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ORIGINAL ARTICLE

Integrated Phytochemical and Enzyme Inhibition Studies of Sequentially Fractionated *Andrographis paniculata* Leaves: Insights into Antidiabetic Mechanisms

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ABSTRACT
Andrographis paniculata has gained attention as potential source of bioactive compounds for Diabetes mellitus. This study aimed to integrate the phytochemical profiling, thin layer chromatography (TLC) finger printing, anti- oxidant evaluation, and in- vitro enzyme inhibition assays to evaluate the anti-diabetic potential of sequential fractionated leaf extracts. Andrographis paniculata leaves are subjected to cold maceration using ethanol and further fractionated using solvents of increasing polarity. Distinct fingerprint across the fractions in TLC analysis revealed the efficient separation of phytoconstituents. The aqueous fraction showed well resolved bands confirming the enrichment of flavonoids and phenolics. Quantitative analysis revealed the highest flavonoid content (245.66 µg/ml), phenolic content (67.90 mg GAE/g)) and total anti- oxidant capacity (361.09 mg TE/g) in the aqueous fraction. In- vitro anti-diabetic activity showed potent inhibition of α- amylase and β- glucosidase compared to other fractions. Correlation analysis revealed a strong positive relationship between TPC/TFC and enzyme inhibition demonstrating a direct contribution of polyphenolic constituents to bioactivity. Mechanistically, the attenuation of carbohydrate digestive enzymes is likely mediated through competitive or mixed type of inhibition pattern of phenolic compounds to the enzyme active sites, which plays a role in reducing postprandial glucose release. Overall, bioactivity guided fractionation enriched bioactive constituents in Andrographis paniculata leaves, resulting in improved anti-oxidant and enzyme inhibition activities. These findings support the mechanistic evidence for anti-diabetic potential and highlight the aqueous fraction as suitable candidate for bioassay guided isolation and in-vivo evaluation.
KEYWORDS: *Andrographis paniculata, sequential fractionation, TLC fingerprinting, phytochemical profiling, total phenol content, total flavonoid content, total anti-oxidant capacity, α- amylase inhibition, β- glucosidase inhibition, anti-diabetic activity, polyphenols.*

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INTRODUCTION

Andrographis paniculata (Burm. f.) Nees is a widely used medicinal plant for its diverse pharmacological activities, including anti-oxidant, anti-inflammatory, and anti-diabetic effects. Bioactive constituents such as flavonoids, polyphenols, diterpenoid lactones (Andrographolide) in the *Andrographis paniculata* contribute to its therapeutic effects [1,2].

Recent studies demonstrated the potential of *Andrographis paniculata* in influencing the metabolic pathways through enzyme inhibition and molecular interactions. Computational and experimental studies revealed the presence of various phytoconstituents capable of suppressing carbohydrate metabolising enzymes suggesting strong anti- diabetic activity [3,4]. Diterpene lactones isolated from the *Andrographis paniculata* leaves exhibited, α- amylase and β- glucosidase inhibition which confirmed the anti- diabetic potential of the plant. Although these findings are promising, majority of the research has been directed toward crude extract or isolated compounds with limited exploration of systematic fractionation and the association between phytochemical profiling and bioactivity. Sequential solvent

fractionation helps separate phytoconstituents based on polarity which helps to concentrate bioactive compounds facilitating a clear understanding of their biological effects. Despite this, the role of solvent polarity in selective enrichment of bioactive components in *Andrographis paniculata* has not been adequately investigated.

Ethnomedical importance of *Andrographis paniculata*

Andrographis paniculata is an annual herb distributed throughout tropical and subtropical regions of Asia. The plant is commonly known as Nilavembu, Kalmegh or King of bitters [5,6]. It is traditionally used in Ayurveda, Siddha, Chinese medicine and folk medicine. The scientific investigations of Andrographis paniculata has identified several bioactive compounds such as andrographolide, neoandrographolide and flavonoids. These constituents have demonstrated its use in the treatment of fever, diabetes, jaundice, dyspepsia, diarrhoea, bronchitis, intestinal worm infestations, rheumatism, parasitic infections, eczema (7,8).

