



# Evaluation of *in vitro* Anti-Diabetic Potential of Aster (*Callistephus chinensis*) Flower Waste Using Alpha-Amylase Inhibitory Assay and Glucose Uptake Assay

HN Nagaonkar<sup>1</sup>, PP Dhawal<sup>2</sup>, SS Barve<sup>2</sup> and SA Kakodkar<sup>1\*</sup>

<sup>1</sup>Department of Biotechnology, KET's V. G. Vaze College of Arts Science and Commerce (Autonomous), Mulund, Mumbai - 400081, Maharashtra, India.

<sup>2</sup>KET's Scientific Research Centre, KET's V. G. Vaze College Campus, Mulund, Mumbai - 400081, Maharashtra, India.

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\*Corresponding Author Email: [shrutibaadkar3@gmail.com](mailto:shrutibaadkar3@gmail.com)

## Abstract

Diabetes mellitus is a major chronic disease that predisposes an individual to severe health complications. The modes of action against diabetes involve inhibiting activities of enzymes such as alpha-amylase, alpha-glucosidase and suppression of oxidative stress emerging due to hyperglycemia. The conventional drugs used for treating diabetes may result in severe side effects. In wake of this, several studies are now exploring the option of using phytochemicals extracted from plants as an alternative method for treating diabetes. The current study investigated the anti-diabetic property of Aster (*Callistephus chinensis*) flower waste (AFW) extract using *in-vitro* assays such as alpha-amylase inhibitory assay and glucose uptake by yeast cells. Additionally, antioxidant activity of the extract was evaluated using ABTS assay. The IC<sub>50</sub> of AFW extract in alpha-amylase inhibitory assay was found to be 1.37 mg/ml, which reflects its promising salivary alpha-amylase inhibitory activity. The glucose uptake assay revealed that 0.5mg/ml extract facilitated maximum increase in glucose uptake (96.875 %) by yeast cells. The rise in percent glucose uptake in yeast cells (91.303%) elicited in the presence of 2 mg/ml AFW extract and 25 mM glucose system was found to be comparable to that observed for 2 mg/ml standard metformin (92.307%). The IC<sub>50</sub> value of AFW extract in ABTS assay was found to be 0.195 mg/ml, which corroborates its strong antioxidant property. In conclusion, *in-vitro* assays showed that AFW extract exhibits favorable *in-vitro* anti-diabetic and antioxidant properties. Our study attempted to highlight the use of organic flower waste for extracting valuable phytochemicals of therapeutic value.

## Keywords

*Callistephus chinensis*, flower waste, anti-diabetic, alpha-amylase, glucose uptake, ABTS assay.

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## INTRODUCTION:

Diabetes mellitus (DM) is a serious complex metabolic disease that occurs due to chronic hyperglycemia associated with failure of insulin

function, secretion or both<sup>[1]</sup>. Damage to health due to oxidative stress is strongly associated with pathophysiology of DM<sup>[2]</sup>. Chronic hyperglycemia causes tremendous oxidative stress owing to the rise

in the level of free radicals resulting from glucose-oxidation, non-enzymatic protein glycation and their oxidative degradation [3]. This oxidative stress eventually causes damage to crucial structural components of cells, DNA, lipids and proteins [4]. Thus, abnormal rise in glucose level due to diabetes culminates into improper functioning of various organs. Due to its overwhelming complex effects on human metabolism, it is considered to be one of the leading causes of death in the world [5-7]. In India, it is estimated that 77 million individuals suffer from diabetes [7]. Further, it is projected that the prevalence of diabetes in adult population will rise to 101 million by the year 2030 [7]. Type-2 DM is said to account for 90% of the total diabetic population [7]. Biguanides, sulfonylureas and thiazolidinediones are the major classes of oral agents reported in treating type-2 diabetes [8]. These agents cause depletion in blood glucose by either decreasing fasting glucose levels, increasing insulin release by triggering beta cells or sensitizing insulin [8]. However, these agents are reported to exert undesirable side effects such as severe gastrointestinal problems, hypoglycemia and hepatotoxicity, among others [8]. On account of this, traditional approach involving use of herbal medicines has gained immense focus as an alternative for addressing the adverse effects of diabetes due to their safe and effective nature [9].

