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Research Article

Antioxidant Activities of The Root Extract of *Gliricidia Sepium*: Correlation with Phenolic and Flavonoid Content

Punyashree H S*, T. Tamizh Mani

Department of Pharmacognosy, Bharathi College of Pharmacy, Bharathinagara, Mandya, Karnataka, India
571422

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ABSTRACT

The leguminous plant *G. sepium*, rich in flavonoids, phenols, and alkaloids, might have compounds that are beneficial for health. The goal of the current study is to examine the plant's antioxidant properties. The antioxidant activity of aqueous extract of *G. sepium* was determined by following assay namely DPPH free radical scavenging assay, nitric oxide scavenging assay, phosphomolybdate assay and ferric reducing antioxidant power assay. In DPPH assay, the IC₅₀ value was found to be 59.27 µg. In nitric oxide radical scavenging activity, the IC₅₀ value was found to be 65.50 µg. In phosphomolybdate assay, the plant extract shown anti-oxidant activity which is equivalent to ascorbic acid at the concentration of 37.50 µg. In ferric reducing antioxidant power assay, the plant extract shown anti-oxidant activity which is equivalent to ascorbic acid at the concentration of 20.22 µg. The plant contained considerable amount of saponin, phenol, alkaloids and flavonoids. The total phenolic content and total flavonoid content of the plant extract (100 µg) which is equivalent to gallic acid and rutin at the concentration of 37.47 µg and 41.73 µg.

INTRODUCTION

Despite the fact that around 80% of people on the planet use a wide range of medicinal plants, most of the time no scientific research has been done to assess their effectiveness. Oxidative stress is highly sensitive to the human immune system. In the event of specific illnesses and as people age, it

is necessary to boost antioxidant capacity in order to strengthen the immune system¹. Reactive oxygen species (ROS) are produced as a result of the oxygen consumption that occurs naturally during cell growth². They are constantly created by the body's regular oxygen consumption processes, including breathing and certain cell-mediated

*Corresponding Author: Punyashree H S

Address: Department of Pharmacognosy, Bharathi College of Pharmacy, Bharathinagara, Mandya, Karnataka, India 571422

Email ✉: punyaruchitha@gmail.com

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immunological responses. ROS comprise non-free radical species like hydrogen peroxide (H_2O_2) and singlet oxygen (1O_2) as well as free radicals such superoxide anion radicals ($O_2^{\bullet-}$) and hydroxyl radicals ($OH^{\bullet}$)³. Normal physiological processes constantly generate ROS, which can readily start the peroxidation of membrane lipids and cause lipid peroxides to build up. Additionally, ROS can harm important biomolecules like nucleic acids, carbohydrates, proteins, and fats, and may harm DNA, which may result in mutations. Diseases develop when ROS are not efficiently scavenged by cellular components. Over 100 diseases have been linked to ROS⁴. Despite the fact that around 80% of people on the planet use a wide range of medicinal plants, most of the time no scientific research has been done to assess their effectiveness. The immune system of humans is extremely sensitive to oxidative stress in the event of a specific illness and in old age. Additionally, as people age, their antioxidant capacities must be strengthened in order to strengthen their immune system⁵. The human body constantly produces reactive free radicals, which can seriously harm cells and tissues during infections, as well as in a number of degenerative conditions like heart disease, aging, and neurodegenerative conditions like Alzheimer's disease, mutation and malignancies^{6,7,8}. Dietary antioxidants found in a variety of fruits and vegetables can stop oxidative damage, which can cause cancer. These vegetables and fruits have the power to scavenge oxidative agents, stimulate immunological system, hormone metabolism, control of gene expression in cell division and apoptosis, and antiviral and antibacterial properties⁹. Studies on aqueous extracts of *G. sepium*, a plant abundant in secondary metabolites, are the main focus of the current goal. The plant's antioxidant potential was demonstrated by these tests, and more research might be done to maximize its therapeutic potential.

MATERIALS AND METHOD:

Collection of Plant Material:

In Dec 2024, the plant material was gathered from Malavalli in the Mandya region of Karnataka, India. The Dr. V. Rama Rao, Research Officer (Botany), Central Ayurveda Research Institute, Bengaluru, identified and verified the plant. For future use, a herbarium voucher specimen was kept in the Pharmacognosy department of the Bharathi College of Pharmacy in Bharathinagar.