RATIONALE AND OBJECTIVE OF THE STUDY

Andrographis paniculata leaves was traditionally used in the management of Diabetes mellitus [9]. The bioactive constituents of the seeds such as flavonoids, terpenoids are potential anti-hyperglycaemic and anti-oxidants [10]. The plant derived anti- oxidants play an important role in the glycaemic regulation by reducing oxidative stress which is a major contributor of diabetic complications. Hence, the present study is to evaluate the anti-diabetic activity of the extract and fractions of *Andrographis paniculata* leaves for scientific validation of its ethnomedical importance.

MATERIAL AND METHODS

Plant Material Collection and Authentication:

Andrographis paniculata leaves were collected from Tamil Nadu Agriculture university, Coimbatore and authenticated (BSI/SRC/5/23/2024/Tech- 1) by the scientist at Botanical Survey of India, Southern Region Centre, T.N.A.U. Campus, Coimbatore.

Preparation of Crude Extract

Leaves of *Andrographis paniculata* are shade dried and pulverized into fine powder. The leaf powder was then subjected to cold maceration using ethanol for 72 hours. The ethanolic extract was then dried using rotary evaporator [11].

Sequential Solvent- Solvent Fractionation

Solvent- solvent fractionation was carried out for the dried ethanolic extract. Solvents of different polarity were used for fractionation process viz., petroleum ether, chloroform, ethyl acetate, and demineralised water. Solvent fractions are stored in a desiccator for further studies [12].

Preliminary Phytochemical Screening

The solvent fractions were evaluated for preliminary phytochemical screening to identify various phytoconstituents like alkaloids, flavonoids, glycosides, saponins, tannins, phenolic compounds, terpenoids and steroids using standard qualitative phytochemical method [13-15].

Thin Layer Chromatography (TLC) Analysis

TLC analysis of extract and the fractions of *Andrographis paniculata* leaves was carried out using Silica gel 60 F_{254} plates using Toluene: Ethyl Acetate: Formic acid as a mobile phase (5.0 :4.0: 0.2). The developed TLC plates were visualised at 254 nm and 540 nm under UV light. Rf values are calculated to identify the isolated compounds [16].

Determination of Total Flavonoid Content

Total flavonoid content was determined by Aluminium chloride assay [17,18,19]. 1ml of each solvent fraction was individually dissolved in 0.1 ml of 10% Aluminium chloride solution, 0.1 ml of sodium potassium tartarate followed by 2.8 ml of distilled water in test tubes. The mixtures are then kept undisturbed at room temperature for 30 minutes and the absorbance was measured at 415nm using UV-Visible spectrophotometer. Blank was maintained without adding sample. Quercetin was used as a standard to calculate the mg/g of the flavonoid content.

Determination of Total Phenolic Content

Total phenolic content of the solvent fraction was studied using Folin- Ciocalteau's reagent method with modifications [20,21]. 2.5 ml of 0.2N Foli- Ciocalteau's reagent was added to 0.5ml of the solvent fractions, mixed gently and kept undisturbed at room temperature for 10 minutes. 2ml of 2 %w/v sodium carbonate solution was added and the mixture was allowed to stand at 30^0 C for 20 minutes. Three replicates were maintained for each solvent fraction. The absorbance of the samples is measured at 765nm using UV-Visible spectrophotometer (Labtronics LT 291, microprocessor). The phenolic content was estimated from the standard curve of Gallic acid and expressed in Gallic Acid Equivalent (GAE)/g.

Determination of Total Anti-Oxidant Capacity (TAC)

Total anti-oxidant capacity was analysed using phospho molybdenum method with slight modification [22,23,24]. 0.5 ml of solvent fractions were individually mixed with 0.5ml of reaction mixture containing 06. M H2SO4, 28mM sodium phosphate and 4mM ammonium molybdate reagent. The solutions are incubated at 50 ^{0} C for 90 minutes with blank. After incubation, the samples were normalised to room temperature and the absorbance was measured at 695nm using UV-Visible spectrophotometer. Ascorbic acid was used as a standard to calculate the mg/g of the total anti- oxidant activity.