Bioactive compounds such as phenols and flavonoids are considered as candidate phytochemicals in the remediation of serious health disorders like diabetes by virtue of their promising antioxidant and anti-diabetic properties [10-12]. Antioxidant properties of phytochemicals enable them to scavenge free radicals and reactive oxygen species that lead to oxidative stress. Plant extracts obtained using different solvent systems and extraction procedures have exhibited tremendous applications in the field of therapeutics. Currently, phyto-compounds present in olive oil, stevioside, ginseng, bitter melon, garlic, cinnamon, onion, *Gymnema sylvestre*, fenugreek, mushrooms, oriental melon seed etc. are gaining interests due to their noteworthy roles in treatment of diabetes [13-17]. Studies have shown that phytochemicals such as phenols and flavonoids function in delaying carbohydrate breakdown by inhibiting alpha-amylase enzyme [18]. Amylase acts by digesting complex carbohydrates into glucose. Amylase inhibitors disrupt the enzyme's carbohydrate digestion process thereby, causing decrease in the rate of glucose absorption in the body [9]. This, in turn, suppresses the rise in postprandial plasma glucose level, an outcome that is highly desired in DM patients. Acarbose is a synthetic amylase inhibitor used in treatment of

diabetes. Though acarbose is effective, it demonstrates severe gastrointestinal unrest and hence, is not desirable for long-term treatment [19]. In view of this, several research studies have attempted to explore the possible effectiveness of plants in inhibiting alpha-amylase activity [20-24]. Anti-diabetic potential of plant extracts is assessed using *in-vitro* indicator assays involving study of glucose uptake across cell membrane of yeast cells, adipose cells or muscle cells and inhibition of alpha-amylase and alpha-glucosidase activities [25].

Our previous study on aster (*Callistephus chinensis*) floral waste revealed the presence of notable phytochemicals *viz.* phenols and flavonoids [26]. In the same study, results of DPPH and FRAP assays revealed that hydroethanolic extract of aster flower waste had strong antioxidant property. However, further investigation using a more sensitive method such as ABTS assay is of importance as it can detect hydrophilic and lipophilic antioxidant properties which are not noticeable using DPPH assay [27]. Previous research studies on fresh *Callistephus chinensis* flowers have reported their alpha-glucosidase inhibitory activity and hepatoprotective abilities [28,29]. However, to our knowledge, a detailed study evaluating its potential alpha-amylase inhibitory activity and function in facilitating glucose uptake in yeast cells has not been explored. In India, flowers are widely used as offerings in temples and several traditional festivities. Most of these flowers after their use are often disposed inappropriately [30]. Accumulation of such improperly discarded floral waste can severely affect the ecosystem. As mentioned earlier, fresh plants have been the focal point of numerous research studies for extracting phytochemicals of medicinal interest. However, currently there is an urgent need to address the unbecoming burden of organic waste especially floral waste on the ecosystem.

Thus, the present study was initiated to investigate the anti-diabetic property of aster floral waste using salivary alpha-amylase inhibitory assay and glucose uptake assay. Further, the study also evaluated the antioxidant property of the extract using ABTS assay. The rationale of the present study is to emphasize the idea of exploring waste flowers that are abundantly available for obtaining prospective phytotherapeutic agents.

## MATERIALS AND METHODS:

### Preparation of aster flower waste (AFW) extract

AFW extract was prepared as per the procedure mentioned in our previous study [31]. Pink hued aster (*Callistephus chinensis*) flowers were collected from local markets in Mumbai and used for extraction

after minimum of 3 days. The wasted aster petals were then plucked and further used for extracting phytoconstituents using Soxhlet extraction procedure as previously described [31]. AFW extract obtained was dried in hot air oven for 3 days and later stored at 4 °C until further use.

