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In-vitro Antidiabetic and Antioxidant Potential of *Annona reticulata* L. Leaves

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ABSTRACT

Annona reticulata L. leaf decoction has ethnopharmacological relevance as it is administered orally with cow's milk to treat diabetes in west Godavari district of Andhra Pradesh, India. The prime objective of the study was to evaluate the antidiabetic and antioxidant capacity of *Annona reticulata* L. leaf extract in various solvents. Ethanol, methanol, acetone, DMSO, and aqueous extract were prepared by cold extraction method. Antidiabetic and antioxidant activity was evaluated by invitro glucose uptake activity by yeast cells, alpha-amylase inhibition assay, alpha-glucosidase inhibition assay, DPPH free radical scavenging assay, and CUPRAC antioxidant activity. In the yeast glucose uptake assay, the glucose uptake % ranges from 57.52±0.11(15 mM glucose) to 71.74±0.11 (5 mM glucose). The glucose uptake percent increased with increasing extract concentration (1-5 mg/ml), whereas it decreased with increasing glucose concentration (5 mM, 10 mM, and 15 mM). The ethanol extract demonstrated the highest α-amylase inhibitory activity, as indicated by the IC_{50} value of 41.64±1.43 µg/ml, and methanol extract exhibited the highest α-glucosidase inhibitory activity with an IC_{50} value of 18.26±0.03 µg/ml. In the DPPH antioxidant assay, the acetone extract has displayed the highest level of free radical scavenging activity at 20 µg/ml (64.12%) with an IC50 value of 16.58±0.07 µg/ml. *Annona reticulata* L. leaf extract possesses significant antidiabetic and antioxidant characteristic as evidenced by in vitro antidiabetic assay, DPPH, and CUPRAC antioxidant assay. The results of the study support the ethnopharmacological use of the plant for the treatment of diabetes.

Keywords: Glucose uptake assay; Yeast cells; DPPH assay; Free radical scavenger; CUPRAC; α-amylase, α-glucosidase

NOMENCLATURE

GC-MS	Gas Chromatography-Mass Spectroscopy
ANR	*Annona reticulata* L.
DPPH	2,2-diphenyl-1-picrylhydrazyl
CUPRAC	Cupric ion Reducing Antioxidant Capacity
CNS	Central Nervous System
DMSO	Dimethyl Sulfoxide
Abs	Absorbance
DNSA	3,5-Dinitrosalicyclic acid
pNPG	p-Nitrophenyl-β-D-glucopyranoside
ANOVA	Analysis of Variance
RT	Room temperature
ROS	Reactive Oxygen Species

1. INTRODUCTION

Diabetes mellitus is a disorder related to endocrine metabolism depicted by inflated levels of glucose in the blood stream brought about by inadequate production of insulin hormone from β-cells of the pancreas[1]. And this condition of prevailing upraised blood glucose level is clinically termed hyperglycemia. As mentioned by WHO, diabetes mellitus is a leading cause of death ranking at 5th position among all diseases around the world[2]. Diabetes is not just limited to glucose intolerance but it also leads to many more complications resulting in higher risk and genesis of disorder such as heart attacks, kidney failure, blindness, stroke, and lower limb impairment. The number of persons affected with diabetes is increasing rapidly in low and middle-income countries. Regular check-ups and medication are required for the treatment of diabetes, and for that various synthetic drugs have been discovered and are being used. But these synthetic drugs have some sort-of adverse effects that make them less reliable for patients; therefore, the search for new and reliable medication with no side effects is needed now. Novel drugs developed from plants are the answer to the present problem of side effects of synthetic drugs. In the present study, *A. reticulata* L. was selected for in vitro antidiabetic assay, DPPH, and CUPRAC antioxidant assay.

