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Phytochemical analysis of selected Nepalese medicinal plants, screening for antioxidant activity and determination of total phenol and total flavonoid content

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Abstract
Background: Oxidative stress has been demonstrated to participate in the progression of diabetes including impairment of insulin action and elevation of the complication incidences. Antioxidants have already shown to be prospective in the treatment of diabetes both type I and type II diabetes.
Methods: The study was conducted to determine the total phenolic and flavonoid content of the ethanolic extracts of four Nepalese medicinal plants by the Folin Ciocalteu reagent and Aluminium chloride colorimetric method respectively. The antioxidant activity of the ethanolic extract was determined by the DPPH (2, 2-diphenyl-1-picryl-hydrazyl) radical scavenging method using ascorbic acid as the positive control was measured at different concentration (1, 10, 100 μg/mL).
Results: The phytochemical analysis revealed presence of phenols and flavonoids in *Magnolia grandiflora* and *Mangifera indica*. The total phenolic content and flavonoid content were 353.81± 0.84 mgGAE/g and 310.54±0. 0 mgQE/g dry extract weight for unripe *Mangifera indica* respectively. The extracts of unripe *Mangifera indica* showed antioxidant activity with the IC_{50} values of 3.87 μg/mL compared with the standard ascorbic acid IC_{50} value of 4.22 μg/mL.
Conclusion: In this study showed potential antioxidant, with present of Flavonoids and Phenols. These data are important because they contribute to the recording of traditional knowledge used for the antioxidant activities.

Keywords: antioxidant, phytochemical, traditional

1. Introduction

1.1 Background of Study

Every culture on the Earth, through written or oral tradition, has relied on the vast variety of plants for their therapeutic properties. The majority of medicinal plant products available today originated from the traditional medicinal formulas or ingredients. The benefit of plant-based cures is that they are cheap, easily obtainable and we can get them directly from the nature. Plant-based medicines are preferable as mainly non-toxic, having typically fewer side effects, better compatibility with physiological flora, and availability at affordable prices. The World Health Organization (WHO) estimates that 4 billion people, 80% of the world's population, according to 2014 data plant-based medicine for some aspect of their primary health care. WHO has listed 21,000 plants used for medicinal purposes around the world (Parikh *et al*., 2014) [14].

Oxidative stress has been demonstrated to participate in the progression of diabetes including impairment of insulin action and elevation of the complication incidences. Antioxidant is a substance whose availability, even in minute concentration inhibits or delays the oxidation of a substrate. There are several species or molecules, endogenous (internally synthesized) or exogenous (consumed), that play a role in antioxidant defense and may be considered as biomarkers of oxidative stress. Antioxidants have already shown to be prospective in the treatment of diabetes both type I and type II. Increase in the levels of oxygen and nitrogen free radicals (ROS/RNS) has been linked with lipid peroxidation, non-enzymatic glycation of proteins and oxidation of glucose which contributes toward diabetes mellitus and its complications (Pradhan *et al.*, 2013) [16]. Plants often contain substantial amounts of antioxidants, which implies that antioxidant action may be an important property of plant medicines associated with diabetes (Akintayo *et al*., 2017) [2].

2. Methods

2.1 Collection and identification of plant materials

Different parts of the selected plants were collected and processed for preservation and further examination. Plants were thoroughly cleaned with tap water and chopped into fine pieces. The chopped plant materials were dried under shade in the drying room of laboratory of Pharmacognosy. Completely dried plant materials were preserved in air tight bottles and subsequently used for extraction. The herbaria were prepared and identified with the help of experts in Botanical Garden, Godawori and by comparison with literature.

2.2 Extraction

Extraction was performed by double maceration method using 100% ethanol as solvent at room temperature. Completely dried plant samples 50 g were taken and macerated with 100% ethanol in the ratio of 1:10 w/v for 24 hrs in a closed vessel with occasional shaking. The extracts were filtered using Whattman-1 filter paper to obtain ethanolic extract. The residue left was subjected to second successive maceration with 100% ethanol (1:10 w/v) for another 24 h under previous conditions. The extracts thus obtained were evaporated in rotatory evaporator at 175-100 mbar pressure, 80-150 rpm and 40°C temperature. Further

drying was done in vacuum dessicator at 50-60 mbar pressure for few days. The dried extracts were stored in refrigerator and used for further experiment.