#### **Salivary alpha-amylase inhibition assay**

A modified salivary alpha-amylase inhibition assay was carried out for AFW extract in 96-well plate. The assay mixture consisted of 20 µl extract of varied concentrations (0.5, 1, 2, 3, 4 and 5 mg/ml), 20 µl salivary alpha-amylase, 20 µl of 1% starch and 20 µl Phosphate buffered saline pH 7.4 (PBS). For color blank, the same additions were made but in absence of amylase enzyme. Maximum enzyme activity was obtained by preparing enzyme-substrate control consisting of 20 µl enzyme, 20 µl of starch substrate and 40 µl PBS. The 96-well plate was incubated at 37°C for 15 min.

The breakdown of starch into reducing sugars by alpha-amylase can be estimated using colorimetric or spectrophotometric based assays that employ chromogenic reagent 3,5-Dinitrosalicylic acid (DNSA). Standard DNSA assay was conducted with slight modifications for analyzing alpha-amylase activity in presence of AFW extract [32, 33]. In this, 20 µl of DNSA was added to 96-well plate. The plate was placed in boiling water bath for 10 min and later cooled at room temperature. The final volume in all the wells was made up to 100 µl using PBS. The plate was read at 540 nm wavelength using ELISA Well Plate Reader (MRX revelation, Thermo Lab systems). The ethanolic extract of *Terminalia arjuna* powder (10 g in 100 ml ethanol, cold extraction/24 h) was used as a positive control as it is known to possess strong alpha-amylase inhibitory activity. All experimental sets were conducted in triplicates. The percent inhibition of alpha-amylase activity was calculated using the following formula: Percent alpha-amylase inhibition = (Abs Control - Abs Sample) \* 100 / Abs Control

#### **Glucose uptake by yeast cells assay**

##### **Preparation of Yeast Cell Suspension**

Commercial baker's yeast pellets (2-3) were added to 50 ml of 0.9% saline solution and incubated overnight. The cells were washed with saline and centrifuged for 10 min at 3000 rpm. The process was repeated twice. The supernatant was discarded and the pellet was re-suspended in saline to give 1 ml of 10% (v/v) [34]. This suspension was used to count yeast cells using Trypan blue dye exclusion principle. Yeast cell suspension 10 µl was added to 10 µl of 0.4% Trypan blue. The hemocytometer slide with Neubauer Chamber was charged with 10 µl dye-cell

solution and, the cell viability along with density was measured [35].

##### **Study of glucose uptake by yeast cells**

Yeast cell suspension (10% v/v) consisting of 2-5 x 10<sup>6</sup> cells/ml was used to study glucose uptake assay for AFW extract. For test solution, 1 ml AFW extract (0.5, 1 and 2 mg/ml) was added to 50 µl yeast suspension. Three different concentrations of glucose (10, 25 and 50mM) were used and 450 µl was added to the respective test suspension. Color blank for each test was prepared by adding 1 ml of respective AFW extract concentrations to 50 µl of 0.9 % saline and 450 µl glucose solution. Initial glucose concentration was measured by preparing glucose control consisting of 1.05 ml of saline and 450 µl of glucose solution. For study of glucose uptake by yeast cells in absence of AFW extract, negative control was prepared by adding 1 ml of saline to 50 µl yeast suspension and 450 µl glucose solution. The reaction and control tubes were incubated for 1 hour at 37°C. After incubation, the pellet was recovered by centrifugation and, the amount of glucose present in the supernatant was tested using DNSA quantification method. The same experiment was performed using metformin as a standard [36]. Supernatant collected was allowed to react with 0.5 ml DNSA reagent in boiling water bath for 10 min. The tubes were allowed to cool and, later the volume was made up to 10 ml. The absorbance of the solution was measured spectrophotometrically at 540 nm.

##### **ABTS [2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] Antioxidant Assay**

ABTS antioxidant assay was performed to confirm the antioxidant nature of AFW extract. The hydrogen donating ability of the antioxidants present in AFW extract was estimated by monitoring their ability to scavenge ABTS<sup>•+</sup> radical generated by the interaction of ABTS with potassium persulfate [37].

##### **Preparation of ABTS stock solution**

ABTS radical cation (ABTS<sup>•+</sup>) solution was prepared by reacting 7 mM ABTS prepared in distilled water with 2.45 mM potassium persulfate. The mixture was incubated in dark for 16 hours before use. The solution was further diluted with distilled water to obtain an absorbance of 0.7 (±0.02) at 734 nm. This solution was considered as ABTS working solution [37].