Extraction Procedure¹⁰:

Each thimble of the Soxhlet extractor was charged with 150 gm of powdered medication and extracted using petroleum. Use ether, chloroform, ethyl acetate, and methanol in ascending sequence of polarity. Marc was squeezed to eliminate leftover solvent before extracting with the next solvent. The extract was then concentrated. The proportion was computed based on the air-dried weight of plant material. The Soxhlet equipment was used to extract powdered herbal material using increasing polarity solutions. The % yield was calculated.

Antioxidant Assays¹¹:

Antioxidant activity was assessed using various invitro techniques. The free radical scavenging activity of methanolic root extract at different doses. The plant and ascorbic acid were analyzed for total phenolic and flavonoid content, DPPH scavenging, phosphomolybdate, ferric reducing power, hydrogen peroxide, and nitric oxide scavenging.

Determination of Total Phenolic Contents:¹²

The extract's total phenolic content was determined using the Folin-Ciocalteu technique, with some modifications. To prepare, dilute 0.1



mL of crude extract (1 mg/mL) with distilled water to 3 mL. Mix carefully with 0.5 mL of Folin-Ciocalteu reagent for 3 minutes, then add 2 mL of 20% (w/v) sodium carbonate. After 60 minutes in the dark, the mixture's absorbance at 650 nm was measured. Rutin was used to create the calibration curve. The absorbance of the mixes was measured at 650nm using a spectrophotometer.

Determination of Total Flavonoid Contents¹³:

The total flavonoid content of *G. sepium* was determined using the aluminum chloride calorimetric method, which was modified from the methods reported by Fifty et al. in 2012. Mix 0.1ml of plant extract (1mg/ml) with 1ml of 10% aluminum chloride, 1ml of potassium acetate (1M), and 2.5ml of purified water. Rutin was used to create the calibration curve. The absorbance of the mixes was measured at 415nm using a spectrophotometer.

DPPH free Radical Scavenging Activity^{14,15}:

Principle: 1, 1-diphenyl-2-picrylhydrazyl (α , α -diphenyl- β -picryl hydrazyl; DPPH) is a stable free radical due to the delocalization of the spare electron over the entire molecule, preventing dimerization, unlike other free radicals. Delocalization produces a rich violet color with an absorption band around 520 nm in ethanol/methanol solution. When DPPH is coupled with a chemical that may donate a hydrogen atom, the reduced form is formed, resulting in the loss of the violet color. However, the picryl group still contributes a pale-yellow color. Z• represents the DPPH radical, while AH represents the donor molecule. The fundamental reaction is $Z\bullet + AH = ZH + A\bullet$.

Procedure:

DPPH (2,2-diphenylpicrylhydrazyl) free radical scavenging activity was measured using a conventional procedure with minor modifications. Diluting Standard and Plant Extract with DMSO resulted in various concentrations (15, 30, 45, 60, and 75 μ g/ml). In each test tube, 0.1ml of plant extract and standard ascorbic acid were combined with 2.9ml of a methanolic DPPH solution. The control was made by combining 0.1 ml of DMSO and 2.9 ml of DPPH. To prevent light exposure, the test tubes were covered with aluminum foil and stored in a dark room for 30 minutes. Absorbance was measured at 517nm with a UV-visible spectrophotometer.

The percentage of inhibition was estimated using the formula below and compared to typical ascorbic acid values.

$$\% \text{ DPPH radical scavenging activity} = [(A_0 - A_1) / A_0] \times 100$$

Where A0 is the absorbance of control and A1 is the absorbance of the extract/standard.

Nitric Oxide Scavenging Activity^{16,17}:

Principle: When oxygen is present, nitric oxide is a highly unstable species. It produces the stable products nitrate and nitrite when it interacts with O₂. Through N₂O₄, N₃O₄, and NO₂ intermediates. The Griess reagent is used to estimate it. The amount of nitrous acid drops when the test substance, a scavenger, is present. The degree of scavenging is reflected in the level of decline.

