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In Vitro Enzyme Inhibition and Thin Layer Chromatography Profiling of Leaf Extract of *Phyllanthus emblica* Linn against Type-II Diabetes

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ABSTRACT

Phyllanthus emblica Linn commonly known as Indian gooseberry is one of the most important plants used in Ayurveda, Unani and Siddha. In the present study, the leaf methanol extract of *P. emblica* was studied against the carbohydrate metabolizing enzyme alpha amylase and alpha glucosidase. *P. emblica* leaf methanol extract showed a moderate α – amylase inhibitory activity (IC_{50} – 155. 10 ± 0.91µg/ml) and significantly high ($P<0.05$) α - glucosidase inhibitory activity (IC_{50} – 141.99 ± 0.43 µg/ml) compared to the standard drug acarbose (IC_{50} – 254.28 ± 0.88 µg/ml).The kinetics study of the leaf methanol extract of *P. emblica* showed uncompetitive mode of inhibition for both the enzymes. Phytochemical screening reveals the presence of several bioactive groups like phenol/tannin, flavanoid, alkaloid, saponin and terpenoids. The TLC fingerprinting confirms the presence of biomolecules like quercetin and gallic acid in the crude methanol extract of *P. emblica* leaf. The study conclude that presence of these bioactive groups including quercetin and gallic acid, contribute towards the inhibition of carbohydrate metabolizing enzymes like alpha amylase and alpha glucosidase.

Keywords: Post prandial hyperglycemia, α- amylase, α- glucosidase, flavonoids, phenol and thin layer chromatography.

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INTRODUCTION

Postprandial hyperglycemia is a prominent and early defect in diabetes, which can in turn lead to various secondary complications including risk factor for cardiovascular diseases[1]. The control of postprandial blood glucose levels in diabetic patients is considered to be relevant in the treatment of Type-II diabetes mellitus. Phenolic phytochemicals, secondary metabolites found in plants, such as different flavonoids and proanthocyanidins, have been reported to display a pancreatic-alpha-amylase and alpha glucosidase inhibitory activity[2]. Amongst the phyto-constituents that have been investigated, flavonoids are one of them that demonstrate the highest alpha amylase inhibitory activities with the potential of inhibition related to number of hydroxyl groups in the molecule of the compound[3]. Many plant extract and phyto-constituent showed *in vitro* α- glucosidase inhibition effect. Furthermore, herbal treatment is always preferred for diabetes due to lesser side effects and low cost [4]. *Phyllanthus emblica* Linn commonly known as 'Amla' in Indian Ayurveda is a rich source of Vitamin C and contains gallic acid, ellagic acid and flavonoids. It possesses antioxidant, anti hyperglycemic and anti hyperlipidemic properties [5]. Several studies were conducted on the effect of fruit extract of *Phyllanthus emblica* Linn on Type-II diabetes, triglycerides and liver specific enzyme, and it was found that the aqueous and ethanol fruit extract significantly reduce the blood glucose level in alloxan induced diabetic rats[6,7,8,9]. The *in vitro* α – amylase inhibition activity of fruit part of *Phyllanthus emblica* Linn against porcine pancreatic amylase exhibit palpable result with a minimum IC_{50} of 36.05µg/ml when compared to standard drug acarbose 48.92µg/ml[10,11]. The objective of the present study is to evaluate the hypoglycaemic potentiality of the leaf part of *P. emblica in vitro* and the screening of those bioactive potential polyphenols and flavanoid groups via thin layer chromatography.

MATERIALS AND METHOD

Plant material – fresh leaves of the test plant were collected from nearby areas of Gauhati University. The plant was authenticated by Curator - Prof. Gajen Chandra Sarma, Department of Botany. It was identified as *Phyllanthus emblica* Linn. Family – Euphorbiaceae, having accession No. GUBH18030 dated 2/9/2015.

