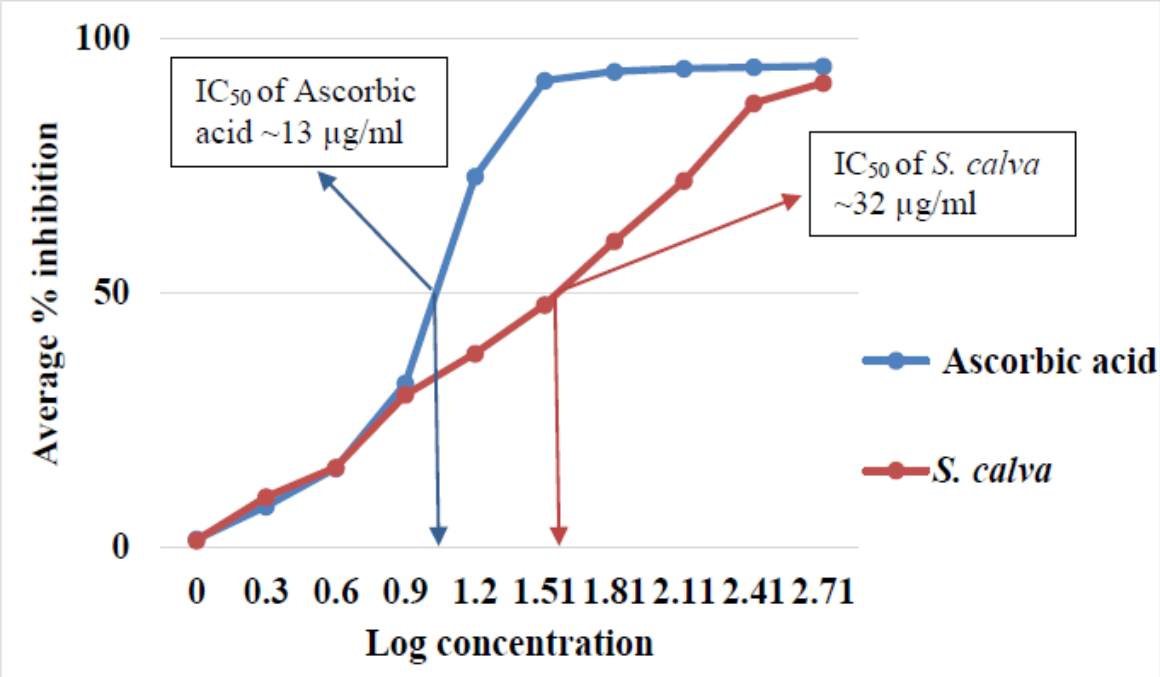
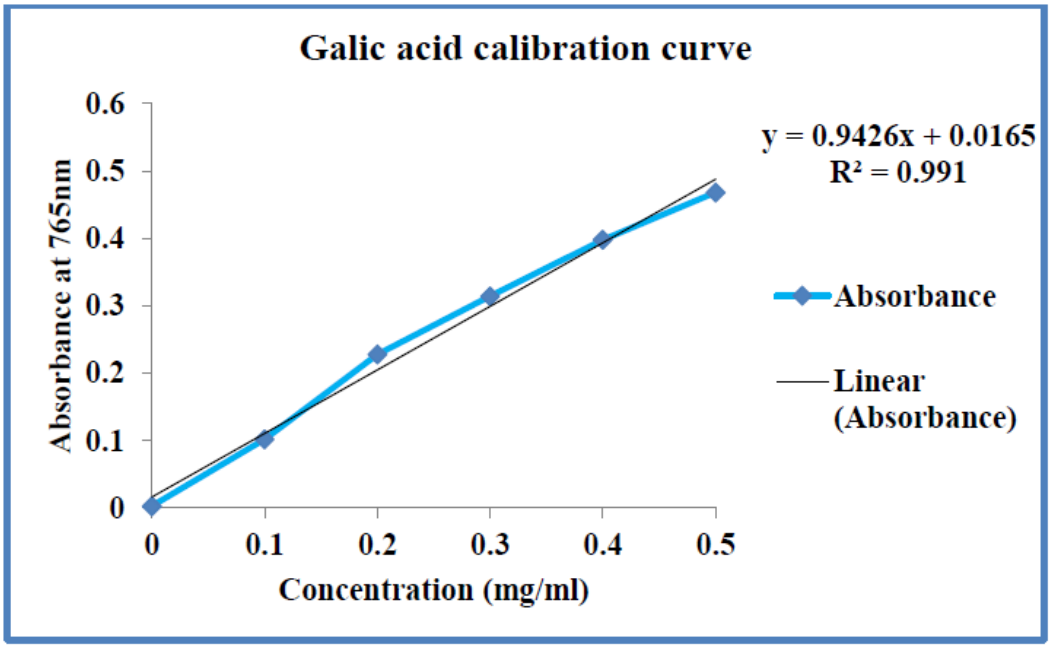