A. reticulata L. is a perennial tree belonging to the family Annonaceae and is autochthonic to West Indies and South and Central America[3]. It is frequently known as Sweetsop, Bullock's Heart, and Ramphal. It possesses antidiabetic activity, antioxidant activity, anti-cancer activity, anti-inflammatory, anthelmintic, analgesic, anthelmintic, insecticidal, and CNS depressant properties[4,5]. Every plant part is medicinally important; that includes the leaf, fruit, fruit peel, seeds, stem, stem bark, and roots. It is used ethnomedicinally for the regimen of fever, indigestion, diarrhoea, dysentery, toothache, malaria, epilepsy, inflammation, and in destroying lice[6]. In ethnomedicinal survey conducted in the west Godavari

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District of Andhra Pradesh, India, it has been stated that oral administration of *A. reticulata* L. leaf decoction with cow's milk used as remedy for diabetes treatment[7]. Its dried leaves are also used to make herbal tea[8].

Annonaceous acetogenins, a type of secondary metabolite in Annonaceae family, are characterized by long-chain fatty acids (C32 and C34) with a terminal γ-lactone structure[9]. These molecules may also include functional groups such as epoxide, hydroxyl, ketone, tetrahydrofuran, and tetrahydropyran. *Annona reticulata* L. contain acetogenins like cis or trans Isomurisolin, which are mono tetrahydrofuran compounds. Retaculatacin, a bioactive acetogenin isolated from this plant, it possesses a range of pharmacological effects, including deterring pests, combating microbes, targeting parasites, inhibiting tumour growth, suppressing the immune system, and acting as pesticides[10]. The diversity in their chemical structure and pharmacological effects has sparked global interest in using them as focal points in synthetic organic and medicinal chemistry. These compounds act as inhibitors of complex I in the respiratory chain of tumour cells, leading to cell death, apoptosis, and autophagy[11,12]. In GC-MS studies on *A. reticulata* L. leaf extract two antidiabetic phytochemicals gamma-Sitosterol and Lup-20(29)-en-3-one; and several antioxidant compounds Hexadecanoic acid, methyl ester; Phytol; Squalene; alpha-Tocospiro B; gamma-tocopherol; Stigmasta-5,22-dien-3-ol, acetate (3beta); Vitamin E and Ergost-5-en-3-ol (3, beta,24R) were reported[13]. The presence of antioxidant and antidiabetic phytochemicals in the leaves of *A. reticulata* L. was the reason for testing its antidiabetic and antioxidant characteristics observed through in vitro experiments.

2. MATERIAL AND METHODS

2.1 Collection of Plant Material and Preparation of Extracts

Fresh leaves of *A. reticulata* L. were gathered in December from Ranchi, Jharkhand, India (23°14'55.1"N and 85°17'45.7"E). The plant has been identified and authenticated by Dr Sunita Garg, CSIR-NIScPR, New Delhi, India, and the herbarium was deposited (Authentication no. NIScPR/RHMD/Consult/2022/4259-60). The extract was prepared by cold extraction method using methanol as the primary solvent and further suspension of dried extract in five different solvents (ethanol, methanol, acetone, DMSO, and distilled water).

2.2 Assessment of Glucose Uptake Capacity in Yeast Cells

In-vitro antidiabetic assay was carried out by the procedure proposed by Cirillo[13,14]. 1% w/v yeast suspension was prepared in distilled water and kept at RT overnight. The suspension was centrifuged repeatedly at 4200 rpm for 5 minutes there-after suspension (10% v/v) was prepared using the supernatant. 1ml glucose solution (5, 10, and 15 mM) was incubated with extract concentrations at 1, 2, 3, 4, and 5 mg/ml at 37 °C for 10 minutes. After incubation, 100 µL of yeast suspension (10%) was added, vortexed for 5 minutes, and then incubated for 60 minutes at 37 °C. After incubation, the reaction mixture centrifuged at 3800 rpm for 5 minutes. Using a spectrophotometer, absorbance at 520 nm was measured, and the formula below was used to compute the percentage elevation in glucose uptake by yeast cells:

$$\text{Activity \%} = \frac{\text{Abs control} - \text{Abs sample}}{Abs\ control} \times 100$$

