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In-vitro Anti-Oxidant and alpha-Amylase Inhibitory Activity of Isolated Fractions from Methanolic Extract of *Asystasia dalzelliana* Leaves

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Abstract: The prevalence of diabetes mellitus is on the increase and needs to be addressed appropriately. In this study area, herbal remedies are considered convenient for management of diabetes with postprandial hyperglycemia due to their traditional acceptability and availability, low costs, lesser side effects. In developing countries, where the per capita income is low, it is necessary to seek affordable alternative therapies. The little epidemiological evidence is available on the role of dietary antioxidant intake in prevention of type 2 diabetes. The present study concern about isolation of anti oxidant and α-amylase inhibitory constituents from methanolic extract of *Asystasia dalzelliana* leaves. The antioxidant activity is done using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging, Nitric Oxide Scavenging, reducing power methods and also screened for anti-amylase activity. Methanolic extract upon the column chromatography yielded five fractions named (AD-01, AD-02, AD-03, AD-04, and AD-05) and were screened for their anti-oxidant and α-amylase inhibitory activity. Among the five fractions tested AD-04, AD-05 fraction shown more activity than AD-03 in both the cases of anti-oxidant and α-amylase inhibitory activity compared with standard gallic acid and acarbose respectively. EC_{50} values of DPPH free radical for AD-03, AD-04, AD-05 and gallic acid standard was found to be 168.76, 117.80, 138.30, 89.59µg/ml and EC_{50} values of Nitric Oxide scavenging activity for AD-03, AD-04, AD-05 and gallic acid standard were found to be and 172.24, 113.46, 128.56, 90.46µg/ml respectively. The AD-03, AD-04, AD-05 fraction exhibited appreciable α-amylase inhibitory activity with an IC_{50} values 25.78, 52.78, and 56.46µg/ml respectively, when compared with acarbose (IC_{50} value 80.34µg/ml). Therefore, our studies support the use of active constituents from *Asystasia dalzelliana* leaves for diabetes mellitus management.
Key words: *Asystasia dalzelliana*, column chromatography, Anti oxidant, anti-amylase.

Introduction

The little epidemiological evidence is available on the role of dietary antioxidant intake in prevention of type 2 diabetes. Previously comparison of various extract of *Asystasia dalzelliana* leaves was reported for anti oxidant activity. Herbs, spices and medicinal plants have been cherished by many ancient cultures for their use in curing common ailments and promoting good health[1]. Although obesity and physical inactivity are known to be major risk factors for type 2 diabetes, recent evidence suggests that oxidative stress may contribute to the pathogenesis of type 2 diabetes by increasing insulin resistance or impairing insulin secretion[2]. Dietary antioxidants have been hypothesized to have a protective effect against the development of diabetes by inhibiting peroxidation chain reactions[3]. It seems plausible that a sufficient intake of antioxidants plays an important role in protection against type 2 diabetes. Type 2 diabetes mellitus is a major metabolic disorder that is increasing worldwide. Normally, the entry of glucose into pancreatic â-cells stimulates the release of insulin into the blood, where it stimulates peripheral cells to take up glucose. But in Type 2 diabetes, insulin is

secreted in inadequate or inappropriate amounts and/ or peripheral cells become resistant to the action of insulin, resulting in hyperglycemia[4]. Long-term hyperglycemia can lead to damage in cells that cannot block sugar from entering. In these cells (especially those lining blood vessels), as mitochondria utilize entering sugars, harmful byproducts accumulate.

Much research has focused on glycosidase inhibitors to control hyperglycemia, but many forms of starch are also digested as rapidly as glucose absorption[5-9]. Slowing the digestion and breakdown of starch may have beneficial effects on insulin resistance and glycemic index control in people with diabetes[4, 6]. Inhibitory activity against amylase by flavonoid and anthocyanins has been reported[10, 11]

Asystasia dalzelliana commonly known as violet Asystasia (Marathi: Neelkanth) belongs to family Acanthaceae. It is a perennial branched herb, about 60-100 m. Stem quadrangular, swollen at nodes. Leaves are opposite, elliptic-oblanceolate acute apex truncate at base; petiole 2cm long[12]. The whole plant is used in Indian folk medicine as antioxidant, anti-inflammatory, anti venom a novel whose pharmacology yet to be proved[13]. In-vitro methods play important role for the preclinical studies for any activity, which makes support to the in-vivo studies.

The present work was planned to evaluate the effect of isolated fractions on oxidative stress events by anti-oxidant and α-amylase inhibition in in-vitro pharmacological models. The significance of plant-based amylase (PPA) inhibitors for oxidation-linked disease modulation was hypothesized in this study.