##### **ABTS Assay**

ABTS assay, which is highly time-dependent, was conducted in a 96-well microtiter plate since microplate method is considered to be efficient in reducing assay time [38]. A modified ABTS assay was performed in the current study [39]. Different concentrations of AFW extract i.e. 0.2, 0.4, 0.6, 0.8

and 1.0 mg/ml were prepared in methanol. Test reaction mixture was prepared in microtiter plate by adding 100  $\mu$ l ABTS working solution to 20  $\mu$ l AFW extract. Color blank was set by substituting 100  $\mu$ l ABTS reagent with methanol. Further, a solution consisting of 20  $\mu$ l methanol and 100  $\mu$ l ABTS served as a negative control. The plate was incubated in dark for 30 min. The experiment set was performed in triplicates. The plate was read immediately within 5 min after incubation at 734 nm using ELISA plate reader. Percent ABTS radical scavenging activity was calculated using the formula, Percent ABTS Scavenging Activity =  $(A - A_0) \times 100 / A_0$ , where A is the absorbance of test solution and  $A_0$  is the absorbance of negative control.

### RESULTS AND DISCUSSION:

In recent years, exhaustive research has been conducted on fresh plants for extracting phytochemicals with medicinal applications. However, it should be noted that the large-scale application of these phytochemicals in therapeutics would entail their bulk extraction thereby, resulting in utilization of large quantities of fresh plants. This may consequently impose an inadvertent pressure on the environment. It is reported that approximately 1450 tonnes of flowers are used as temple offerings in India [40]. Incorrect disposal of these flowers has become a serious situation. Hence, several research studies have explored their alternate applications in the production of value-added products such as composts, biofuels, natural dyes, bio surfactants etc. [41]. On a similar line, the current work aimed to utilize organic AFW for recovering vital phytochemicals of medicinal value. The study intended to exhibit that utilization of organic floral waste for obtaining phytochemicals could potentially reduce the overwhelming dependence imposed on their fresh counterparts.

#### Salivary alpha-amylase inhibition assay

Previous studies on fresh flower extracts such as hydroethanolic extracts of *Hibiscus rosasinensis*, *Michelia champaca* flower buds and methanolic extract of *Punica granatum* have reported their promising anti-diabetic properties [42-44]. Inhibition of salivary and pancreatic amylase enzymes have been strongly implicated in reducing post-prandial rise in blood glucose and hence have been widely suggested as a strategy to manage diabetes [45,46]. The present study examined the salivary alpha-amylase inhibition activity of AFW extract. The percent inhibition of amylase activity at varied concentrations of AFW extract is shown in Table 1. A graph of percent salivary alpha-amylase inhibition activity versus concentration of AFW extract was plotted. The linear

regression equation obtained for the graph was  $y = 10.979x + 34.856$  ( $R^2 = 0.8392$ ) (Fig. 1). Based on the equation, the  $IC_{50}$  value was found to be 1.37 mg/ml. *T. arjuna* extract was selected as a positive control in the study due to its strong amylase inhibitory activity [47]. Percent inhibition of salivary alpha-amylase for *T. arjuna* extract is displayed in Table 2. Based on the linear regression equation,  $y = 32.935x + 20.632$  ( $R^2 = 0.9079$ ), the  $IC_{50}$  value calculated for the extract was 0.891 mg/ml which further affirms its strong salivary alpha-amylase inhibitory activity (Fig. 2). This result also validates the modified method employed in the present study for assessing salivary alpha-amylase inhibitory activity of AFW extract. In summation, our study revealed the application of ethanolic extract of AFW in inhibiting salivary amylase activity. Further research providing insight regarding the effect of AFW extract on pancreatic amylase activity is suggested.