Preparation of Plant Extract

The collected leaves were washed under running tap water to remove all foliar contaminants, shade dried and pulverized to fine powder using a mixture. These powdered samples (25g) were extracted successively from non polar to polar using petroleum ether, acetone and methanol respectively in a Soxhlet apparatus. The resulting extracts were filtered and concentrated using

rotary evaporator, under reduced pressure. The semisolid plant extracts were then stored at 4ºC until enzyme inhibitory assay.

α – Amylase inhibition assay

α - amylase inhibitory activity of each plant was evaluated using Bernfield method[12] with little modification. 100µl of test extract was allowed to react with 200µl of porcine pancreatic alpha amylase enzyme (Sigma Aldrich-3176) and 100 µl of 2mM of phosphate buffer (pH 6.9). After 20 min of incubation, 100µl of 1% potato starch solution was added. The same was performed for the control where 200µl of enzyme was replaced by buffer. After incubation for 15 min, 500µl of 3,5-dinitrosalicyclic acid reagent was added to both control and test. They were kept in boiling water bath for 5 - 10min. The absorbance was recorded in 540nm using a UV – VIS spectrophotometer and the percentage of inhibition of alpha amylase enzyme was calculated using the formula:

$$\text{Inhibition percentage (\%)} = 100\left(\frac{Control-Test}{Control}\right)$$

α - Glucosidase inhibition assay

α - glucosidase inhibitory assay was conducted according to N Artanti et al., 2012[13] with little modification. 100µl of plant extract was added to a test tube containing 100µl of 20mM pNPG (*p*-Nitrophenyl-α-D-glucopyranoside, Sigma) and 2.2 ml of 100mM phosphate buffer at pH 7.0, and then incubated for 10min at 37℃. The reaction was initiated by addition of 100µl of alpha glucosidase from *Sacchromyces cereviseae* (Sigma, G5003) solution (1mg/0.1ml) followed by 15min incubation at 37℃. The reaction was stopped by addition of 2.5ml of 200mM Na_2CO_3. The absorbance of p-Nitrophenol released from PNPG at 400nm was measured in Spectrophotometer. Percentage of inhibition on the α-glucosidase activity was calculated by the equation:

$$\text{Inhibition percentage (\%)} = 100\left(\frac{Control-Test}{Control}\right)$$

Suitable reagent blank and inhibitor controls were simultaneously carried out and subtracted. Dose dependent alpha amylase and alpha glucosidase inhibitory activity was measured using 50, 100, 200, 400 and 800µg /ml of different extract. The IC_{50} value denotes the concentration of sample required to inhibit 50% of enzyme activity.

α - amylase inhibition kinetic study by the crude methanol extract of *Phyllanthus emblica* Linn

The mode of inhibition for the sample was conducted using the modified method described by Kazeem et al[14]. The extract with the lowest IC_{50} value was taken. Two sets were prepared – one for the sample extract and another for standard blank. In set one 5µL of extract (5mg/ml) was pre incubated with 250µL of α – amylase solution for 10 mins. In another set of tubes α – amylase was

pre-incubated with 250µL phosphate buffer (pH 6.9). At an increasing concentration (0.2 – 2mg/ml) of 250µL of starch was added to both the reaction mixture to start the reaction. The mixture was then incubated for 15mins at 25°C. 500µL of DNS reagent was added and boiled for 5mins to stop the reaction. The amount of reducing sugar released was determined spectrophotometrically using a maltose standard curve and converted to reaction velocities. A double reciprocal plot (1/V versus 1/*[S]*) where V is the reaction velocity and *[S]* is the substrate concentration was plotted. The mode of inhibition of crude extract on α – amylase activity was determined by analysis of the double reciprocal (Lineweaver Burk) plot using Michaelis-Menten kinetics.

