Research Article

Evaluation of Antioxidant Properties and Total Phenolic Content of *Gardenia gummifera* Linn.

Vindhya K*, Leelavathi S
[1]Department of Studies in Botany, University of Mysore, Karnataka, India.
***Corresponding author's E-mail:** vindhyakumar88@gmail.com

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ABSTRACT

Gardenia gummifera Linn. of the family Rubiaceae has been considered as an important plant in the traditional Ayurveda. The *Gardenia gummifera* Linn. leaf was extracted serially by the solvents of increasing polarity (Petroleum ether, ethyl acetate and ethanol). The present investigation was to examine the *in vitro* antioxidant potential of various extracts of leaf of *Gardenia gummifera* Linn. The antioxidant activity was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, superoxide radical scavenging assay, reducing power assay, cerium (IV) amaranth dye assay, Ferric Reducing Antioxidant Property (FRAP) assay and total antioxidant capacity. In all the assays, the ethanolic extract of *Gardenia gummifera* Linn. is more effective in free radical scavenging activity than that of other two extracts. So, the *in vitro* studies clearly showed that the ethanolic extract of *Gardenia gummifera* Linn. has significant antioxidant activity. The quantitative estimation showed that ethanolic extract contains higher phenolic content than the other extracts.

Keywords: *Gardenia gummifera* Linn., Rubiaceae, Antioxidant activities, Reducing power, Total phenolic content.

INTRODUCTION

Nature is serving as man's primary source for the cure of his ailments. However, the potential of higher plants as sources for new drugs is still largely unexpected. It is widely accepted that antioxidants are radical scavengers, which protect the human body against free radicals that may cause pathological conditions such as ischemia, anaemia, asthma, arthritis, inflammation, neuro-degeneration, Parkinson's diseases, mongolism, ageing process and perhaps dementias.[1]

Plant-based antioxidants are now preferred to the synthetic ones because of safety concerns.[2] They are potential sources of natural antioxidants. They absorb the sun's radiation and generate high levels of oxygen as secondary metabolites of photosynthesis. On the other hand, Oxygen is easily activated by Ultra Violet (UV) radiation and heat from the sunlight to produce toxic Reactive Oxygen Species (ROS).[3]

These ROS are highly reactive because they can interact with a number of cellular molecules and metabolites thereby leading to a number of destructive processes causing cellular damage.[4] Plants produce various antioxidative enzymes and non-enzymes compounds to counteract and detoxify these ROS in order to survive. Hence, naturally occurring phytocompounds possessing antioxidative and anti-inflammatory properties appear to contribute to their chemopreventive or chemoprotective activity which in turn, by the alternative medicine, has been used to the benefit of human beings.[5]

Gardenia gummifera Linn. belongs to the family Rubiaceae. It is commonly known as gummy gardenia. It is found in dry forests of Karnataka, Tamil Nadu, Andhra Pradesh and Kerala. *Gardenia gummifera* is claimed to have a number of medicinal properties which include anthelmintic, antispasmodic, carminative, diaphoretic, expectorant, potentiation of pentobarbitone induced sleep, Antiepileptic, peripheral and central Analgesic, Cardiotonic, Antioxidant, and Antihyperlipidemic. It is also claimed to be useful in dyspepsia, flatulence for cleaning foul ulcers and wounds, and to keep off flies from wounds in veterinary practice.[6-8]

The aim of this study was to evaluate *in vitro* antioxidant potential of the leaf extracts of *Gardenia gummifera* Linn. growing in Mandya region, Karnataka, India.

MATERIALS AND METHODS

Collection of Plant Material

The fresh plant material (leaves) were collected from Melkote, Mandya district, Karnataka state, India in the month of December 2012. The plant was identified with the help of Flora of Presidency of Madras.[9]

Processing of Plant Material

The leaves of *Gardenia gummifera* Linn. were cleaned and shade dried. The dried material was powdered using mechanical method and resulting powder was sieved with sieve of 0.3mm aperture size and stored in the airtight container.

Extraction of Plant Material[10]

The powdered plant leaf material was subjected to successive solvent extraction taking from non-polar to polar solvents like petroleum ether, ethyl acetate and ethanol. 50gms of powdered plant material was subjected to soxhlet extraction for 12-16 hrs with 300ml of the various solvents each at a time. The extracts obtained were later kept for evaporation to remove the

excessive solvents. These extracts were stored in airtight container and a cool dry place.

DPPH Assay[11]

The DPPH free radical is reduced to a corresponding hydrazine when it reacts with hydrogen donors. The DPPH radical is purple in colour and upon reaction with hydrogen donor changes to yellow colour. It is a discoloration assay, which is evaluated by the addition of the antioxidant to a DPPH solution in ethanol or methanol and the decrease in absorbance was measured at 490nm.

Reagents

2, 2-Diphenly 1-picryl hydrazyl solution (DPPH, 100 μM): 22 mg of DPPH was accurately weighed and dissolved in 100 ml of methanol. From this stock solution, 18 ml was taken and diluted to 100 ml using methanol to obtain 100 μM DPPH solution.