#### Evaluation of glucose uptake by yeast cells

Glucose uptake by yeast cells in absence of AFW extract was found to be negligible. Maximum uptake of glucose (96.875%) was observed in presence of 0.5 mg/ml AFW extract and 50 mM glucose in the test system (Table 3). It was seen that 0.5 mg/ml and 1 mg/ml extract did not result in any noticeable trend in uptake of glucose (Fig. 3). On the contrary, in the presence of 0.5 and 1 mg/ml AFW extract, as the concentration of glucose increased from 10 mM to 25mM in the system, the uptake by yeast cells decreased. Further, when the glucose concentration in the system was increased to 50 mM, AFW extract of 0.5 and 1 mg/ml concentrations triggered an increase in glucose uptake in yeast cells. Interestingly, extract concentration of 2 mg/ml exhibited a dose dependent rise in glucose uptake for different glucose concentrations i.e. 10 mM, 25 mM, 50 mM. It was seen that increase in concentration of AFW extract in a test system with 25 mM glucose, elicited a gradual increase in glucose uptake (Fig. 3). Hence, 25 mM glucose concentration was considered for further studying the effect of standard metformin on glucose uptake. Maximum rise in glucose uptake of 92.307% was observed in the presence of 2 mg/ml metformin. Interestingly, as seen in Table 3, with 25 mM glucose in the test system, 2 mg/ml AFW extract facilitated 91.303% increase in glucose uptake in yeast cells. This indicates that the glucose uptake elicited by 2 mg/ml metformin and 2 mg/ml AFW extract is comparable. This interesting finding suggests a possible role of AFW extract in effective transfer of glucose across yeast cell membrane. Further *in-vitro* studies are warranted to investigate the influence of AFW extract on glucose uptake in cells belonging to higher eukaryotic organisms.

In the current study, phytochemicals were extracted using 50% ethanol in water solvent. However, it should be noted that the proper choice of solvent dictates the nature and concentration of phytochemicals extracted. Earlier study on fresh aster flowers showed that ethyl acetate (EtOAc) extract displayed higher alpha-glucosidase inhibitory activity in comparison to the crude extract acquired using 70% ethanol and other fractions of hexane, n-butanol and dichloromethane [28]. EtOAc extract was found to possess high content of flavonoids, and displayed significant alpha-glucosidase inhibitory activity [28]. Thus, the selection of suitable solvent is essential to enable optimum extraction of phytoconstituents with highest intended bioactivities. Certainly, considering these points, it will be interesting to explore and compare the profile of phytochemicals extracted from AFW using different solvents with varied polarities. In conclusion, the results of the present study have provided an information base of evidence supporting the role of AFW extract in glucose uptake in yeast cells and inhibition of salivary alpha-amylase activity.

#### ABTS radical scavenging activity

Antioxidant status of cells and occurrence of human diseases are known to display an inverse relationship [48]. Due to this, secondary metabolites that are known to be strong antioxidants have crucial applications in phytotherapeutics. As mentioned earlier, oxidative stress plays a major role in the pathogenesis of DM. Our previous work reported promising antioxidant activity of AFW extract using DPPH assay [26]. In continuation, the present study

made an attempt to confirm the same using ABTS assay, which is a more sensitive method for detecting antioxidants in biological systems. ABTS assay allows the measurement of hydrophilic and lipophilic antioxidant systems whereas; DPPH assay facilitates measurement of hydrophobic antioxidant systems [27,49]. In ABTS assay, antioxidant property of a phytochemical is governed by its ability to scavenge ABTS<sup>•+</sup> radical generated by the interaction of ABTS with potassium persulfate. In the current study, ABTS activity was analyzed by plotting a graph of percent ABTS<sup>•+</sup> radical scavenging ability versus concentration of AFW extract.

The results of ABTS assay for AFW extract supported our previous result that was based on DPPH assay [26]. The ABTS radical scavenging ability increased with increase in AFW extract concentration (Table 4). Based on the linear regression equation  $y = 45.739x + 41.07$  ( $R^2 = 0.9665$ ), the  $IC_{50}$  value of AFW extract for scavenging ABTS<sup>•+</sup> radical was found to be 0.195 mg/ml (Fig. 4). Interestingly, as per our previous findings, the concentration of AFW extract needed to inhibit 50% DPPH radical activity i.e.  $IC_{50}$  value was 0.37 mg/ml. This observed difference in  $IC_{50}$  values for the two assays reinforces the findings that suggest the sensitive nature of ABTS assay [27,49]. Considering these results, further research work focusing on identification and separation of different types of phenolic compounds in AFW extract is recommended. This could in turn enable the development of suitable phytotherapeutic agents for treatment of various free radicals associated health ailments.