2.3 *In Vitro* Alpha-Amylase Inhibitory Assay

This assay was carried out by the procedure given by Kazeem,[15-17] *et al*. 200 µL of the extract was taken in a tube along with 200 µL of enzyme solution (0.5 mg/ml) and incubated for 10 minutes at 25 °C. The termination of reaction was done by the addition of 200 µL DNSA reagent. The samples were subsequently incubated in boiling water for 5 minutes and, after cooling to room temperature, were diluted with 5 mL of distilled water. Acarbose was utilized as a positive control, and the absorbance was assessed at 540 nm using a spectrophotometer.

$$\text{Inhibition \%} = \frac{Control\ Absorbance - Plant\ Extract\ Absorbance}{Control\ Absorbance} \times 100$$

Control: 200 µL enzyme solution, 200 µL1% starch solution, 200 µL DNSA reagent, and 200µL distilled water.

Blank: 200 µL each of enzyme solution and DNSA reagent was used.

2.4 *In Vitro* Alpha-Glucosidase Inhibitory Assay

This assay was performed by the procedure given by Kazeem, *et al*.[17], 100 µL of α-Glucosidase solution (0.01 mg/ml) was added to 50 µL of extract and incubated at 37 °C for 10 minutes. 50 µL of 3 mM pNPG was added to initiate the reaction and incubation at 37°C for 20 minutes. The reaction was aborted by adding 2 mL of 0.1M Na_2CO_3 and absorbance was taken at 405 nm using a spectrophotometer and acarbose was employed as a positive control.

$$\text{Inhibition \%} = \frac{Control\ Absorbance - Plant\ Extract\ Absorbance}{Control\ Absorbance} \times 100$$

Control: 100 µL enzyme solution, 50 µL mM pNPG, and 2 ml 0.1M Na2CO3.

Blank: 1 ml enzyme solution.

2.5 DPPH Antioxidant Assay

DPPH antioxidant assay was detected by using the protocol given by Burits & Bucar, 2000[18]. Freshly prepared 0.5 mM DPPH in methanol was used for testing the free radical scavenging potential of *A. reticulata* L. extract. 1 ml of DPPH solution was mixed with extract (5, 10, 15, and 20 µg/ml) and 30 minutes incubation in dark. A spectrophotometer was used to measure the absorbance at 517 nm, and the DPPH free radical scavenging activity was determined using the formula:

$$\text{Inhibition\%} = \frac{Control\ Absorbance - Plant\ Extract\ Absorbance}{Control\ Absorbance} \times 100$$

2.6 CUPRAC Antioxidant Assay

This assay was performed by the procedure given by Callaghan[19], *et al.* 2013. 150 µL of the extract was mixed with 150µL of each 1mM ammonium acetate, 7.5 mM neocuproine, and 10 mM copper II chloride dihydrate. Following a 30 minute incubation at 37 °C, the absorbance at 450 nm was measured using a spectrophotometer. The ascorbic acid standard curve was used to calculate the extract's antioxidant capability.

3. RESULTS

3.1 Effect of *Annona reticulata* L. Extract on Glucose Uptake Capacity by Yeast Cells

The glucose uptake across the plasma membrane of yeast cells has increased in response to leaf extract of ANR (Figures 1, 2, and 3). The glucose uptake capacity at glucose concentration 5 mM ranged from 62 % to 64.96 % at extract concentration 1 mg/ml which has reached 66.44 % to 71.74 % at the extract concentration 5 mg/ml. This indicates that the increase in the concentration of extract has increased the capacity of glucose uptake by yeast cells from the environment. Figure and Table 1, 2, and 3 shows that the incremental rise in extract concentration results in a proportional increase in the uptake of glucose by yeast cells. However, the increase in glucose concentration from 5 mM to 15 mM has shown a decrease in glucose uptake showing the inverse relationship between the glucose concentration and glucose uptake capacity by yeast cells. The results for glucose uptake capacity by yeast cells are significantly different ($P=7.22\times10^{22}$ for 5 mM, $P=2.5\times10^{13}$ for 10 mM, and $P=8.3\times10^{13}$ for 15 mM, one-way ANOVA).