***In- vitro* α- Amylase Inhibition assay**

The alpha amylase inhibition assay was carried out to determine the ability of the plant extract and its fractions to modulate carbohydrate metabolism [25]. A reaction mixture containing 500 µL of the test (25,50,75,100µg/ml) sample and 0.25 mL of 0.1% starch solution (in 16mM sodium acetate buffer, pH 6.8) was prepared. 100 µLof alpha amylase enzyme solution was added and the mixture was incubated at 25 ^{0} C for 10 minutes under alkaline conditions. After incubation, 0.25mL of sodium potassium tartaric acid and 96 mL of 3, 5 – dinitro salicylic acid (DNS) reagent is added to cease the reaction. The absorbance of the samples was measured at 540 nm using UV-Visible spectrophotometer [23]. The percentage inhibition was calculated using the formula.

$$\% \text{ of inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

***In- vitro* β- Glucosidase Inhibition assay**

The alpha glucosidase inhibition assay was performed to assess the potential of the plant extract and its solvent fractions in regulating postprandial glucose levels. A reaction mixture containing 500 µL of the test sample (25,50,75,100µg/ml) of Tris buffer (pH 8.0) was added and the samples were incubated at 37 ^{0} C for 15 minutes. After incubation, 100 µL of alpha glucosidase enzyme solution was added and incubated at 35 ^{0} C for 45 minutes The reaction was ceased by adding 6N HCl and the absorbance was recorded at 540nm using UV-Visible spectrophotometer [26]. The percentage inhibition was calculated using the formula mentioned as above in the alpha amylase inhibition assay.

IC_{50} Determination

α- Amylase Inhibition assay

α- Amylase inhibitory activity of *Andrographis paniculata* leaves was evaluated at concentrations ranging from 25 -100 µg/ml. Percentage inhibition was calculated relative to the control, and IC_{50} values were determined by linear regression analysis of concentration versus percentage inhibition.

Statistical Analysis

All measurements were performed in triplicate and expressed as mean ± standard deviation (SD). IC_{50} values were calculated from concentration- response curves by regression analysis. Statistical significance was considered at $p < 0.05$.

RESULTS AND DISCUSSION

Phytochemical Screening

The phytochemical screening results (Table 1) revealed the presence of several bioactive constituents distributed among different solvent fractions. Alkaloids and terpenoids were detected in most fractions except the ethanolic extract, while phenolics and sugars were predominantly present in the ethyl acetate and aqueous fractions. Saponins were observed only in the aqueous fraction, reflecting their polar nature. Flavonoids were detected in the ethanolic extract, ethyl acetate fraction, and aqueous fraction, whereas quinones were present only in the ethanolic extract. Proteins were identified in the petroleum ether, ethyl acetate, and aqueous fractions, while steroids were detected in all fractions. The abundance of phenolics, flavonoids, alkaloids, and terpenoids suggests that the extract possesses significant biological potential, particularly antioxidant and therapeutic activities. Overall, the ethyl acetate and aqueous fractions exhibited a richer phytochemical profile compared to the other fractions (Table 1).

Table 1: Phytochemical screening results of extract and its solvent fractions

Chemical constituent	Chemical tests	Extract/ Fractions				
		Ethanolic extract	Petroleum ether fraction	Chloroform fraction	Ethyl acetate fraction	Aqueous fraction
Alkaloids	Mayer's test	–	+	+	+	+
Terpenoids	Sulphuric acid test	+	+	+	+	+
Phenol	Ferric chloride test	–	–	–	+	+
Sugar	Fehling's test	–	–	–	+	+
Saponins	Foam test	–	–	–	–	+
Flavonoids	Shinoda test	+	–	–	+	+
Quinones	Alkaline Reagent Test	+	–	–	–	–
Protein	Xanthoproteic Test	–	+	–	+	+
Steroids	Salkowski Test	+	+	+	+	+