**Table 1: Percent salivary alpha-amylase inhibition in presence of AFW extract at different concentrations**

Concentration of AFW extract (mg/ml)	Percent salivary alpha-amylase inhibition
0.5	32.619 ± 0.075
1	35.889 ± 0.070
2	59.013 ± 0.020
3	60.151 ± 0.053
4	89.996 ± 0.043
5	83.633 ± 0.043

**Table 2: Percent salivary alpha-amylase inhibition in presence of *T. arjuna* extract at different concentrations**

Concentration of <i>T. arjuna</i> extract (mg/ml)	Percent salivary alpha-amylase inhibition
0.1	18.970 ± 0.028
0.2	27.081 ± 0.040
0.4	38.870 ± 0.027
0.5	39.887 ± 0.013
0.6	40.904 ± 0.004
0.8	46.636 ± 0.056
1.0	50.638 ± 0.017

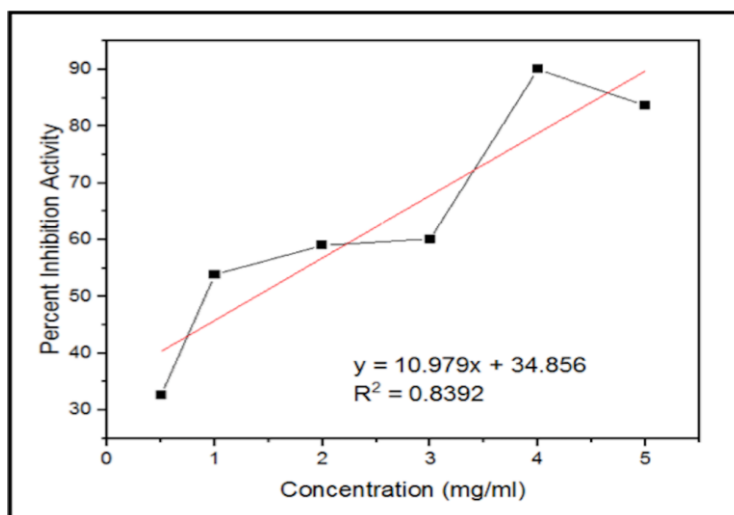
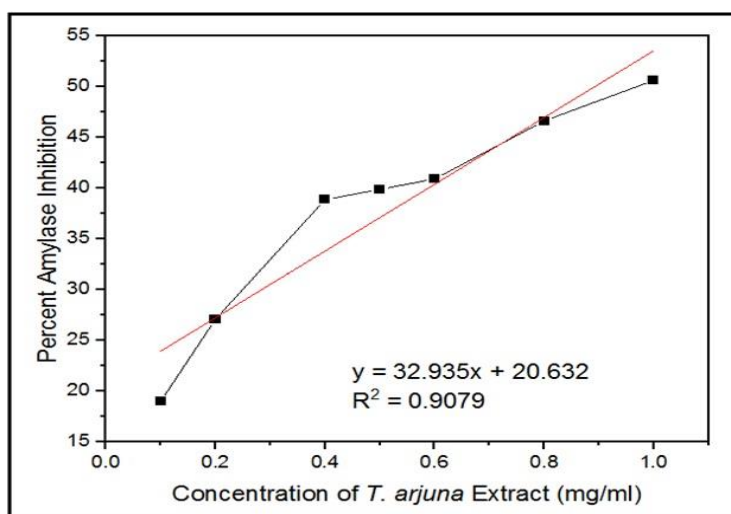