α - glucosidase inhibition kinetic study by the crude methanol extract of *Phyllanthus emblica* Linn

The mode of inhibition of α – glucosidase by *P. emblica* leaf was conducted using the modified method described by MI Kazeem et al., 2013[14]. The methanol extract having the lowest IC_{50} value was taken. Two sets were prepared – one for the sample extract and another for standard blank. In set one 5µL of Extract (5mg/mL) was preincubated with 100µL of α – glucosidase solution for 15mins at 25ºC. In another set of tubes α – glucosidase was pre incubated with 100µL phosphate buffer (pH 6.9). At an increasing concentration (15 – 100µg/ml) of 50µL of PNPG was added to both the reaction mixture to start the reaction. The mixture was then incubated for 15mins at 25°C. 500µL of Na_2CO_3 was added and boiled for 5mins to stop the reaction. The amount of reducing sugar released was determined spectrophotometrically using a PNPG standard curve and converted to reaction velocities. A double reciprocal plot (*1/V* versus *1/[S]*) where [V] is the reaction velocity and [S] is the substrate concentration was plotted. The mode of inhibition of the sample on α – glucosidase activity was determined by analysis of the double reciprocal (Lineweaver Burk) plot using Michaelis-Menten kinetics.

Phytochemical screening of the three extracts of *Phyllanthus emblica* Linn

A preliminary phytochemical analysis (qualitative tests) of petroleum ether, acetone and methanol extract of *Phyllanthus emblica* Linn was carried out by using standard test/methods[15, 16, 17].

Test for Carbohydrate

Benedict test was performed. 2mL of Benedict's reagent was taken and 2-3 drops of the extract was added to it. The solution was then boiled for 5 minutes in a water bath and allowed to cool. Formation of green colour confirms the presence of carbohydrates in the sample.

Test for Protein

Biuret test was performed. To 2mL of test extract solution, 2mL of 10% NaOH and 2 drops of 0.1% $CuSO_4$ was added. Violet or pink colour formation confirms the presence of protein in the sample.

Test for Alkaloid

2mL of HCl was added to crude extract and heated gently. Mayer's and Wagner's reagent were added and turbidity of the resultant precipitate was taken as evidence for the presence of alkaloids.

Test for Saponin

5mL of Distilled water was added to crude extract and shaken vigorously. Formation of stable foam indicated the presence of saponins.

Test for Phenols:

2mL of 2% solution of $FeCl_3$ was mixed to crude extract. Blue-green or black colour indicated the presence of phenols.

Test for Flavonoid

Alkaline reagent test was performed, where crude extract was mixed with 2mL of 2% solution of NaOH. An intense yellow colour which turns colourless on adding a few drops of diluted acid indicated the presence of flavonoid.

Test for Terpenoid

Crude extract was dissolved in 2mL chloroform and evaporated to dryness. To this, 2mL of conc. H_2SO_4 was added and heated for about 2 minutes. A reddish brown precipitate confirms the presence of terpenoids.

Thin Layer Chromatography

The methanol extract of *P. emblica* was screened for the detection of quercetin and gallic acid by means of thin layer chromatography using silica gel 60 F_{254} (Merck).

Preparation of Standard and Plant Sample for Thin Layer Chromatography

Quercetin (RM 6191, 25G, Himedia) and Gallic acid (RM233, Himedia) were accurately weighted in a 10ml volumetric flask and dissolved in methanol (1mg/ml). The methanol leaf extract of *P. emblica* was weighed and dissolved in methanol (100mg/10ml) [18].

Development of TLC Chamber

The solvent system (toluene: ethyl acetate: formic acid) in a ratio of (5.8: 4: 0.8) was poured to a depth of 0.5cm in a rectangular chromatographic glass chamber. The chamber was lined with a piece of filter paper to ensure proper saturation. The spots of extract were spotted on the ready-made silica gel 60 F_{254}TLC plate using micro capillary tubes. The distance between two spots was

kept approximately 2.0cm. The applied spots were dried at room temperature and the plate was gently placed inside the glass chamber. The chromatogram was developed till the solvent front migrated to about 10cm.

Visualization of Spots

The plate was taken out and the solvent front was marked. The plate was dried at room temperature and inspected either under visible light, UV light or using iodine vapour inside an iodine chamber.