TLC Fingerprinting of Extract and Fractions

TLC profiling of the ethanol extract and its solvent fractions revealed the presence of diverse phytochemical constituents with varying polarities (Table 2). The ethanol extract exhibited three distinct spots with Rf values of 2.7, 3.5, and 4.0, indicating the presence of flavonoids, phenolics, and diterpenoid lactones. Similarly, the petroleum ether and chloroform fractions produced three spots each, suggesting the enrichment of non-polar compounds such as lipids, fatty acids, phytosterols, terpenoids, and triterpenoids.

The ethyl acetate and aqueous fractions showed two spots each, corresponding mainly to flavonoids, phenolic acids, glycosides, tannins, and sugars. Comparison with the quercetin standard revealed similar Rf values in some fractions, indicating the possible presence of quercetin-related flavonoid compounds. The observed variation in spot numbers and Rf values among fractions confirms the successful separation of phytoconstituents according to their polarity. Overall, the TLC results support the phytochemical screening findings and demonstrate that the ethanol extract and its fractions contain a broad range of bioactive metabolites (Table 2).

Table 2: TLC profiling of ethanol extract and its solvent fractions

Fraction	Number of spots	Rf value	Probable compounds
Ethanol extract	3	2.7, 3.5, 4.0	Flavonoids, phenolics, diterpenoid lactones
Petroleum ether fraction	3	2.0, 3.0, 5.5	Lipids, fatty acids, phytosterols, terpenoids
Chloroform fraction	3	1.8, 2.7, 4.5	Diterpenoids, phytosterols, triterpenoids
Ethyl acetate fraction	2	1.9, 3.1	Flavonoids, phenolic acids
Aqueous fraction	2	2.8, 3.5	Phenolic glycosides, tannins, sugars, water soluble flavonoids
Standard	2	2.9, 3.6	Quercetin

Total Flavonoid Content

As shown in Table 3, the ethanolic extract exhibited the highest flavonoid content (348.04 µg/ml) among all fractions, followed by the aqueous fraction (245.66 µg/ml). The ethyl acetate and chloroform fractions showed moderate flavonoid levels, while the petroleum ether fraction contained the lowest amount (45.66 µg/ml). The higher flavonoid content in the ethanolic extract and aqueous fraction indicates the efficient extraction of flavonoids by polar solvents, supporting their potential antioxidant and therapeutic activities. (Table 3).

Table 3: Total flavonoid content in extract and its solvent fractions

Total flavonoid content (µg/ ml)					
Ethanolic extract	Petroleum ether fraction	Chloroform fraction	Ethyl acetate fraction	Aqueous fraction	Standard (Quercetin)
348.04	45.66	103.28	116.61	245.66	529.36

Total Phenol Content

The total phenolic content increased with increasing concentration in all extracts and fractions (Table 4). Among the tested samples, the aqueous fraction exhibited the highest phenolic content (67.90 mg GAE/g at 100 µg/ml), followed by the ethanolic extract (62.40 mg GAE/g). The ethyl acetate and chloroform

fractions showed moderate phenolic levels, while the petroleum ether fraction displayed the lowest content. The higher phenolic content in the aqueous and ethanolic extracts suggests a greater abundance of polar phenolic compounds, which may contribute to their antioxidant potential (Table 4).

Table 4: Total phenolic content in extract and its solvent fractions

Concentration µg/ ml	Total phenolic content (mg GAE/g)					
	Ethanolic extract	Petroleum ether fraction	Chloroform fraction	Ethyl acetate fraction	Aqueous fraction	Standard (Gallic acid)
25	26.49	12.94	14.79	21.04	31.04	32.38
50	34.89	19.73	19.24	34.94	42.87	59.64
75	56.14	24.40	26.88	49.86	59.33	86.19
100	62.40	25.73	34.90	50.50	67.90	127.03