2.3. Phytochemical Screening

Phytochemical analysis of the four ethanolic extracts were carried out as per the procedure described by Trease and Evans, 1989 and Yadav *et al*., 2014 for detection of active components

2.3.1 Procedure of screening for phytochemical composition

All assessments were carried according to standard procedures. The processes are briefly given below-

Test for flavonoids

Alkaline Reagent Test

Few drops of dilute sodium hydroxide (20% w/v) was added to 1ml of the extract. When the intense yellow color produced goes away by adding a little acid, a positive result is considered.

Test for Saponins

Foam test, the extract was taken in a test tube it was vigorously shaken and was left to stand for 10 minutes. If foam produced persists for ten minutes, it indicates the presence of saponin.

Tannin

2 mg of each plant extracts were dissolved in ethanol and treated with 5 mL of 1% w/v gelatin solution containing 10% sodium chloride. Formation of white precipitate indicates the presence of tannin.

Test for alkaloids

50 mg of the extract is mixed with 5 mL of dilute hydrochloric acid and was filtered. Various alkaloidal reagents were used to test the filtrate as follows:

Mayers's test

One or two drops of Mayer's reagent was added to 5-6 mL of the filtrate sloping by the corner of the test tube. The test is indicated as a positive when white or creamy precipitate occurs.

Mayer's reagent: In 60 mL of distilled water 1-3 g of mercury was dissolved and in a separate container potassium iodide was dissolved in 10 mL of distilled water. The solutions were mixed together and 100 mL solution was marked up by adding water.

Wagner's test

2-4 drops of Wagner's reagent was added to the filtrate by the side of the test tube. Positive indication is shown by a reddish-brown precipitation.

Wagner's reagent: in 5 mL of distilled water 1.27 g of iodine and 2g of potassium iodide was dissolved and was made 100 mL by adding more water.

Carbohydrates

2 mg of each plant extracts were dissolved in 2 mL of distilled water and filtered.

Molish's Test

Filtrates were treated with 2 drops of alcoholic α-naphthol solution and 2 mL of conc. sulphuric acid was added carefully along the sides of the test tube. Formation of violet ring at the junction indicates the presence of carbohydrates.

Molish's Reagent

0.5 g of 1-napthol, 3 g of sodium hydroxide and 8 g of sodium carbonate were dissolved in 50 mL of distilled water to prepare Molish's reagent.

Benedict's Test

Filtrates were treated with Benedict's reagent and heated on water bath. Formation of orange-red precipitate indicates the presence of reducing sugars.

Glycosides

2 mg of each plant extracts were taken and hydrolysed with 10 mL of dil. hydrochloric acid (10% v/v).

Modified Borntrager's test

Extracts were treated with few drops of ferric chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and shaken with equal volume of benzene. Then, benzene layer was separated and treated with ammonia solution. Formation of rose-pink color in the ammonia layer indicates the presence of anthranol glycosides.

Phenols

Ferric Chloride Test

2 mg of each plant extracts were dissolved in ethanol and treated with few drops of ferric chloride (15% w/v) solution. Formation of bluish black color indicates the presence of phenols

Terpenoids

Salkowski Test

2 mg of each plant extracts were mixed with chloroform and 3 mL of conc. sulphuric acid was added from sides of test tubes to form a layer. A reddish brown precipitate at the interface formed indicates the presences of terpenoids.

2.3.2 Determination of Antioxidant Activity

DPPH (2, 2-Diphenyl-1-Picrylhydrazyl) Free Radical Scavenging Assay

Preparation of stock solution

Stock solution of each plant extract and ascorbic acid were prepared by dissolving the required amount in ethanol making the concentration of 1mg/ml (1000 μg/mL).

Preparation of test samples

Different concentrations of extract/ascorbic acid 1 μg/mL, 10 μg/mL, 100 μg/mL were prepared by serial dilution from stock solution (1000 μg/mL).

Preparation of DPPH solution

4.72 mg of DPPH (MW: 394.32 g/mol) was dissolved in 200 mL of ethanol in order to prepare 60 μM DPPH solution.

Experimental procedure

The radical scavenging activity of five plant extracts against DPPH was determined spectrophotometrically by the method of (Brand Williams *et al* 1995 and Yadav *et al*., 2013). In brief, 2 mL of different concentration of extract solution (1 μg/mL, 10 μg/mL and 100 μg/mL) of each plant

sample were mixed with 2 mL of DPPH solution (60 μM). Then the tubes were incubated for 30 min in dark condition at room temperature. The formed yellow colour chromophore was measured at 517 nm using UV spectrophotometer in terms of color change from violet to pale yellow. Ascorbic acid was used as a standard for comparison.