Total Phenolic Content Estimation

The total amount of phenol in the aqueous extract was determined by Folin – Ciocalteu reagent method[19] which uses folin – ciocalteu as the oxidizing reagent and gallic acid as standard. 0.5ml of *P. emblica* methanol extract was mixed with 2.5mL of 2N Folin-Ciocaltaeu reagent for 5 mins and then 2ml of 75g/ml Na_2CO_3 was then added. The mixture was incubated for 20mins at room temperature and the absorbance of the reaction mixture was measured at 760nm against a methanol blank. The result was determined from the gallic standard curve having concentration ranging 25-150µg/ml and the total phenolic content of the extract was expressed as gallic acid equivalent (mg/g of extracted compound).

Total Flavonoid Content Estimation

Total flavonoid in the extract was determined, using quercetin as a standard reference compound. A volume of 1ml of plant extracts (200µg/mL) was mixed with 1mL $AlCl_3$in methanol (20 mg/ml) and a drop of acetic acid. The mixture was then diluted with 25ml methanol. The absorbance was measured at 415nm after 40mins incubation. Blank samples were prepared from 1ml plant extract and a drop of acetic acid, diluted with 25ml methanol. The total flavonoids content was determined from the quercetin standard curve having concentration ranging 12.5-100µg/mL and the total flavanoid content of the extract was expressed as quercetin equivalent (mg/g of extracted compound)[20].

Statistical Analysis

Statistical analysis was performed using Graph pad Prism 6 statistical tool. IC_{50} values were calculated using non linear regression analysis with normalized data. All results are expressed as mean ± S.D of triplicates. The level of significance was taken at 5% confidence interval ($P < 0.05$).

RESULTS AND DISCUSSION

In the present study, five different concentrations viz., 50, 100, 200, 400, 800 µg/ml of methanol, acetone and petroleum ether extract of *P. emblica* (Table 1) leaf part were investigated for their

potentiality to inhibit pancreatic α – amylase and yeast α – glucosidase enzymes. Among all the selected extracts, the highest α - amylase inhibitory activities (Figure 1), was shown by methanol extract i.e. 97.88% with IC_{50} value 155.10 ± 0.91µg/ml, at a concentration of 800µg/ml .The alpha amylase inhibition of the methanol extract was not significantly ($p>0.05$) less than the standard drug acarbose i.e. 90.33%with IC_{50} value 161.80 ± 1.59µg/ml. On the other hand, highest α glucosidase inhibitory activity was exhibited by the methanol extract of *Phyllanthus emblica* leaves (Figure 2). It showed 99% inhibition with a minimum IC_{50} value of 141.99 ± 0.43µg/ml at a concentration of 800µg/ml which was significantly less ($p<0.05$) than standard drug acarbose having 98.34% with IC_{50} 254.28±0.88µg/ml. (Table 2).