Table 1. Ability of yeast cells to take up glucose at a glucose concentration of 5 mM

Concentration (mg/ml)	Glucose Uptake (%)					
	Ethanol Extract	Methanol Extract	Acetone Extract	DMSO Extract	Aqueous Extract	Metformin
1	64.96±0.15	62±0.11	63.22±0.07	62.15±0.09	61.59±0.13	99.15±0.02
2	67.26±0.13	62.74±0.14	65.81±0.13	63.89±0.13	62.52±0.06	99.19±0.18
3	68.19±0.15	64±0.16	66.67±0.16	65.04±0.11	64.04±0.11	99.74±0.09
4	70.04±0.15	65.04±0.09	68.19±0.13	66.52±0.14	64.7±0.04	99.8±0.03
5	71.74±0.11	66.93±0.15	68.52±0.18	67.63±0.15	66.44±0.04	99.81±0.04

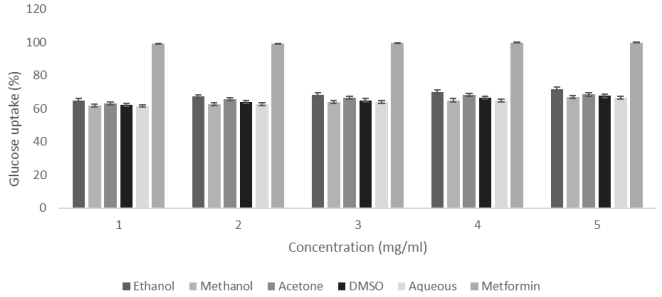

Figure 1. Glucose uptake capacity of yeast cells in the presence of *A. reticulata* L. leaf extract at a glucose concentration of 5 mM.

Table 2. Ability of yeast cells to take up glucose at a glucose concentration of 10 mM

Concentration (mg/ml)	Glucose Uptake (%)					
	Ethanol Extract	Methanol Extract	Acetone Extract	DMSO Extract	Aqueous Extract	Metformin
1	64.22±0.11	58.96±0.09	62.11±0.11	60.56±0.13	60.07±0.06	83.18±0.92
2	65.41±0.15	60.78±0.1	63.7±0.06	62.74±0.14	60.74±0.21	87.99±0.69
3	65.89±0.15	61.41±0.14	64.85±0.08	63.96±0.15	62.04±0.13	95.5±0.3
4	68.63±0.15	62.81±0.14	66.07±0.12	64.52±0.08	63±0.13	97±0.46
5	69.41±0.14	64.3±0.13	67.44±0.19	65.89±0.17	63.81±0.12	98.5±0.35

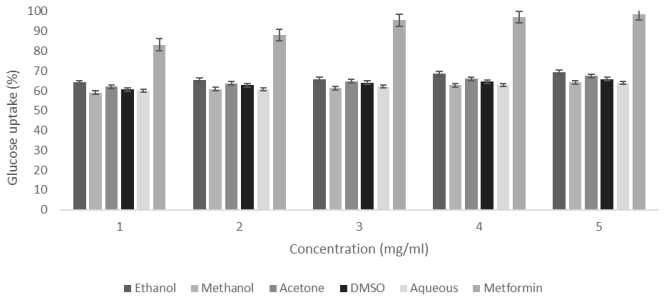

Figure 2. Glucose uptake capacity of yeast cells in the presence of A. reticulata L. leaf extract at a glucose concentration of 10 mM.