Procedure: Using sodium nitroprusside and the Griess-Ilosvay reaction, the nitric oxide radical scavenging capacity of the plant extract and standard ascorbic acid was assessed. By diluting with DMSO, various concentrations of standard and plant extract, such as 15, 30, 45, 60, and 75 μ g/ml, were created. 2ml of sodium nitroprusside (10 mM), 0.5 ml of phosphate buffer (pH-7.4), and



0.5 ml of various concentrations of plant extract and standard ascorbic acid were combined, and the mixture was incubated for 150 minutes at 25 °C. Following the incubation period, 1 mL of sulfanilic acid reagent (0.33% sulfanilic acid in 2% glacial acetic acid) was pipetted into 0.5 mL of nitrite, which was then left for 5 minutes. After that, 1 milliliter of 1% naphthyl ethylene diamine dihydrochloride (NEDD) was added, and the mixture was let to stand at 25 degrees Celsius for 30 minutes. At 540 nm, the solution's pink absorbance was measured. The percentage of nitric oxide inhibition was calculated using the following equation:

Percentage (%) of nitric oxide radical scavenging assay = $[(A_0 - A_1)/A_0] \times 100$.

Where A₀ was the absorbance of control, and A₁ was the absorbance of the treated sample.

Hydrogen Peroxide Scavenging Assay^{11,18}:

Principle: At 230 nm, it is simple to detect hydrogen peroxide's absorption of UV radiation. The absorbance drops at this wavelength as the plant extracts scavenge hydrogen peroxide.

Procedure: The Ruch et al. method was used to measure the plant extract's radical scavenging ability against hydrogen peroxide, with some alteration. By diluting with DMSO, various concentrations of the standard and plant extract—such as 15, 30, 45, 60, and 75 µg/ml—were created. Plant extract and standard ascorbic acid samples with varying concentrations were added to 3.4 ml of phosphate buffer solution (pH 7.4) and combined with 0.6 ml of 43 mM hydrogen peroxide solution. The absorbance of the reaction mixture was measured at 230 nm after 10 minutes. The blank was the reaction mixture devoid of sample. As a reference substance, ascorbic acid

was employed. The inhibitory activity as a percentage was computed.

Phosphomolybdate Assay¹⁹:

Principle: The basis of this assay is the reduction of the phosphomolybdate ion in the presence of an antioxidant, which produces a green phosphate/MoV complex that can be detected by spectrophotometry.

Procedure: By diluting with DMSO, various concentrations of the standard and plant extract such as 15, 30, 45, 60, and 75 µg/ml were created. 1ml of phosphomolybdate reagent solution (0.6M sulfuric acid, 28mM sodium phosphate, and 4mM ammonium molybdate) was mixed with 0.1ml of various concentrations of plant extract and standard ascorbic acid. After being covered, the test tubes were incubated for 90 minutes at 95 °C in a water bath. The mixture's absorbance was measured at 765 nm once the samples had cooled. The standard was ascorbic acid. The following formula was used to assess the antioxidant capacity.

Total antioxidant capacity (%) = $[(\text{Abs. of control} - \text{Abs. of sample}) / (\text{Abs. of control})] \times 100$

Ferric Reducing Antioxidant Power Assay^{11,14,20}:

Principle: The fundamental idea behind the assay is that, in the presence of an antioxidant at an acidic pH, the ferric complex of Fe (TPTZ) ³⁺, or tripyridyl triazine, an analog of ferroin, is reduced to the iron-blue Fe (TPTZ) ²⁺ complex. At 593 nm, the absorbance value will increase.

Procedure: Plant extracts' reducing power was ascertained using a modified version of Oyaizu's (1986) methodology. By measuring the absorbance at 700 nm following incubation, the



extract's ability to convert the ferric-ferricyanide complex to the ferrous-ferricyanide complex of Prussian blue was ascertained. The standard and plant extract were made at various concentrations (15, 30, 45, 60, and 75 µg/ml) by diluting them with DMSO. They were then combined with 2.5 ml of 0.2M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. After 20 minutes of incubation at 50°C, the resultant mixture was quickly chilled. After that, 2.5 ml of 10% tri-chloro acetic acid was added, and the mixture was

centrifuged for 10 minutes at 3000 rpm. One milliliter of 0.1% ferric chloride and 2.5 milliliters of deionized water were combined with the supernatant. At 700 nm, the absorbance was then measured.