The absorbance of ascorbic acid was measured following the same procedure as mentioned above. Radical scavenging activity of each sample/ascorbic acid against DPPH was calculated using the following equation:

Radical Scavenging Activity (%) = [(Ac - As) / Ac] x 100

Where, A_c = Absorbance of control and A_S= Absorbance of sample.

The percentage of scavenging activity was then plotted against concentration. From the graph obtained the inhibitory concentration (IC_{50}) value was calculated by using linear regression analysis with Microsoft Office Excel 2007.

2.3.3 Determination of Total Phenolic

The determination of total phenolic content (TPC) was performed according to the Folin-Ciocalteu method with slight modifications (Singleton *et al*., 1965) [19].

Preparation of Gallic Acid Solutions and Test Samples

In this study, gallic acid was taken as standard phenolic compound. The different concentrations of gallic acid (50 mg/L, 100 mg/L, 200 mg/L, 300 mg/L, 400 mg/L and 500 mg/L) were prepared using stock solution (1 mg/kg) initially prepared. Extracts of plants solution of 1 mg/mL of each plants extract were prepared using ethanol.

Preparation of Folin Reagent

Folin reagent of strength 2N was used. (15 mL of folin reagent was mixed with 30 mL of distilled water in order to prepare solution).

Preparation of Sodium Carbonate

Sodium carbonate 5 g was dissolved in distilled water and volume was made up to 50 mL to make 10% of solution.

Total Phenolic Content Analysis

Aliquot of all extracts each extract (1 mL) added to 1 mL of 2N Folin-Ciocalteu reagent followed by the addition of 5 mL distilled water and mixed for 5 minutes. Then, 1 mL of 10% Sodium carbonate was added and incubated at room temperature for 1 hour. Finally, the absorbance of the reaction mixture was measured at 725 nm against a blank. The total phenol content was expressed as mg gallic acid equivalents (GAE)/g of dry weight of extracts, using the calibration curve of Gallic acid (50-500 mg/mL) standards.

2.3.4 Determination of Flavonoids

The total flavonoids content was determined using the method adopted by (Arvouet- Grand *et al*., 1994) [5].

Preparation of Quercetin and Test Samples

Quercetin was taken as standard flavonoid compound. Different concentrations of quercetin (50 mg/L, 100 mg/L, 200 mg/L, 300 mg/L, 400 mg/L and 500 mg/L) were prepared from the stock solution (1 mg/mL) using DMSO as solvent. 1 mg/mL concentration of the ethanolic extracts of plants were prepared.

Preparation of Sodium Nitrite Solution

5 percentage of Sodium nitrite 2.5 g was dissolved in 50 mL of distilled water according to Indian Pharmacopoeia.

Preparation of Aluminium Chloride

5 g of Aluminium chloride was dissolved in distilled water and diluted up to 25 mL to make 20% of Aluminium chloride solution according to Indian Pharmacopoeia.

Preparation of 1M Sodium Hydroxide Solution

Sodium hydroxide 2 g was dissolved in 50 mL of distilled water to make 1 M sodium hydroxide solution according to Indian Pharmacopoeia.

Determination of Total Flavonoids

Different extracts of plants (1 mL of 1mg/mL) was mixed with 4 ml of distilled water and 0.3 mL of 5% Sodium nitrite. After 5 minutes, 0.3 mL of 20% Alumunium chloride was added and again incubated for 5 minutes. 2 ml of 1M Sodium hydroxide was then added to the solution. After 30 minutes, the absorption reading against a blank solution and absorbance was measured at 510 nm using UV spectrophotometer. The calibration curve was prepared by plotting absorbance against different concentrations (50 mg/L-500 mg/L) of quercetin. Total flavonoid content was calculated from the calibration curve and results were expressed as mg quercetin equivalent (QE) per gram dry extract weight.

3. Results

3.1 Extraction Yield Value

The extraction of plant materials with 100% ethanol by double maceration method resulted in various extraction yield values. The detail of extraction yield values is shown in Table 1.

Table 1: Extraction yield values of the selected plant samples

S.N.	Plants	Parts used	Wt. of dry extract (g)	Wt. of crude sample (g)	Yield (%)
1	*Nephrolepis cardifolia*	Fruit	11.63	50	23.26
2	*Mangifera indica*	Unripe fruit	23.20	50	46.4
3	*Magnolia grandiflora*	Leaves	7.77	50	15.54
4	*Tinospora smilacina*	Vine	3.07	50	6.14

% Yield = (Weight of dry extract / Weight of crude sample taken) *100%

Phytochemical Screening

Preliminary phytochemical screening performed as per the standard procedure showed the presence of varied degree of phytoconstituents such as alkaloid, flavonoid, reducing sugar, saponin, terpenoid and tannin. The presence or absence of phytochemicals in extracts was determined on the basis of color and precipitate formation. The detail of phytochemical screening is shown in the Table 2.