Table 3. Ability of yeast cells to take up glucose at a glucose concentration of 15 mM

Concentration (mg/ml)	Glucose Uptake (%)					
	Ethanol Extract	Methanol Extract	Acetone Extract	DMSO Extract	Aqueous Extract	Metformin
1	61.44±0.04	57.52±0.11	60.44±0.13	58.96±0.09	58.59±0.19	76.99±0.51
2	62.78±0.15	58.15±0.02	61.85±0.12	61.04±0.14	59.85±0.08	78.17±0.17
3	63.56±0.1	59.52±0.15	63±0.21	62±0.1	60.85±0.18	79.94±0.17
4	66.44±0.15	60.56±0.13	63.93±0.11	62.81±0.09	62.04±0.18	80.63±0.25
5	67.44±0.06	62.59±0.17	66±0.3	63.96±0.13	62.85±0.15	83.78±0.34

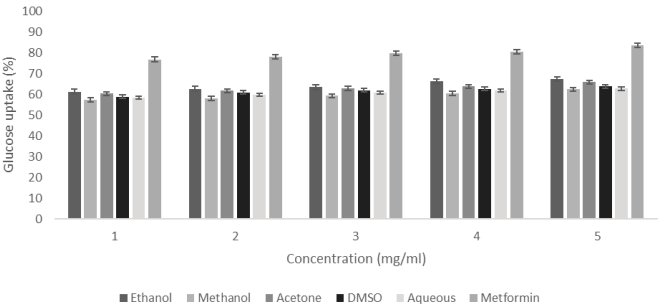

Figure 3. Glucose uptake capacity of yeast cells in the presence of *A. reticulata* L. leaf extract at a glucose concentration of 15 mM.

3.2 In Vitro Alpha-Amylase Inhibitory Assay

Among all the extracts, ethanol extract exhibited maximum α-amylase inhibition with 83.51±0.1 % (IC_{50} 41.64±1.43 µg/ml) whereas minimum inhibition is shown by aqueous extract with 62.19±1.74 % (IC_{50} 74.74±1.26 µg/ml) (Table 4). The inhibition percent has gradually increased

with increasing extract concentration (Fig. 4). The IC_{50} values of all the extracts were found to be significantly different (P = 1.64 X 10^{-5}, one-way ANOVA).

Table 4. Percentage of α-amylase inhibitory effects and IC_{50} values of *A. reticulata* L. leaf extract

Alpha Amylase Inhibitory Assay						
Concentration (µg/ml)	20	40	60	80	100	IC_{50} (µg/ml)
Ethanol	30.47±2.54	50.36±0.1	69.53±0.1	74.91±0.37	83.51±0.1	41.64±1.43
Methanol	25.09±2	42.11±2.7	53.05±1.74	71.51±1.79	77.6±0.21	53.93±1.35
Acetone	29.21±3.5	48.75±0.27	67.2±0.18	77.6±1.8	81±0.21	43.11±1.61
DMSO	23.3±1.8	43.91±0.72	55.2±0.1	70.07±0.83	75.09±1.05	54.5±1.36
Aqueous	18.64±1.8	31.18±2.64	41.04±3.41	55.91±2.76	62.19±1.74	74.74±1.26
Acarbose	51.37±0.41	69.85±0.05	73.48±0.09	84.76±0.15	96.49±0.52	12.01±0.8

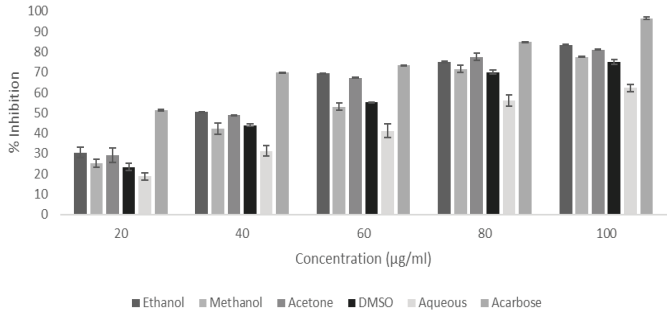

Figure 4. α-amylase inhibitory activity of *A. reticulata* L. leaf extract.