RESULTS AND DISCUSSION:

Percentage Yield of Extracts Obtained by Successive Solvent Extraction:

Table 1: Percentage yield values

Plant Name	Part used	Method of Extraction	Solvents	Wt of the drug taken (gm)	Wt of the extract (gm)	Colour of the extract	Percentage yield of extracts (%w/w)
<i>G. sepium</i>	Root	Continuous hot Percolation by Soxhlet Apparatus	Petroleum ether	65	0.8	Brownish and sticky with oily mass	1.23
			Chloroform	65	0.91	Brownish black	1.4
			Ethyl acetate	65	3.69	Dark brown	5.67
			Methanol	65	4.01	Brownish black	6.16

In-vitro Anti-oxidant Study:

Total Phenolic Content Determination:

Table 2: Concentration and absorbance value of ascorbic acid and extract

Sample	Concentration(µg)	Absorbance
Standard	20	0.247

(Gallic acid)	40	0.422
	60	0.634
	80	0.816
	100	0.999
Plant Extract (methanolic extarct)	100	0.356

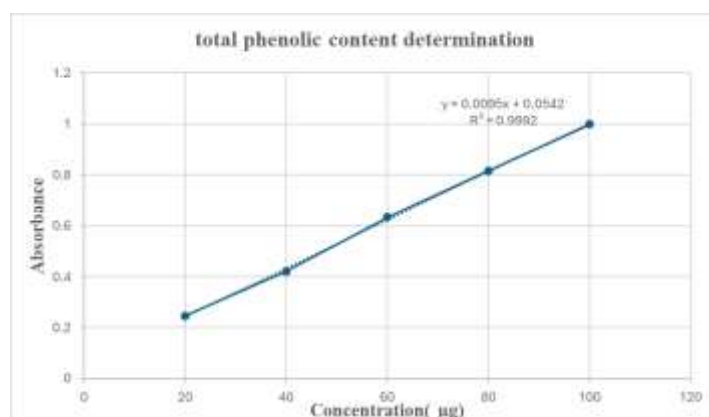


Fig. 1: Standard graph of ascorbic acid for estimation of total phenolic content

Table 3: Total phenolic content of methanolic root extract of *G. sepium*

Extracts 100µg/ml	Phenolic content Ascorbic acid equivalent µg/ml
Plant extract (Methanolic extract)	37.47

Total Flavonoid Content Determination:

Table 4: Concentration and absorbance values of rutin and extract

Sample	Concentration(µg)	Absorbance
Standard	20	0.341

(Rutin)	40	0.440
	60	0.534
	80	0.604
	100	0.714
Plant Extract (Methanolic extract)	100	0.192

Table 5: Total flavonoid content of methanolic root extract of *G. Sepium*

Extracts 100µg/ml	Phenolic content Ascorbic acid equivalent µg/ml
Plant extract (Methanolic extract)	41.73

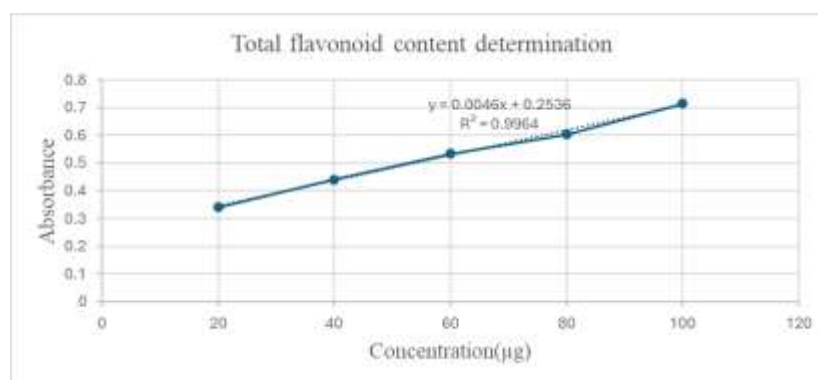


Fig. 2: Standard graph of rutin for estimation of total flavonoid content

DPPH Free Radical Scavenging Activity:

DPPH is stable free radical which get reduced by accepting hydrogen or electron from source. Plant extract demonstrates exceptional scavenging activity when compared with ordinary ascorbic acid. DPPH scavenging activity of both plant extract and standard ascorbic acid was shown to be increase in dose dependent way. The IC₅₀ value was calculated to identify the concentration of the sample required to block 50% of radical. The lower the IC₅₀ value, the higher the antioxidant activity of samples. IC₅₀ value of standard ascorbic acid was found to be 11.38 mcg/ml and it demonstrate very high antioxidant activity where

as IC₅₀ value of methanolic extract of *G. sepium* was found to be 59.27 mcg/ml and it exhibit strong antioxidant activity. The results demonstrated a significant decrease in the concentration of DPPH radical due to the scavenging activity of extract as compared to standard (ascorbic acid). In the present study support, plant extract has good antioxidant and scavenges DPPH radicals.