Total Anti-Oxidant Capacity

The total antioxidant capacity of the extract and its fractions increased in a concentration-dependent manner (Table 5, Figure 1). Among the tested samples, the aqueous fraction exhibited the highest antioxidant activity (361.09 mg TE/g at 100 µg/ml), followed by the ethanolic extract (324.72 mg TE/g). Moderate activity was observed in the ethyl acetate and chloroform fractions, whereas the petroleum ether fraction showed the lowest antioxidant potential. The higher antioxidant activity of the aqueous and ethanolic extracts may be attributed to their greater phenolic and flavonoid contents. As expected, ascorbic acid exhibited the highest antioxidant activity among all samples (482.39 mg TE/g at 100 µg/ml). These findings indicate that the polar fractions are rich sources of antioxidant phytoconstituents (Table 5, Figure 1).

Table 5: Anti- oxidant activity of extract and its fractions

Concentration µg/ ml	Anti- oxidant capacity (mg TE/g)					
	Ethanolic extract	Petroleum ether fraction	Chloroform fraction	Ethyl acetate fraction	Aqueous fraction	Standard (Ascorbic acid)
25	186.39	24.77	103.34	94.83	194.96	229.61
50	204.14	39.87	122.71	186.33	226.84	359.48
75	284.19	66.64	154.39	190.04	303.49	416.59
100	324.72	83.36	161.09	202.54	361.09	482.39

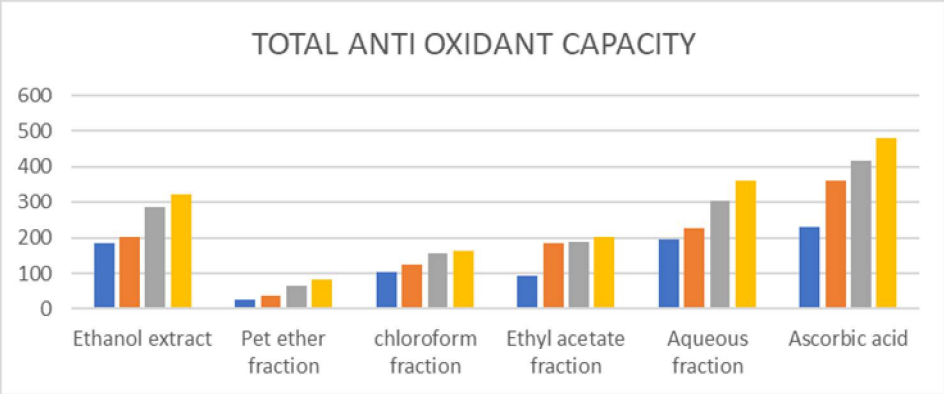

Figure 1: Total anti- oxidant capacity of extract and its fractions

***In- Vitro* Enzyme Inhibition Studies**

A- Amylase Inhibition Activity

The α-amylase inhibition activity increased with increasing concentration in all extracts and fractions (Table 6, Figure 2). Among the tested samples, the aqueous fraction exhibited the highest inhibitory activity (66.38% at 100 µg/ml), followed by the ethanolic extract (63.48%) and ethyl acetate fraction (60.75%). The chloroform and petroleum ether fractions showed comparatively lower inhibition. The standard drug acarbose demonstrated the highest α-amylase inhibitory activity (72.67% at 100 µg/ml). The significant inhibition observed in the aqueous and ethanolic extracts may be attributed to their higher content of phenolic and flavonoid compounds. These findings suggest that the extract possesses promising antidiabetic potential through inhibition of carbohydrate-hydrolyzing enzymes (Table 6, Figure 2).