3.3 In Vitro Alpha-Glucosidase Inhibitory Assay

The methanol extract exhibited maximum α-glucosidase inhibition with 81.95±0.08 % (IC_{50} 18.26±0.03 µg/ml) followed by DMSO extract 73.66±0.68 % (IC_{50} 23.56±0.07 µg/ml) and minimum by aqueous extract 69.85±0.51 % (IC_{50} 30.12±0.1 µg/ml) (Table 5). The IC_{50} values of extracts in different solvents were reported to be significantly different (P = 14.04 X 10^{-12}, one-way ANOVA).

Table 5. Percentage of α-glucosidase inhibitory effects and IC_{50} values of *A. reticulata* L. leaf extract

Alpha Glucosidase Inhibitory Assay						
Concentration (µg/ml)	10	20	30	40	50	IC50 (µg/ml)
Ethanol	23.26±0.1	41.76±0.03	53.63±0.05	65.26±0.03	70.81±0.03	29.2±0.02
Methanol	34.8±0.06	57.84±0.03	66.35±0.05	73.69±0.14	81.95±0.08	18.26±0.03
Acetone	24.42±0.28	40.98±0.06	59.27±0.03	65.55±0.53	72.75±0.65	27.88±0.21
DMSO	25.85±0.09	57.78±0.03	61.05±0.05	64.68±0.26	73.66±0.68	23.56±0.07
Aqueous	20.68±0.2	46.97±0.07	54.26±0.03	57.62±0.06	69.85±0.51	30.12±0.1
Acarbose	47.97±0.12	61.99±0.33	72.62±0.18	82.15±0.28	91.83±0.11	10.24±0.14

3.4 Antioxidant Capacity of *Annona Reticulata* L. Leaf Extract

To test the antioxidant capacity of ANR leaf extract, a DPPH assay was performed. At the inceptive concentration of 5 µg/ml of the extract, the free radical scavenging activity percent ranged from 11.09 % to 23.65 % but reached up to 64.12 % at an extract concentration of 20 µg/ml (Table 6). The highest free radical scavenging activity was shown by acetone extract at concentration 20 µg/ml (64.12 %) with an IC_{50} value of 16.58±0.07 µg/ml, and aqueous extract had shown minimum free radical scavenging activity at 20 µg/ml (49.49 %) with IC_{50} value 22.41±0.04 µg/ml.

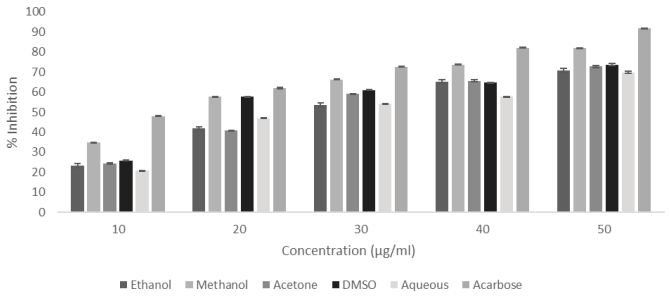

Figure 5. Percentage of α-glucosidase inhibitory effects of *A. reticulata* L. leaf extract.

A progressive increase in extract concentration has resulted in a step-by-step increase in free radical scavenging activity % as shown in Figure 6. The study revealed that the ANR leaf extract has a significant free radical scavenging ability. The results for the DPPH antioxidant assay were significantly different (P = 0.0004, one-way ANOVA).