Table 2: Phytochemical screening of the selected plant samples

Phytochemical Constituents	Specific Tests	*T. smilacina* vine S1	*M. grandiflora* leaf S2	*N. cardifolia* fruit S3	*M. Indica* Unripe fruit S4
Alkaloid	Mayer	+	+	+	+
	Wagner	-	-	+	+
Carbohydrate	Molish	+	-	+	++
	Benedict	+	-	++	+++
Glycoside	Modified Borntrager		-	-	-
Saponin	Foam	-	-	-	+
Phenol	Ferric Chloride	-	-	-	++
Flavonoid	Alkaline Reagent	+	-	+	++
Tannin	Gelatin	-	-	-	-
Terpenoid	Salwoskii	++	+	-	+

(+)* presence of phytochemical and (-)* absence of phytochemical

3.2 DPPH Radical Scavenging Activity

The antioxidant activity of the ethanolic extract was determined by the DPPH (2, 2-diphenyl-1-picryl-hydrazyl) radical scavenging method using ascorbic acid as the positive control. The antioxidant activity of the plant extracts and ascorbic acid was measured at different concentrations (1, 10, 100 μg/mL) and IC_{50} value was calculated. The results of DPPH radical scavenging assay is shown in Table 3 and Figure 1.

Table 3: DPPH radical scavenging activity and IC_{50} value of selected plant samples

	% Radical Scavenging Activity			IC_{50} (μg/mL)
	1 μg/mL	10 μg/mL	100 μg/mL	
T. smilacina (S1)	30.07± 15.38	30.75 ± 1.42	85.08 ± 0.79	41.94
M. grandiflora (S2)	27.45 ± 1.88	58.09 ± 5.82	91.80 ± 0.59	7.63
N. cordifolia (S3)	20.27 ± 2.33	30.41 ± 10.48	61.62 ± 0.71	66.65
M. indica (S5)	37.13 ± 7.40	77.56 ± 0.71	94.08 ± 0.20	5.91
Ascorbic acid	25.06 ± 5.30	94.87± 0.00	97.70±0.18	4.22

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

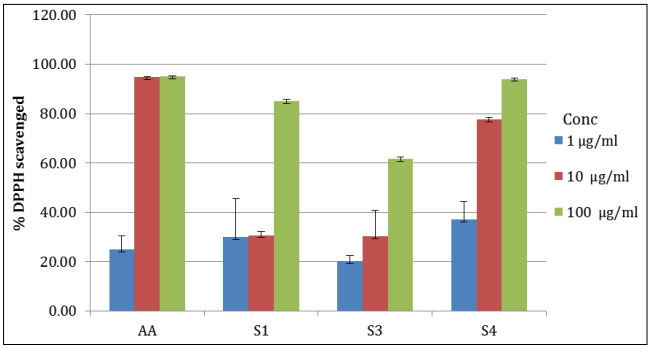

Fig 1: Percentage scavenging activity of selected plant samples with reference to standard ascorbic acid. Here, S1 = *T. smilacina* vine, S2 = *M. grandiflora* leaf, S3 = *N. cordifolia* fruit, and S4 = *M. indica* unripe fruit.

3.3 Total Phenol Content

The total phenolic content in different plant extracts was determined by Folin-Ciocalteu's reagent in terms of gallic acid equivalent (GAE) with standard curve equation (y = 3.8266x - 0.0488, R^2 = 0.999). The calibration curve of gallic acid at different concentration (50-500 mg/L) is shown in Figure 4.