Material and Methods

Plant material

The fresh leaves were collected from Bidar (District of Karnataka state, India) and authenticated by Dr. Siddamallaya Regional Research Institute, Bangalore. The voucher specimen (RRI/BNG/SMP/2009-10/717) was deposited in the same institute. Prior to use, it was ensured that the leaves were free from contamination, sand and had no microbial growth.

Preparation of Methanolic Extract

Dried leaves of *Asystasia dalzelliana* macerated for 24 hrs with methanol (1:4) at room temperature, followed by two washes (refluxes) with methanol ratio 1:3 and filtered. Filtrates were combined, concentrated under vacuum at temperature not more than 70^0C and dried in vacuum tray drier.

Fractionation and Isolation

50g of methanolic extract was chromatographed on a silica gel (60-120 mesh) column using gradient elution starting with n-hexane and ending with methanol to obtain 5 fractions. Fractions were concentrated to 1/10th of their volume and kept for crystallization at room temperature for 7 day. Three out of five fractions showed crystal formation and named as AD-01 (90% hexane in ethyl acetate), AD-02 (60:40 methanol and ethyl acetate), AD-03 (50:50 methanol and ethyl acetate) AD-04 (75:25 methanol and ethyl acetate), AD-05 (100% methanol). Further purification was done by re-crystallization. The compounds were confirmed for the single constituent by running TLC using different solvent systems and finally the spots were separated as single in ethyl acetate: formic acid: glacial acetic acid: water (10:1.1:1.1:2.6) system and observed in UV at 254 nm.

Antioxidant activity

DPPH radical scavenging assay[14, 15]

DPPH scavenging activity was measured by the slightly modified spectrophotometric method. A solution of DPPH in methanol (6 x 10^{-5} M) was prepared freshly. A 3 ml aliquot of this solution was mixed with 100 µl of the samples at varying concentrations (50–250 µg/ml). The solutions in the test tubes were shaken well and incubated in the dark light for 15 min at room temperature. The decrease in absorbance was measured at 517nm. The percentage inhibition of the radicals due to the antioxidant property of the isolated fractions was calculated using the formula,

$[(A_0–A_1)/A_0]$ x100,

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the extract/ standard. Blank is the absorbance of the control reaction (containing all reagents except the test compound). The % scavenging activity of different concentration of isolated fraction was tabulated in Table.1 & Fig: 1.

Tab: 1. EC_{50} values (µg/ml) of isolated fractions in DDPH assay method.

Sl. No	Isolated Fractions	EC_{50} µg/ml
1	AD-03	168.76
2	AD-04	117.08
3	AD-05	138.30
4	Standard(gallic acid)	89.59

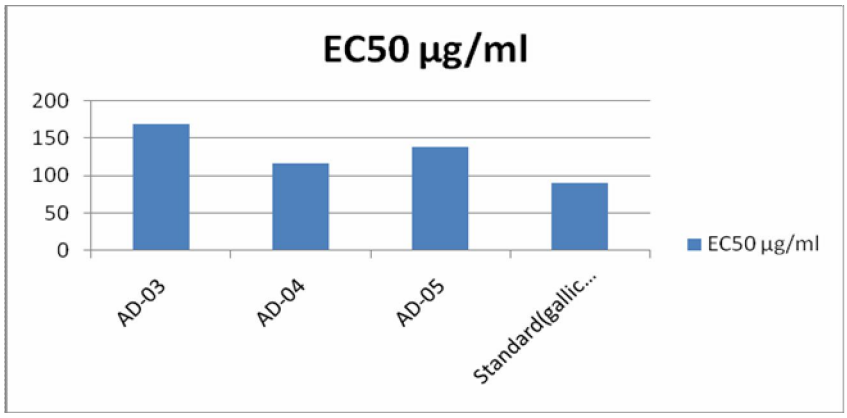

Figure 1. EC_{50} values (µg/ml) of isolated fractions for DDPH assay method

Reducing Power method[16]

The AD-03, AD-04, AD-05 fractions of methanolic extract of dried leaves of *Asystasia dalzelliana* (50µg, 100µg, 150µg and 200µg) in 1 ml of appropriate solvents were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide [$K_3Fe(CN)_6$] (1%) and then the mixture were incubated at 50°C for 30 min. Afterward, 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 mL of the upper layer solution was mixed with 2.5 ml of distilled water and 0.5 mL of $FeCl_3$ (0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixtures indicates the increased reducing power. The reducing power activity for different concentrations of isolated fractions is tabulated in Table.2 & Fig: 2.

Tab: 2. Reducing powers activity of Isolated Fractions.