The extract's antioxidant capability was additionally evaluated through a CUPRAC assay. Total antioxidant activity was calculated using the ascorbic acid standard graph (y = 0.0147x + 0.3275, R^2 = 0.9644) (Fig. 7) of absorbance 450 nm plotted against its concentration. Maximum antioxidant activity potential was reported for ethanol extract (41.39±1.04) at extract concentration 100 µg/ml (Table 7).

Table 6. DPPH Antioxidant assay of *A. reticulata* L. leaf extract

	Free radical (DPPH) scavenging activity %					
Concentration (µg/ml)	Ethanol Extract	Methanol Extract	Acetone Extract	DMSO Extract	Aqueous Extract	Ascorbic Acid
5	11.09±0.74	16.65±0.18	23.65±0.34	14.93±0.1	13.68±0.2	49.87±0.08
10	25.97±0.54	24.94±0.25	26.14±0.21	25.12±0.15	23.82±0.1	75.75±0.14
15	40.2±0.23	39.93±0.07	41.5±0.2	43.62±0.27	26.09±0.11	89.48±0.06
20	59.68±0.63	55.63±0.15	64.12±0.2	61.57±0.34	49.49±0.1	92.66±0.05
IC50 (µg/ml)	17.43±0.1	18.47±0.03	16.58±0.07	16.82±0.08	22.41±0.04	3.02±0.02

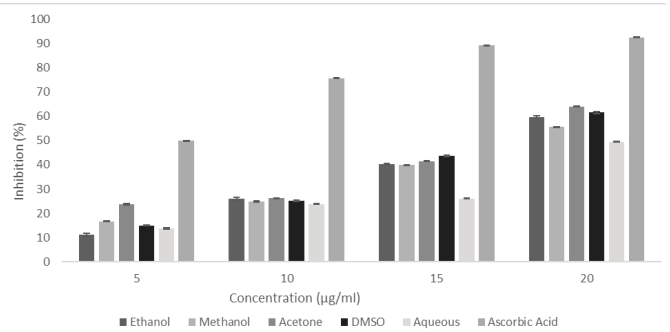

Figure 6. DPPH Antioxidant assay for *A. reticulata* L. leaf extract.

Table 7. *A. reticulata* L. leaf extract CUPRAC antioxidant assay

Ascorbic acid equivalence antioxidant capacity				
Concentration (µg/ml)	25	50	75	100
Ethanol	3.55±0.41	15.63±0.63	29.26±0.9	41.39±1.04
Methanol	4.3±0.1	9.13±0.84	19.47±1.42	34.3±0.79
Acetone	3.21±1.12	9.24±0.68	28.99±0.86	33.8±0.6
DMSO	2.87±0.71	11.12±0.06	24.93±0.1	27.79±0.14
Aqueous	3.55±0.57	6.56±0.08	17.45±0.1	25.52±1.17

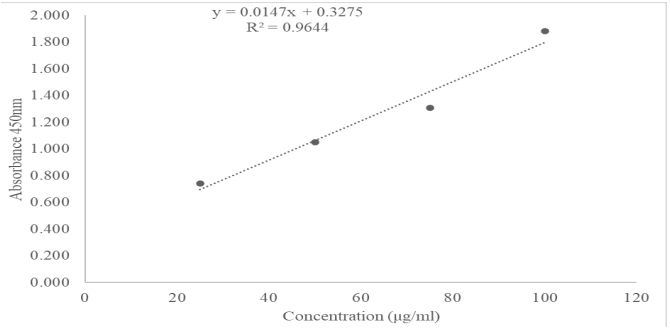

Figure 7. Standard curve of ascorbic acid for CUPRAC antioxidant assay.

4. DISCUSSION

Several ethnomedicinally important plants in existence have been used for the regimen of diabetes mellitus in ayurveda and traditional medicine. *Annona reticulata* L. is also used in the regimen of diabetes, its hyperglycaemic activity has been tested on a mice model in which it significantly decreased serum glucose levels up to 56.1%20.