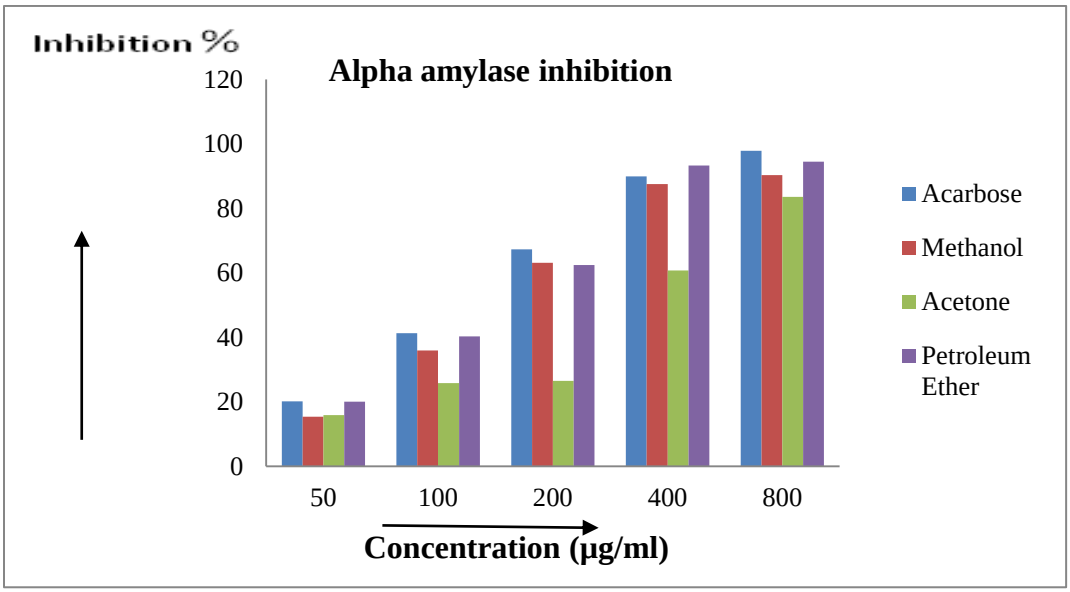

Figure 1: α-Amylase Inhibition of *Phyllanthus emblica* Extracts

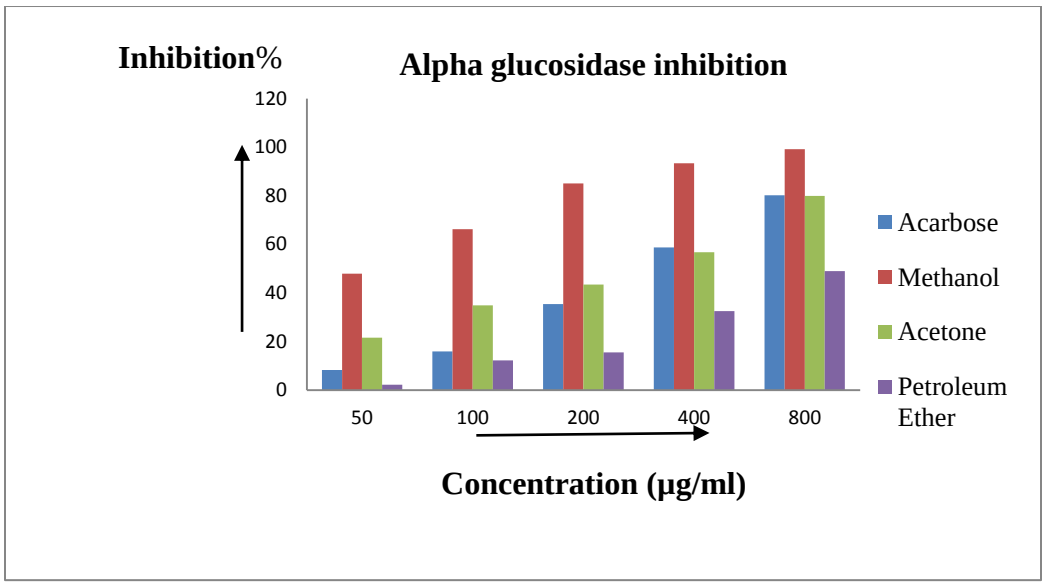

Figure 2: α- Glucosidase Inhibition of *Phyllanthus emblica* Extracts

Table 1: Tabulation of Plant Name, Dry Weight and the Weight of Solvent Extracts

Plant name	Dry Weight yield (in g)	Petroleum ether extract yield (in g)	Acetone Extract yield (in g)	Methanol Extract yield (in g)
Phyllanthus emblica Linn.	25	2.621	6.711	6.160

Table 2: Ic_{50} Values of a Amylase and a Glucosidase Inhibition by *Phyllanthus emblica* Extracts and Standard Drug Acarbose

Analyte	IC – 50 (µg/ml) ± S.D	
P. emblica	α - amylase	α – glucosidase
Petroleum ether	162.87 ± 2.4	274.71 ± 4.18
Acetone	*314.08* ± 3.6	261.66 ± 2.0
Methanol	*155. 10*± 0.91	141.99 ± 0.43*
Acarbose	*161.80* ± 1.59	254.28 ± 0.88*

All the analysis was done in triplicate. Values are mean ± S.D. *P <0.05 was considered significant when compared with the standard drug acarbose.