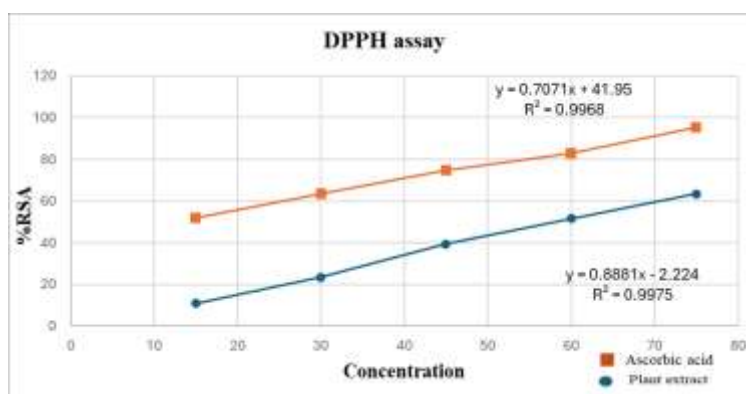
Note:

IC ₅₀ value (mcg/ml)	Category
<50	Very strong
50-100	Strong
100-150	Medium
150-200	Weak

Table 6: Concentration and % RSA, IC₅₀ value of standard ascorbic acid and extract

Sample	Concentration(μ g)	%RSA	IC ₅₀ value (μ g)	Category
Standard (Ascorbic acid)	15	52.06	11.38	Very strong
	30	63.49		
	45	74.93		
	60	83.05		
	75	95.31		
Plant Extract (Methanolic extract)	15	10.90	59.27	Strong
	30	23.48		
	45	39.30		
	60	51.56		
	75	63.47		

%RSA= Percentage radical scavenging activity,
 IC₅₀= Half-maximal inhibitory concentration

**Fig. 3: Dose-response curve of standard ascorbic acid and plant extract**

Nitric Oxide Scavenging Assay:

Figure 4 shows the percentage inhibition of nitric oxide formation by plant extract based on the nitric oxide scavenging activity of *G. sepium* and ascorbic acid plant extracts. The reference was ascorbic acid. Comparing plant extract to regular ascorbic acid reveals excellent scavenging action. It was discovered that the nitric oxide radical scavenging activity of both plant extract and regular ascorbic acid increased in a dose-dependent way. To find the sample concentration needed to inhibit 50% of the radical, the IC₅₀ value

was computed. The greater the antioxidant activity of the samples, the lower the IC₅₀ value. The methanolic extract of *G. sepium* was found to have an IC₅₀ value of 64.50 mcg/ml and to demonstrate strong antioxidant activity, whereas the standard ascorbic acid had an IC₅₀ value of 30.97 mcg/ml and very strong antioxidant activity. Because of the extract's scavenging activity in comparison to the standard (ascorbic acid), the results demonstrated a considerable drop in the levels of nitric oxide. According to the current research, plant extract scavenges nitric oxide and has high antioxidant properties.

Table 7: Concentration and % RSA, IC₅₀ value of standard ascorbic acid and extract

Sample	Concentration(μ g)	%RSA	IC ₅₀ value (μ g)	Category
Standard (Ascorbic acid)	15	40.88	30.97	Very strong
	30	50.17		

Sample (methanolic extract)	45	57.70	64.50	Strong
	60	65.16		
	75	74.80		
	15	12.03		
	30	22.24		
	45	36.23		
	60	45.39		
	75	56.92		

%RSA= Percentage radical scavenging activity,
IC₅₀ = Half-maximal inhibitory concentration.

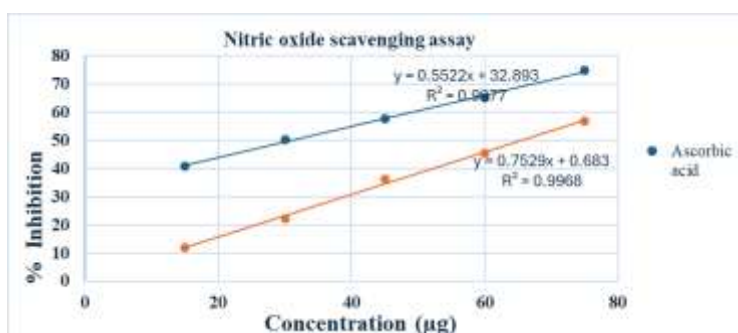


Fig. 4: Dose-response curve of standard ascorbic acid and plant extract

Phosphomolybdate Assay:

The basis of this assay is the reduction of the phosphomolybdate ion in the presence of an antioxidant, which forms a green phosphate/MoV complex that can be detected by spectrophotometry. The figure illustrates the phosphomolybdate ion lowering power potentials of the *G. sepium* methanolic root extract at 765 nm in relation to the standard ascorbic acid. The outcome shows that both plant root extract and regular ascorbic acid had an increase in phosphomolybdate ion reduction activity as their

concentrations rose. At 37.50 mcg/ml, plant extract exhibited antioxidant activity equal to that of ascorbic acid.

Table 8: Concentration and absorbance values of standard ascorbic acid and extract

Sample	Concentration(µg)	Absorbance
Standard (Ascorbic acid)	15	0.069
	30	0.077
	45	0.085
	60	0.094
	75	0.103
Plant Extract (Methanolic extract)	75	0.081

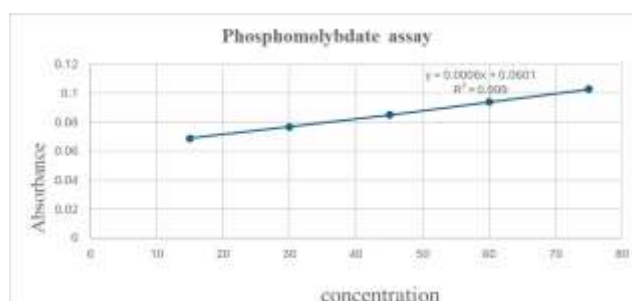


Fig. 5: Dose-response curve of standard ascorbic acid

Ferric Reducing Antioxidant Power Assay:

The figure illustrates the ferrion lowering power potentials of the *Annona glabra* methanolic root extract at 700 nm in relation to the standard ascorbic acid. The outcome shows that when the concentrations of conventional ascorbic acid and plant root extract grew, so did their ferric reduction activity. At 20.22 mcg/ml, the antioxidant activity of plant extract is equal to that of ascorbic acid.

Table 11: Concentration and absorbance values of standard ascorbic acid and extract

Sample	Concentration(μ g)	Absorbance
Standard (Ascorbic acid)	15	0.531
	30	0.623
	45	0.713
	60	0.822
	75	0.915
Plant Extract (Methanolic extract)	75	0.561

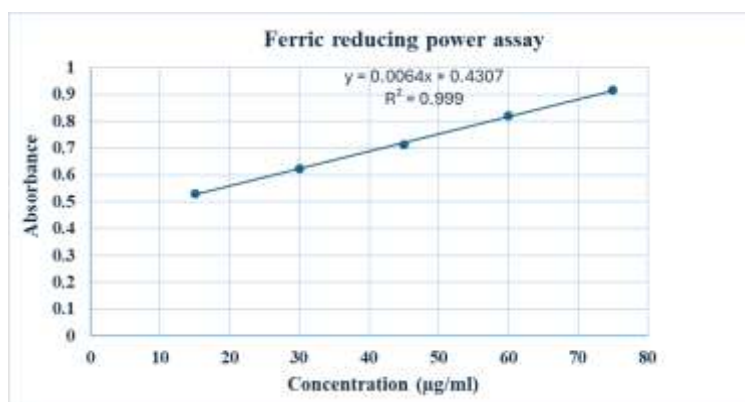


Fig. 6 : Dose-response curve of standard ascorbic acid

CONCLUSION:

The study confirms that *G. sepium* possesses significant antioxidant potential, likely due to the presence of phenolic compounds, flavonoids, and other phytoconstituents. The methanolic extract of *G. sepium* exhibited notable antioxidant potential across multiple invitro assays, including DPPH, nitric oxide scavenging, phosphomolybdate, and FRAP assays. The extract showed concentrate dependent activity with IC₅₀ values comparable to that of ascorbic acid, indicating strong free radical scavenging and re'ducing power. The presnrce of phenolic and flavonoid compounds likely contributes to this activity. These findings suggest that *G. sepium* can serve as a promising natural source of antioxidants and has potential applications in pharmaceutical and cosmetic formulations. Further in vivo studies and compound profiling are recommended for deeper understanding and therapeutic exploration.

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