SI No	Concentration µg/ml	Absorbance at 700nm			
		AD-03	AD-04	AD-05	Std (Gallic acid)
1	5	0.254	0.526	0.345	0.826
2	10	0.386	0.632	0.502	0.964
3	15	0.542	0.786	0.628	1.02
4	20	0.658	0.912	0.738	1.24

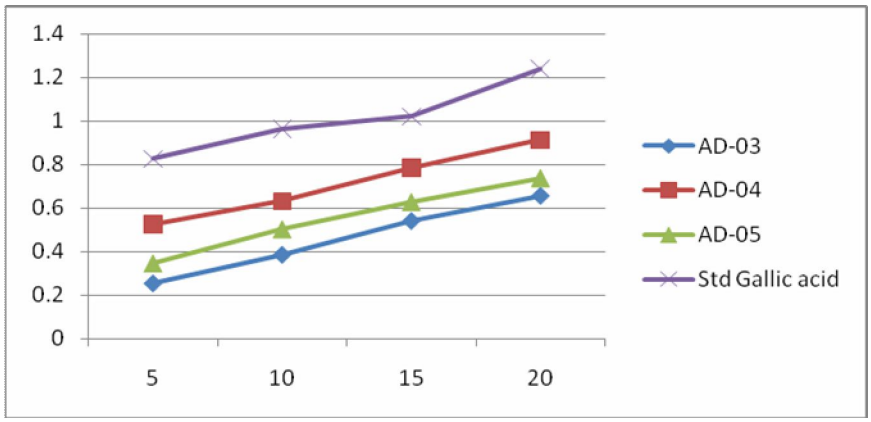

Figure 2. Reducing powers activity of Isolated Fractions.

Nitric oxide generation and assay of Nitric oxide scavenging method[17- 20]

Nitric oxide (NO) was generated from sodium nitroprusside (SNP) and was measured by the Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dichloride and 3% phosphoric acid). SNP in aqueous solution at physiological pH spontaneously generates NO which interacts with oxygen to produce nitrite ions that can be estimated by the use of Griess reagent. Scavengers of NO compete with oxygen leading to reduced production of NO. SNP (10mM) in phosphate buffer saline (PBS) was mixed with different concentration of isolated fraction (100-500µg/ml) of the drug dissolved in ethanol and water then incubated at 25°C for 180 minutes. The samples from the above were made to react with Griess reagent. The absorbance of the chromophores formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine dichloride was read at 546 nm and referred to the absorbance of ascorbic Acid, taken as a positive control treated in the same way with Griess reagent.

Nitric Oxide scavenged (%)

$$= \frac{A_{control} - A_{test}}{A_{control}} \times 100$$

Where, $A_{control}$ = Absorbance of control reaction,

A_{test} = Absorbance in the presence of the samples of isolated fraction.

The % Nitric Oxide scavenged activity of different concentrations of isolated fractions are tabulated in Table.3 & Fig: 3.

***In Vitro* α- Amylase Inhibitory Assay**[21]

Starch azure (2 mg) was suspended in a tube containing 0.2ml of 0.5 M Tris-Hcl buffer (pH 6.9) containing 0.01 M calcium chloride (substrate). The tubes were boiled for 5 min and then pre incubated at 37°C for 5 min. 1ml of 0.1% of dimethyl sulfoxide was used to dissolve 1mg of isolated fractions in order to obtain concentrations of 20, 40, 60, 80 and 100µg/ml. Then 0.2 ml of isolated fraction of a particular concentration was added in the tube containing the substrate solution. 0.1 ml of porcine pancreatic amylase in Tris-Hcl buffer (2units/ml) was added to the tube containing the isolated fraction and substrate solution, all the process was carried out at 37°C for 10 min. The reaction was stopped by adding 0.5 ml of 50% acetic acid in each tube. The reaction mixture was then centrifuged at 3000 rpm for 5 min at 4°C and the absorbance of resulting supernatant was measured at 595 nm spectrometrically.

The α-amylase inhibitory activity = (Ac+) - (Ac-) - (As-Ab) / (Ac+) - (Ac-) X 100

Where, Ac+ is absorbance of 100% enzyme activity (only solvent with enzyme), Ac- is 0% enzyme activity (only solvent without enzyme), As, test sample (with enzyme) and Ab is blank (a test sample without enzyme) respectively. The percentage α-amylase inhibitory activity and IC_{50} value of different concentration of isolated fractions are tabulated in Table.4 & Fig: 4.