The mild inhibition of α-amylase and strong inhibition of α – glucosidase, suggest that the extract could serve as effective therapy for postprandial hyperglycemia with minimal side effects[21, 22].

Enzyme Kinetics study of methanol leaf extract of *Phyllanthus emblica* for α - amylase and α - glucosidase inhibition

The amount of reducing sugar released was determined using a standard curve (maltose and PNPG) and converted to reaction velocities. A double reciprocal plot (*1/V* vs.*1/[S]*) where V is the reaction velocity and [S] is the substrate concentration was plotted. The mode of inhibition of the sample on α – amylase and α – glucosidase activity was determined by the analysis of this double reciprocal (Burk) plot using Michaelis-Menten kinetics (Figure 3 and 4).

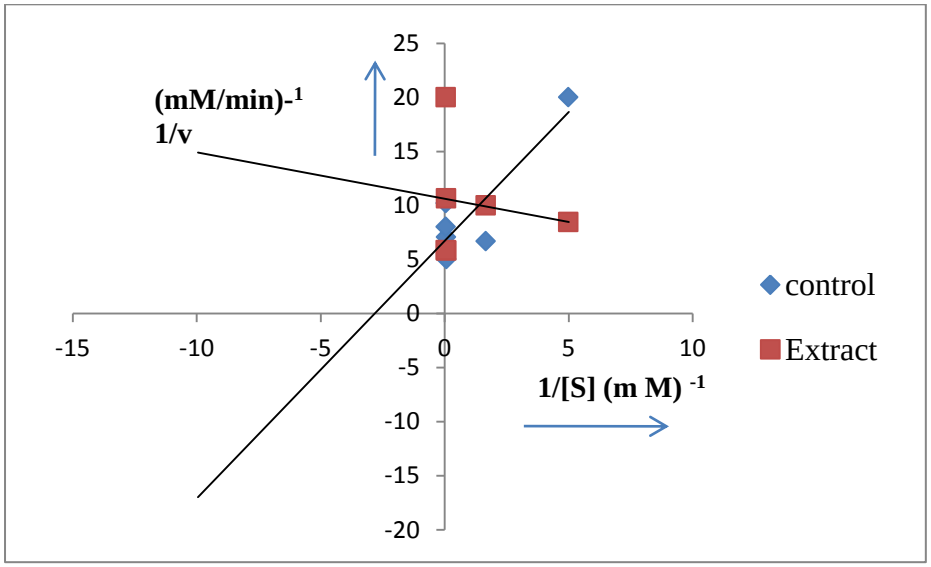

Figure 3: *Phyllanthus emblica:* Mode of Inhibition of α Amylase by Methanol Extract of Leaf