Concentration	α -amylase inhibition activity (%)					
	Ethanolic extract	Petroleum ether fraction	Chloroform fraction	Ethyl acetate fraction	Aqueous fraction	Standard (Acarbose)
25	39.42	9.21	14.67	19.28	46.24	41.57
50	42.32	11.77	16.38	32.59	48.63	50.88
75	56.82	11.94	17.06	43.68	56.14	59.83
100	63.48	12.09	18.14	60.75	66.38	72.67

Table 6: α- amylase inhibition activity of ethanol extract and its solvent fractions

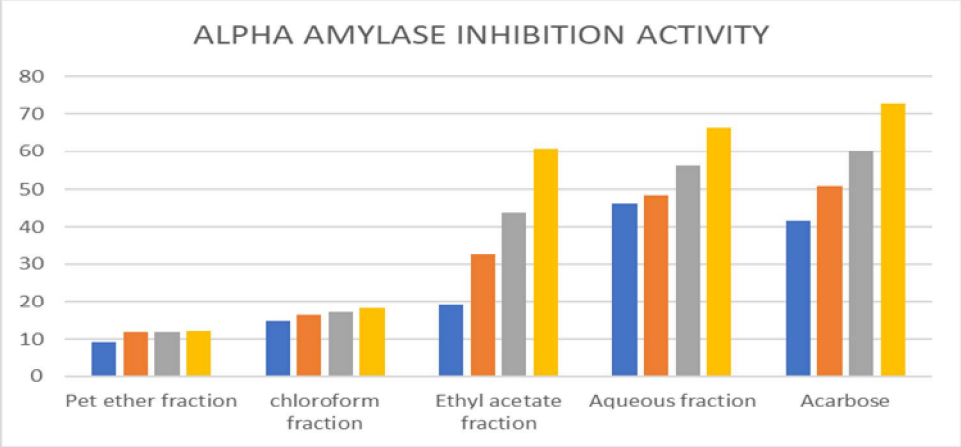

Figure 2: α -amylase inhibition activity of extract and its fractions

Alpha Glucosidase Inhibition Activity

The α-glucosidase inhibition activity increased progressively with increasing concentration in all extracts and fractions (Table 7, Figure 3). At 100 µg/ml, the ethanolic extract exhibited the highest inhibitory activity among the test samples (52.9%), followed by the ethyl acetate fraction (51.6%) and aqueous fraction (48.9%). The chloroform and petroleum ether fractions showed comparatively lower inhibition. The standard drug acarbose demonstrated the greatest α-glucosidase inhibitory activity (72.67% at 100 µg/ml). The notable inhibitory effects of the ethanolic extract, ethyl acetate fraction, and aqueous fraction may be attributed to their higher levels of flavonoids and phenolic compounds. These findings indicate the potential of the extract as a natural source of α-glucosidase inhibitors for the management of postprandial hyperglycemia (Table 7, Figure 3).

Table 7: α -glucosidase inhibition activity of extract and its fractions

Concentration	α -glucosidase inhibition activity (%)					
	Ethanolic extract	Petroleum ether fraction	Chloroform fraction	Ethyl acetate fraction	Aqueous fraction	Standard (Acarbose)
25	21.4	1.4	10.6	20.8	21.9	41.57
50	34.5	2.8	12.8	34.6	36.4	50.88
75	47.6	4.6	14.4	49.4	39.6	59.83
100	52.9	7.4	16.9	51.6	48.9	72.67