Figure 1.3: The calibration curve of Gallic acid to determine total phenolic content



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In Table 1.2, Data to determine Total Phenolic Content of *S. calva* extract

No. of observation	Absorbance at 765nm			Average absorbance	Grand Average of absorbance	Total Phenolic Content (mg GAE/gm of dry extract)
	$_1$st	$_2$nd	$_3$rd			
1	0.468	0.434	0.442	0.448	0.450±0.002	**459.89**
2	0.450	0.448	0.459	0.452		

Values are expressed as grand mean ±SD (n=2)

In figure 1.4, the calibration curve of of plant extract is graphed against quercetin to determine total flavonoids content

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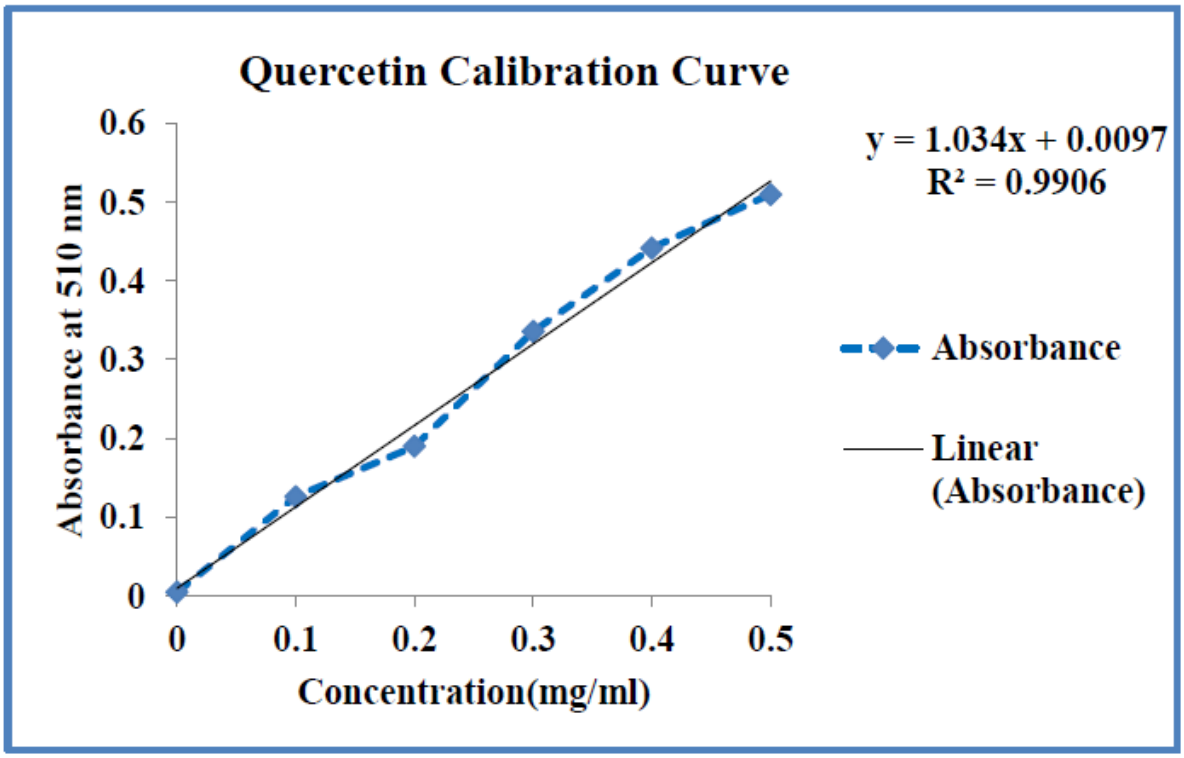