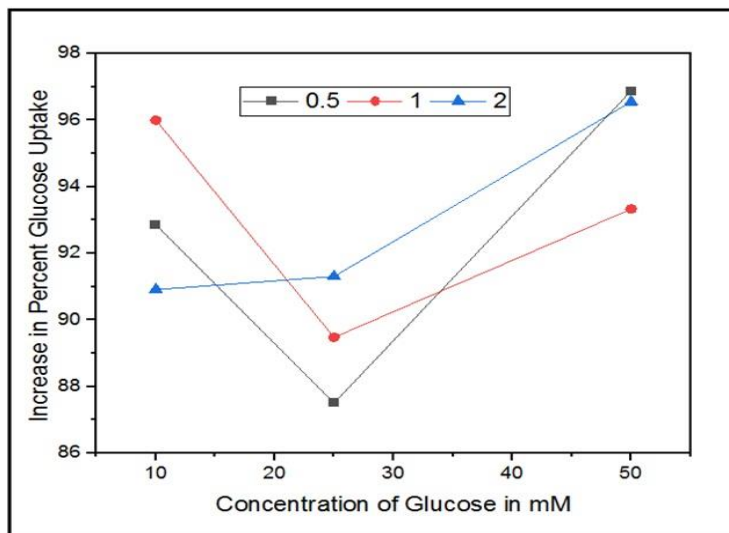
**Table 3: Percentage increase in glucose uptake by yeast cells in the presence of varied AFW extract concentrations and glucose concentrations in the test system**

Concentration of glucose used in the test system (mM)	Concentration of AFW extract (mg/ml)		
	0.5	1	2
10	92.857 %	96.000 %	90.909 %
25	87.500 %	89.473 %	91.303 %
50	96.875 %	93.330 %	96.552 %

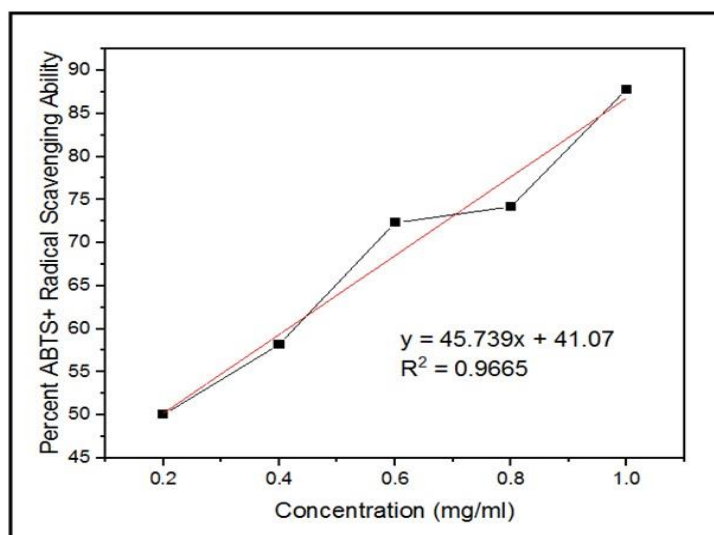
**Table 4: ABTS radical scavenging activity at different concentrations of AFW extract**

Concentration of AFW extract (mg/ml)	ABTS radical Scavenging ability (%)
0.2	50.05 ± 0.004
0.4	58.22 ± 0.003
0.6	72.23 ± 0.006
0.8	74.18 ± 0.004
1.0	87.80 ± 0.001


**Figure 1: Percent alpha-amylase inhibition ability of AFW extract**

**Figure 2: Percent alpha-amylase inhibition Ability of *T. arjuna* extract**



**Figure 3:** Graphical representation of percent increase in glucose uptake by yeast cells in the presence of different concentrations of AFW extract (0.5 mg/ml, 1 mg/ml, 2 mg/l) and glucose (10 mM, 25 mM, 50 mM) in the test system



**Figure 4:** ABTS radical scavenging activity of AFW extract

#### CONCLUSION:

AFW extract displayed promising *in-vitro* anti-diabetic potential and strong ABTS<sup>•+</sup> radical scavenging ability. This highlights that further studies need to be carried out for studying and identifying the candidate phenols and flavonoids in the extract that are responsible for these functions. Our study illustrated medicinal importance of discarded aster flowers for extraction of efficient and inexpensive pharmaceutical inhibitor of alpha-amylase. Current study, to the best of our knowledge, is the first to report the role of hydro-alcoholic extract of aster (*Callistephus chinensis*) flower waste in *in-vitro* enhancement of glucose uptake by yeast cells and salivary alpha-amylase inhibition.

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