Tab: 3. EC_{50} values (µg/ml) of isolated fractions for nitric oxide scavenging assay

SI no	IsolatedFractions	EC_{50} µg/ml
1	AD-03	172.24
2	AD-04	113.46
3	AD-05	128.56
4	Standard(gallic acid)	90.46

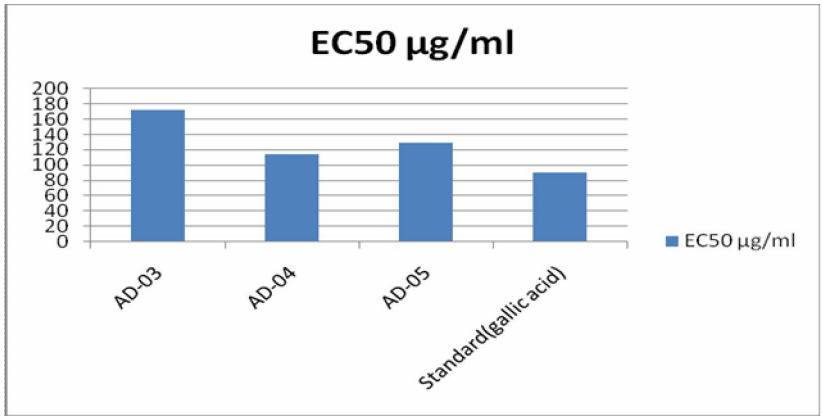

Figure 3. EC_{50} values (µg/ml) of isolated fractions for nitric oxide scavenging method.

Tab: 4 IC_{50} values (µg/ml) of isolated fractions for α-Amylase Inhibitory Assay

SI. No	IsolatedFractions	IC_{50} µg/ml
1	AD-03	25.78
2	AD-04	52.78
3	AD-05	56.46
4	Standard(acarbose)	80.34

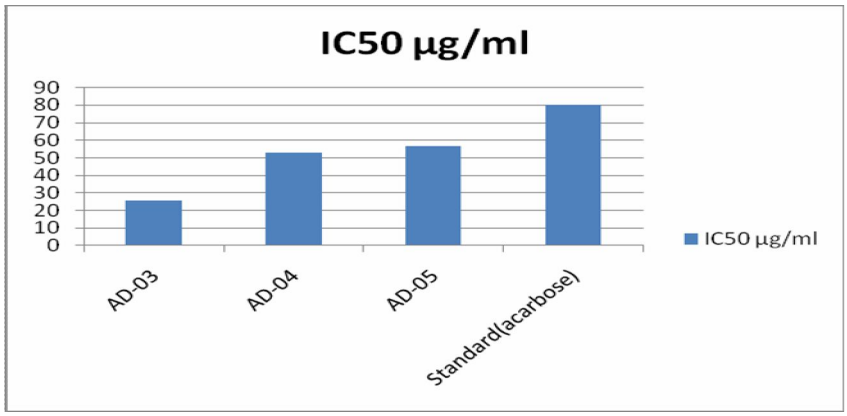

Figure 4. IC_{50} values (µg/ml) of isolated fractions for α-Amylase inhibitory assay

Results

The methanolic extract yielded 5 different fractions named as AD-01, AD-02, AD-03, AD-04, AD-05. The all fractions obtained were screened for its anti-oxidant and alpha-amylase inhibitory actions. The isolated fractions named AD-01, AD-02 are neglected in result part due to their less activity or no activity. The isolated fractions Ad-03, AD-04 and AD-05 were screened by using DPPH, reducing power and nitric oxide scavenger activity. EC_{50} values of DPPH free radical for AD-03, AD-04, AD-05 and gallic acid standard was found to be 168.76, 117.80, 138.30, 89.59µg/ml respectively. The reducing power goes on increases with increase in the concentration resulted were compared to the standard gallic acid as summarized in the table no.2. The standard, acarbose for alpha amylase inhibitory action shown 80.34% inhibition as the AD-05 shown 56.46% inhibition and AD-04 showed 52.78%. The alpha amylase inhibitory table clearly indicates that AD-05 is more active than the AD-03 or AD-04.

Discussion and Conclusion

Oxidative stress has been shown to have a significant effect in the causation of diabetes as well as diabetes related complications in human beings. Oxidative stress may have significant effect in the glucose transport protein (GLUT) or at insulin receptor. Scavengers of oxidative stress may have an effect in reducing the increased serum glucose level in diabetes and may alleviate the diabetes as well as reduce its secondary complications. The DPPH, Scavenger activity and nitric oxide methods confirmed its antioxidant activity. The enzyme pancreatic alpha-amylase plays a major role in the digestion of the starch. Blocking this pathway is one of the ways for antidiabetic action. In vitro anti oxidant and α-amylase inhibitory studies of isolated fractions (AD-03, AD-04, and AD-05) of the leaves of *Asystasia dalzelliana* shown prominent activity. The present study concludes that AD-04 and AD-05 fractions shows more anti-oxidant and α-amylase inhibitory activity compared with standard gallic acid and acarbose respectively. This made proper attempt to isolate the active principles from *Asystasia dalzelliana* leaves which might help in the findings of new lead compounds in the fields of anti-diabetic drug research Therefore, our studies support the use of active constituents from *Asystasia dalzelliana* leaves in diabetes mellitus management.

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