Table 1.3: Data to determine Total Flavonoids Content of *S. calva* extract

No. of observation	Absorbance			Average absorbance	Grand Average of absorbance	Total Flavonoid Content(mg QE/gm dry extract)
	$_1$st	$_2$nd	$_3$rd			
1	0.144	0.137	0.133	0.138	0.1375±0.0005	**123.59**
2	0.137	0.140	0.132	0.137		

Values are expressed as mean ±SD (n=2)

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Figure 1.5: The calibration curve of Gallic acid to determine total tannin content

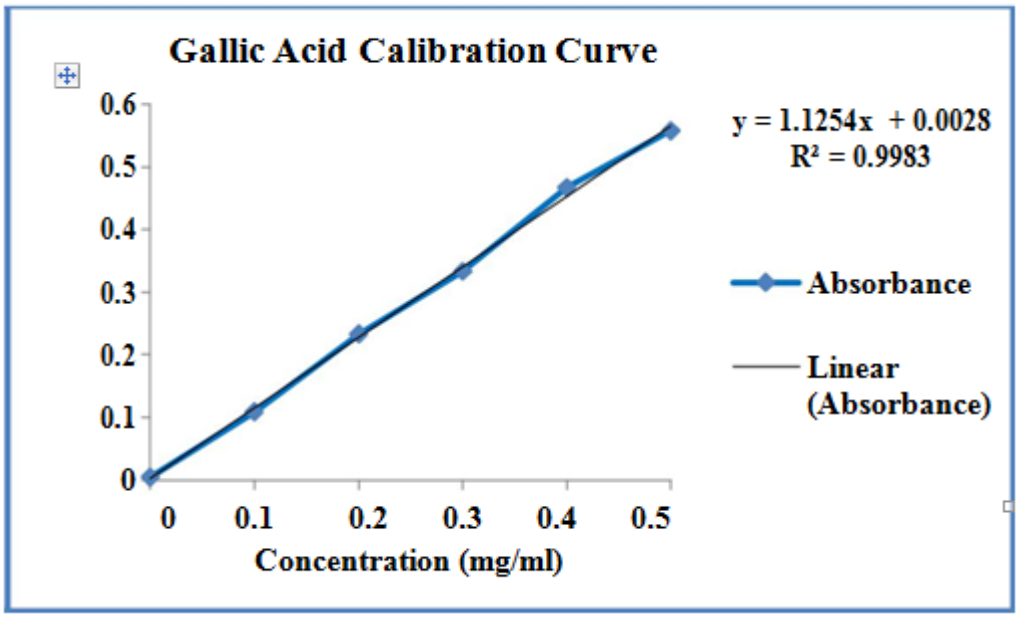

Table 1.4: Data to determine Total Tannin Content of *S. calva* extract.

No. of observation	Absorbance			Average absorbance	Grand Average of absorbance	Total Tannin Content (mg GAE/gm dry extract)
	$_1$st	$_2$nd	$_3$rd			
1	0.024	0.027	0.036	0.029	0.0285±0.0005	**22.83**
2	0.029	0.022	0.033	0.028		

Values are expressed as mean ±SD (n=2)

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