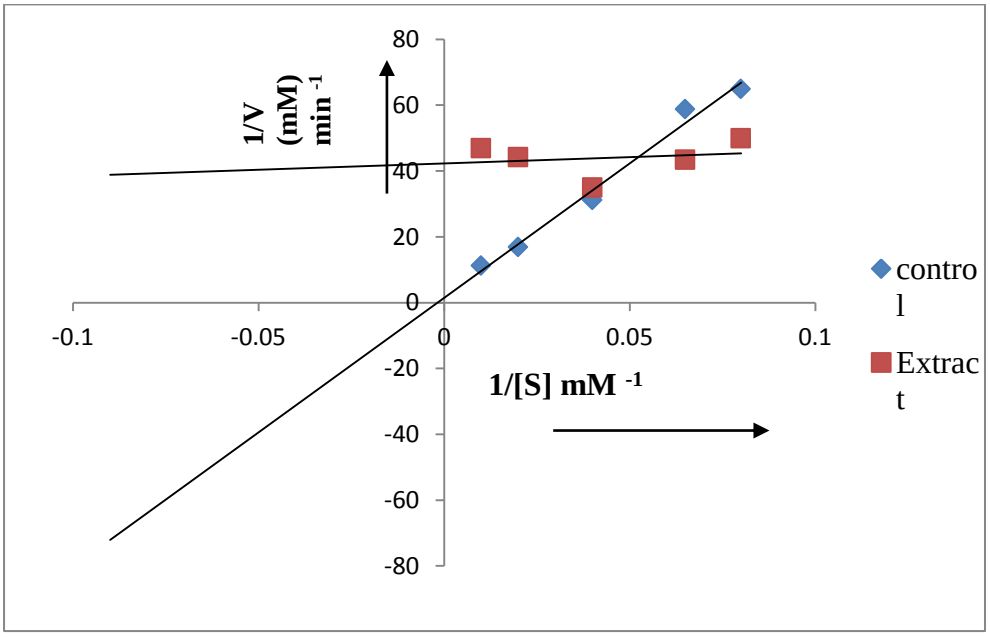

Figure 4: *Phyllanthus emblica:* Mode of Inhibition of α-Glucosidase by Methanol Extract of Leaf

The mode of inhibition of both α – amylase and α-glucosidase as depicted from the Lineweaver Burk plot is a non competitive type. The non competitive inhibition displayed by the methanol extract towards both the enzymes implies that the active component of the extract binds to a site other than the active site of the enzyme and combines with the either free enzyme or the enzyme substrate complex , possibly interfering with the action of both[21, 22]. Plants generally contain secondary metabolites like phenolics, flavonoids, glycosides, coumarins, saponins, terpenoids, alkaloids etc that reveal their specific characteristic properties and attribute to their pharmacological properties[23, 24]. The phytochemical study (Table 3) reveals the presence of several bioactive compounds like phenol, tannin, flavanoid, alkaloid and terpenoid. The methanol extract showed the presence of saponin that is shown absent in petroleum ether and acetone extract. These bioactive compounds, mainly flavanoids and polyphenols are among the natural active antidiabatic agents that are capable of inhibiting the carbohydrate metabolising enzyme like α amylase and α glucosidase.

Table 3: Phytochemical Screening of the Methanol Extract of *Phyllanthus emblica Linn*

Phyllanthusemblica	**Carbohydrate**	**Protein**	**Saponin**	**Phenol**	**Flavanoid**	**Terpenoid**	**Alkaloid**
Powder	++	+	-	+++	+++	+++	-
Petroleum ether	+	++	-	+	++	+	+ ++
Acetone	+++	++	-	+++	+	++	++

Methanol	+++	+++	++	+++	+++	+++	+++

+ Marginally present. ++ Moderately present. +++ Present in high quantity. - Showed absent.

Reports suggested that *P. emblica* contains tannins, alkaloid and phenol. The hydrolysable tannin like emblicanin on hydrolysis yields gallic acid, ellagic acid and glucose. The fruit part of the plant is rich in flavonoids like quercetin, alkaloid like phyllantine and phyllantidine[25, 26]. Flavonoids occur commonly, and are widespread, in the plant kingdom. Quercetin could exert a protective effect against β cell damage by its anti-inflammatory, anti-apoptotic, and antioxidant effects; and aids regeneration of β cells which might through stimulation of the ductal stem cells[27.] Flavonols like quercetin and phenolic acids like caffeic acid and gallic acid are the most well absorbed polyphenols present in plant that posses hypoglycaemic and anti oxidant properties. Studies have found that quercetin can inhibit alpha amylase in dose dependent manner[28]. The TLC profiling (Figure 5 and 6) confirms the presence of anthoxanthins flavonols like quercetin and phenolic hydroxybenzoic acids like gallic acid. Quercetin is a much more potent natural product than acarbose in controlling blood glucose level. Treatment with either insulin or quercetin alone resulted in partial reversal of the biochemical and histopathological signs of nephropathy in diabetic rats[29, 30]. Studies have shown that querctine posses relatively higher inhibitory activity on maltase, glucoamylase, and isomaltase. Peroxyl radical absorbing activity, followed by rutin and isoquercetin than their corresponding their glycoside derivatives like rutin and isoquerctin [31].