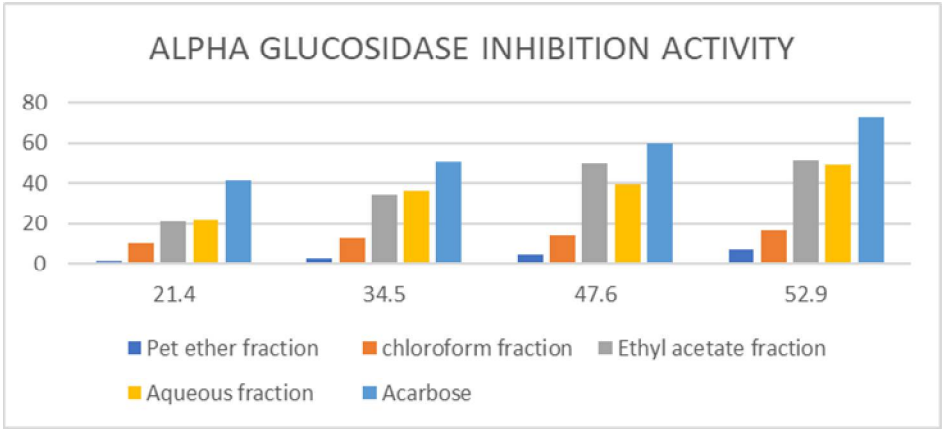

Figure 3: α -glucosidase inhibition activity of extract and its fractions

IC_{50} Analysis and Activity Comparison

In the alpha amylase inhibitory activity, the IC_{50} values of the extract was found to be 62.0 µg/ml and that of the fractions were >100, >100, 86.0 and 54.8 µg/ml for petroleum ether, chloroform, ethyl acetate and aqueous fractions respectively. In the alpha glucosidase inhibitory activity, the IC_{50} values of the extract was found to be 86.3 µg/ml and that of the fractions were >100, >100, 81.8 and > 100 µg/ml for petroleum ether, chloroform, ethyl acetate and aqueous fractions respectively. The IC_{50} value of the standard was found to be 48.2 µg/ml.

Correlation Between Phytochemicals and Enzyme Inhibition

Phytochemical screening of *Andrographis paniculata* leaf extract and its solvent fractions revealed the presence of flavonoids, phenolic compounds, diterpenoid lactones, glycosides, tannins, sterols and terpenoids. Differences in phytochemical composition among fractions appeared to be linked to differences in enzyme inhibitory activity. The aqueous fraction showed highest alpha amylase inhibitory activity (IC_{50}= 54.8 µg/ml) indicating that polar constituents such as phenolics, tannins and glycosides play a crucial role in alpha amylase inhibition. The ethanol extract exhibited significant activity which may be attributed to the presence of several bioactive phytochemicals collectively. The ethyl acetate fraction showed the highest activity as an alpha glucosidase inhibitor (IC_{50} = 81.8 µg/ml), indicating that semi- polar compounds like flavonoids, phenolic acids and diterpenoid lactones play a role in the inhibition of alpha glucosidase activity. The variations in enzyme inhibitory activity among the fractions appeared to be associated with the differences in their phytochemical composition.

Mechanistic Insights into Enzyme Inhibition

The obtained results suggest that the alpha amylase and alpha glucosidase inhibitory activities may be due to the bioactive components like flavonoids, phenolic compounds, tannins and diterpenoid lactones such as andrographolide. These compounds interact with carbohydrate hydrolyzing enzymes through hydrogen bonding, hydrophobic interactions and binding to catalytic amino acid residues. The phenolic compounds and flavonoids can inhibit enzyme activity by competitive binding to the active site. Tannins form stable complexes with proteins resulting in reduced catalytic activity. Diterpenoid lactones like andrographolide bind to the key residues responsible for substrate hydrolysis thereby influencing glucose metabolism.

The stronger enzyme inhibitory activities observed in the aqueous and ethyl acetate fractions indicates that the polar and semi-polar compounds are responsible for anti-diabetic activity. Inhibition of alpha amylase and alpha glucosidase enzymes delays the digestion of carbohydrates and absorption of glucose from the food contributing to the reduction in the post prandial glucose level. Therefore, the anti- diabetic activity of *Andrographis paniculata* may result from the synergistic effect of multiple phytoconstituents modulating the carbohydrate metabolising enzymes.

CONCLUSION

Sequential fractionation of *Andrographis paniculata* leaves enriched phytoconstituents like flavonoids, phenolics exhibited significant anti- oxidant and anti- diabetic activities. Concentration dependent inhibition of alpha amylase and alpha glucosidase enzymes of the fractions suggesting their efficacy to regulate the postprandial glucose levels. A positive correlation observed between the phytochemical content and the enzyme inhibitory activity offers the mechanistic support for the use of *Andrographis paniculata* leaves in the management of Diabetes mellitus. Overall, the study emphasizes the anti- diabetic potential of *Andrographis paniculata* leaves.

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