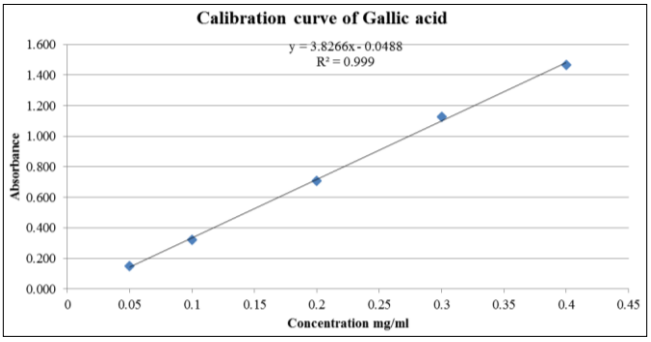

Fig 2: Calibration curve of gallic acid

3.4 Total Flavonoid Content

The total flavonoid content in various plant extracts was determined by aluminum chloride colorimetric assay using quercetin as standard. The content of flavonoid was expressed in terms of quercetin equivalent (QE) with standard curve equation (y=0.3511x+0.0832 R^2 = 0.9956), as mg of QE/g of dry extract weight (Table 5). The calibration curve of quercetin at different concentration (50-500 mg/L) is shown in Figure 3.

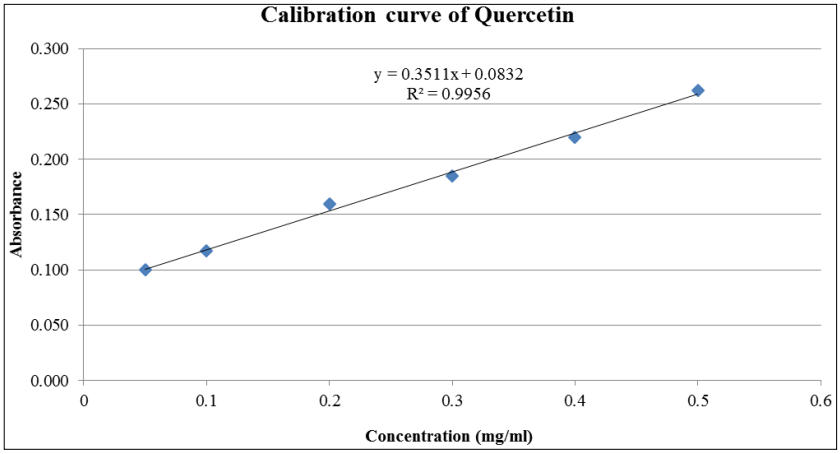

Fig 3: Callibration curve of quercetin

Table 4: Total phenol and flavonoid contents of selected plant samples

Plant Samples	Total Phenol Content (mg GAE/g dry extract weight)	Total Flavonoid Content (mg QE/g dry extract weight)
T. smilacina (S1)	109.08±9.24	54.13 ± 5.70
M. grandiflora (S2)	150.20±6.46	231.72 ± 5.93
N. cordifolia (S3)	92.52± 3.01	25.64 ± 7.54
M. indica (S4)	353.81± 0.84	310.54 ± 0.00

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

4. Discussion

In the present study, antioxidant activity, total phenol and flavonoid content of selected Nepalese medicinal plants were determined by in vitro methods.

In our study preliminary phytochemical screening of ethanolic extract of the selected four different plants revealed the presence of various bioactive components like alkaloids, flavonoids, steroids, carbohydrates, phenols, saponin, terpenoids and tannin in different parts of the selected plants. Glycosides were not detected in any of the selected plants by using the Modified Brontrager's test. According to Suganya *et al*., the phenols and flavonoids are potent water soluble antioxidants and free radical scavengers which prevent oxidative cell damage and have strong anticancer activity (Suganya *et al*., 2014) [20]. Similarly, Hasan *et al*., reported that the phenols and flavonoids have use in a varied range of anti-inflammatory antibacterial, anticancer, antiviral and anti-allergic activities. It is also considered to be highly effective against oxidizing molecules like singlet oxygen and various free radicals responsible for several diseases (Hasan *et al*., 2017) [10].

The extracts of *Tinospora smilacina*, *Magnolia grandiflora*, *Nephrolepis cordifolia* and *Mangifera indica* showed antioxidant activity with the IC_{50} values of 41.94, 7.63, 66.65 and 3.87 μg/mL respectively. The standard ascorbic acid showed IC_{50} value of 4.22 μg/mL. The Sugar free plant and *Mangifera indica* have a better hydroxyl radical scavenging effect compared to standard ascorbic acid. The extracts of *M. indica* have been reported to have several pharmacological activities such as antioxidant (Ribeiro *et al*. 2008; Maisuthisakul and Gordon 2009) [17, 12]. Mohan *et al*., evaluated the antioxidant and free radical scavenging activity in methanolic, ethyl acetate and *n*-butanol extracts of *Mangifera indica* leaf and found significant antioxidant activity with IC_{50} values of 13.37, 3.55 and 14.19 μg/mL respectively compared with reference standard gallic acid with IC_{50} value of 1.88 μg/mL. Similarly, Xuelian *et al*., showed that 70% aqueous ethanol extract of the mango peel had strong antioxidant activity, with 92±4.2% of DPPH radical scavenging rate. The antioxidant activity of leaf and peel of *M. indica* showed potent activity compared with gallic acid and ascorbic acid as reference standard in the above mentioned studies (Mohan *et al*., 2012 and Xuelian Bai *et al*., 2018) [21]. Therefore, it is clear that leaf, peel and unripe fruits extracts of this plant have good antioxidant activities as well as high polyphenolic contents.