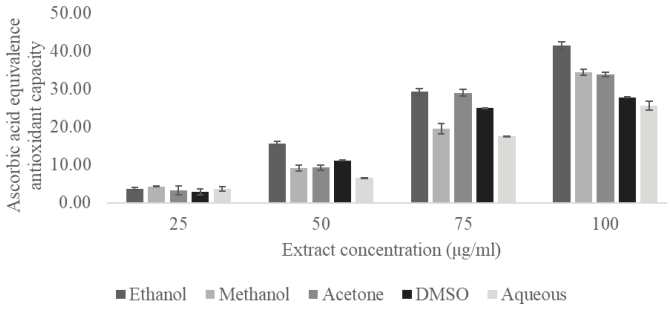

Figure 8. Ascorbic acid equivalence antioxidant capacity of *A. reticulata* L. leaf extract.

The current study was performed to find the possible mechanism that might have contributed to lowering blood serum glucose levels. Three possible mechanisms for lowering blood glucose can be as follows; enhanced release of insulin, elevation in glucose uptake, or inhibition of polysaccharide metabolizing enzymes[21,23]. The findings of the current study indicate that all the extracts have enhanced glucose uptake athwart the plasma membrane. Pulvarthi[24], *et al.* (2020) evaluated the in vitro antidiabetic activity of *Annona reticulata* L. by glucose uptake assay using a yeast model that showed % uptake of glucose by methanol extract 48.55 % at 500 µg/ml. The results of present and prior study support the antidiabetic nature of the *Annona reticulata* L. leaf extract.[22] The extract inhibited the enzymes involved in carbohydrate metabolism, namely α-amylase and α-glucosidase. α-amylase is an enzyme that breaks down oligosaccharides into disaccharides and α-glucosidase converts disaccharides to monosaccharides. Thus, the inhibition of these two enzymes resulted in the delayed rise of postprandial glucose concentration. Since this plant extract has inhibited the enzymes α-amylase and α-glucosidase effectively. So, it can be considered an effective antidiabetic plant.

Modern lifestyle has increased the intake of diet that elevated free radicals and ROS concentration in the body. These free radicals generate oxidative stress and subsequently result in cell damage and diabetic complications. Therefore, the intake of antioxidants is always recommended for a diabetic person to avoid complications related to diabetes[20]. The DPPH and CUPRAC antioxidant assay revealed that all the fractions of extract possess significant antioxidant activity. The results of antioxidant study were also supported by previous study done by Parthiban,[23] *et al.* (2019). The possible reason for the good antioxidant capacity of the extract is the existence of several bioactive phytocompounds that are antioxidant and were identified in GC-MS analysis like Hexadecanoic acid; methyl ester; Phytol; Squalene; alpha-Tocospiro B; gamma-tocopherol; Stigmasta-5,22-dien-3-ol; acetate (3beta); Vitamin E and Ergost-5-en-3-ol (3, beta, 24R)[8].

5. CONCLUSION

A. reticulata L. leaf extract has promoted the glucose uptake athwart the plasma membrane of yeast cells and significantly inhibited enzyme α-amylase and α-glucosidase showing its antidiabetic property. Ethanol extract has shown maximum glucose uptake capacity with an activity percent of 71.74±0.11 %. Maximum α-amylase and α-glucosidase inhibition percent were shown by ethanol extract (83.51±0.1 %, IC_{50} = 41.64±1.43) and methanol extract (81.95±0.08 %, IC_{50} = 18.26±0.03) respectively. All the fraction of extract possesses remarkable antioxidant activity with acetone extract showing the best DPPH scavenging property (64.12±0.2%, IC_{50} = 16.58±0.07). Maximum activity in the CUPRAC assay was shown by ethanol extract. This study revealed that *Annona reticulata* L. leaf extract possesses both antidiabetic and antioxidant properties and thus can be used as a therapeutic drug for the regime of Type II diabetes mellitus.

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