Figure 5: TLC Profile of the Methanol Extract *P. emblica* (R to L) with Standard Gallic Acid and Quercetin in Visible Light. Solvent System used - Toluene: Ethyl Acetate; Formic Acid (5.8: 4: 0.8)

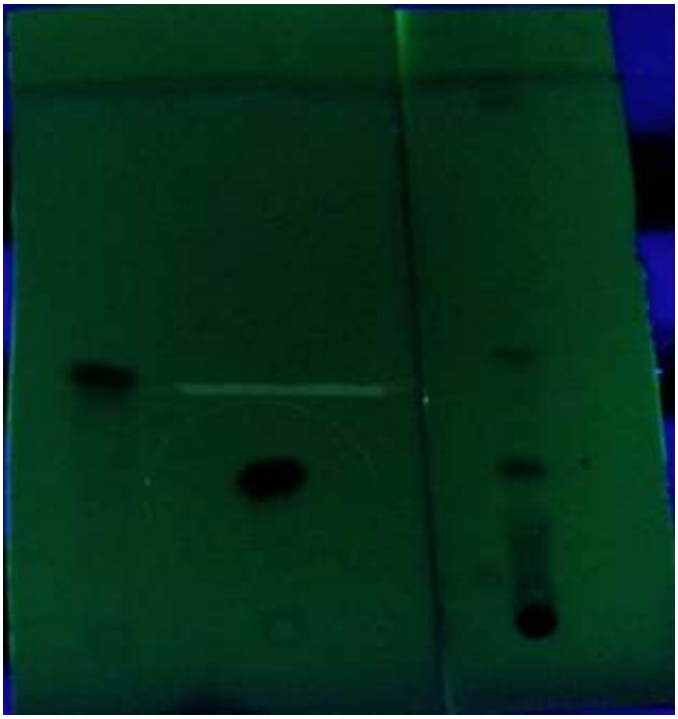

Figure 6: TLC profile of the methanol extract *P. emblica* (R to L) with standard Gallic acid and Quercetin inside an iodine chamber. Solvent system used - toluene: ethyl acetate; formic acid (5.8: 4: 0.8)

After the qualitative conformation by TLC fingerprinting, the quantitative estimation of total amount of phenol and flavonoids present in the methanol extract was determined. The total phenolic content determined was found to be 153.99±1.23µg/ml and the total flavanoid content was found to be 80.03±1.07µg/ml. (Table 4).The plant leaf methanol extract has higher phenolic content compared to flavonoid content.

Table 4: **Total phenol and flavonoids content of Methanol extract of *Phyllanthus emblica* Linn.**

Methanol extract of *Phyllanthus emblica* Linn	**Total phenol content (µg/ml) ± S.D**	**Total flavonoids content (µg/ml) ± S.D**
	153.99± 1.23	80.03± 1.07

CONCLUSION

Studies have shown that plant based α amylase and α glucosidase inhibitors offers a prospective therapeutic approach for management of postprandial hyperglycemia. Compounds such as luteolin, myricetin, and quercetin flavonoids are potent inhibitor of metabolizing enzyme and thus aid to minimize the postprandial hyperglycemia[32, 33]. The fruit part of *P. emblica* is traditionally and clinically proved to be have its medicinal property for numerous diseases. [34] From this study it can

be concluded that, not only the fruit part, the leaf part of *P. emblica* is a potent inhibitor of carbohydrate metabolizing enzymes like alpha amylase and glucosidase and the presence of biomolecules like querctin and gallic acid in the methanol extract of leaves, confirm the contribution of such compounds towards beneficial management of Type II diabetes. In future, the potent methanol extract will be needed to subject column chromatography bioassay guided fraction for the further isolation and characterization of other potent flavonoids and polyphenol conjugates compounds possessing antidiabatic activity from the leaf of *Phyllanthus emblica*.

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