In present study the total phenols content was carried out by Folin-Ciocalteu's reagent in terms of gallic acid equivalent (GAE) as a standard. The total phenols content value of pure ethanolic extracts of *Tinospora smilacina*, *Magnolia grandiflora*, *Nephrolepis cordifolia* and *Mangifera indica* were 109.08±9.24, 150.20±6.46, 92.52± 3.01 and 353.81± 0.84 mgGAE/g dry extract weight respectively. The total phenols content value was highest in *M. indica* unripe fruits and lowest in *Nephrolepis cordifolia* fruits compared to others plants. The total flavonoid content was determined by aluminum chloride colorimetric assay using quercetin as a standard. The total flavonoids content value of ethnolic extracts of *Tinospora smilacina*, *Magnolia grandiflora*, *Nephrolepis cordifolia* and *Mangifera indica* were 54.13 ± 5.70, 231.72 ± 5.93, 25.64±7.54 and 310.54 ± 0.00 mgQE/g dry extract weight respectively. The total flavonoids content value was highest in *Mangifera indica* raw fruit and lowest in *Nephrolepis cordifolia* fruits compared to others plants. Hence, the potent antioxidant activity of pure ethanolic extracts of *Mangifera indica* raw fruit hold high phenols and flavonoids content.

Antioxidant activity is correlated with the total phenolic content and total flavonoids content in the extracts that were likely to contribute to the radical scavenging activity. It is known that only flavonoids of a certain structure and particularly hydroxyl position in the molecule determine antioxidant properties and in general these properties depend on the ability to donate hydrogen or electron to a free radical (Ali *et al*., 2014).

Recently flavonoids and phenols have aroused considerable interest because of their beneficial effects on human health mainly antioxidant activity that depends on their molecular

structure (Parvez *et al* 2004) [15]. Many studies affirmed that flavonoids and phenols offered the highest ability of scavenging activity in medicinal plants (Castaneda-Ovando *et al*., 2009, Jaime *et al*., 2009 and Nithya *et al*., 2016) [7, 11, 13]. Therefore, the excellent antioxidant activity of *M. indica* can be correlated with their high total phenols and flavonoids contents.

Phenolic compounds have diverse biological activities and have been reported to be effective in the treatment and management of degenerative diseases such as diabetes, hypertension and obesity (Graf *et al*., 2005 and Arts *et al*., 2005) [9, 4]. Phenolic compounds have been reported to inhibit α-amylase and α-glucosidase inhibitory activities and exhibit antioxidant properties which are essential in the prevention and management of type 2 diabetes (Saliu *et al*., 2012) [18]. The use of medicinal plants which are rich in polyphenols are often advocated for the management of type 2 diabetic owing to their possible lower risk of side effects compared to contemporary antidiabetic drugs such as acarbose (Felix *et al*., 2017) [8].

5. Conclusion

Among the four studied plant extract *M. indica* unripe fruits showed potent antioxidant activity and higher contents of total phenol and flavonoid. Secondary metabolites are the source of therapeutic potentials in the management of diabetes. The high flavonoid and phenolic contents of extracts from Sugar free plant and *M. indica* also contribute to its antioxidant. These data are important because they contribute to the recording of traditional knowledge used for the treatment of various type of diseases as well as *Nephrolepis cordifolia* (Pani amala) which does not show the activity in dried extract that means the activity may be present only in the water content of this plant.

Abbreviations

μg	Microgram
μl	Microliter
DPPH	2, 2-Dipheny l, 1-picrylhydrazyl
g	Gram
GAE	Gallic acid equivalent
Kg	Kilogram
M	Meter
M	Molarity
Min	Minute
mM	Millimole
N	Normality
Nm	Nanometer
NMR	Nuclear magnetic resonance
QE	Quercetin equivalent
ROS	Reactive oxygen species
Rpm	Revolution per minute
SD	Standard deviation
UV	Ultraviolet
V/v	Volume by volume
W/v	Weight by volume

Declarations

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6. References

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