Preparation of test solutions

21 mg each of the extracts were dissolved in distilled DMSO separately to obtain solutions of 21 mg/ml concentrations. Each of these solutions were serially diluted separately to obtain lower concentrations.

Preparation of standard solution

10 mg of rutin was weighed and dissolved in 0.95 ml of Dimethyl sulfoxide (DMSO) to get 10.5 mg/ml concentration. This solution was serially diluted with DMSO to get lower concentrations.

Procedure

The assay was carried out in a 96 well microtitre plate. To 200 μl of DPPH solution, 10 μl of each of the test sample or the standard solution was added separately in wells of the microtitre plate. The final concentration of the test and standard solutions used were 1000, 500, 250, 125, 62.5, 31.25, 15.62 μg/ml. The plates were incubated at 37^0 C for 30 min and the absorbance of each solution was measured at 490 nm, using a microplate reader. The experiment was repeated thrice. Percent of radical scavenging activity was calculated using the following formula:

$$\% \text{ radical scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Scavenging of Superoxide Radical by Alkaline DMSO Method[12]

In alkaline DMSO method, superoxide radical is generated by the addition of sodium hydroxide to air saturated DMSO. The generated superoxide remains stable in solution, which reduces nitro blue tetrazolium (NBT) in to formazan dye at room temperature and that can be measured at 560 nm. Superoxide scavenger capable of reacting inhibits the formation of a red dye formazan.

Preparation of test and standard solutions

14 mg of each of the extracts were weighed accurately and separately dissolved in 3 ml of DMSO. These solutions were serially diluted with DMSO to obtain the lower dilutions.

Procedure

To the reaction mixture containing 1 ml of alkaline DMSO (1 ml, 1% distilled water, 5 mM NaOH) and 0.3 ml of the extracts in DMSO at various concentrations (1000, 500, 250, 125, 62.5 μg/ml.), 0.1 ml of NBT (1 mg/ml) was added to give a final volume of 1.4 ml. The absorbance was measured at 560 nm.

$$\% \text{ radical scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Cerium (IV) Amaranth Dye Assay[13]

Standardization of Ce (IV) solution

Concentrated sulfuric acid (0.2 ml) was used for the dissolution of 0.016 g of Ce $(SO_4)_2.4H_2O$ which was then diluted to 25 ml; the solution was made homogenous with a magnetic stirrer at room temperature until total dissolution.

This solution was transferred to a 50 ml standard flask, and diluted to the mark with distilled water. The Ce (IV) stock solution was standardized with arsenic (III) oxide by using N-phenyl anthranilic acid indicator.

Procedure

In a final volume of 10 ml, 0.1185 mM Ce (IV) solution, varying concentrations of standard antioxidant solution and 49.6 μM amaranth dye solutions were added.

After shaking well, the absorbance of the reaction mixture was measured at 530 nm against a control without antioxidant. Similarly, 100 μl of different extracts of plant material were subjected to antioxidant assay.

The absorbance was recorded at a wavelength of 530 nm. The antioxidant activity was expressed as μM equivalent of gallic acid from standard curve.

FRAP (1,10-phenonthroline) Method[14]

$NH_4Fe\ (SO_4)_2.12H_2O$ (0.160 g) was added to 2 ml of 1 M HCl. A suitable mass of 1,10-phenonthroline was dissolved in water to get a final concentration of 1.0×10^{-2} M. These two solutions were mixed and diluted to 100 ml with distilled water. This reagent was kept in the dark to protect it from sunlight and was shown to be stable for several weeks.

The stock solution of the tested antioxidants were prepared in 98% ethyl alcohol. To 1 mL of different antioxidant solutions, 1 ml of 1,10-phenonthroline reagent solution and 5 ml of EtOH (96%) were added, diluted to the mark with water in a 25ml standard flask, incubated in a water bath at 50°C for 30 min, cooled to room temperature, and the absorbance measured at 510 nm against the control.

FRAP activity quantified by calibration curve (obtained from known concentrations of Gallic acid standard) and the concentrations were expressed as μM equivalent of

Gallic Acid and all the determinations were performed in triplicates.

Evaluation of Total Antioxidant capacity[15]

The total antioxidant capacity was determined by phosphomolybdenum method and is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compounds and the formation of a green Mo (V) complex which has the maximal absorption at 695 nm.

Preparation of test and standard solutions

Weighed accurately 55 mg of each extracts and the standard, ascorbic acid and dissolved in 5 ml of DMSO. The lower dilutions were made serially with DMSO.

Procedure

An aliquot of 0.1 ml of the sample solution containing a reducing species in DMSO was combined in an eppendorf tube with 1 ml of reagent solution (0.6 M Sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate).

The tubes were capped and incubated in water bath at 95 °C for 90 min. The samples were cooled to room temperature, and the absorbance of each solution was measured at 695 nm. The total antioxidant capacity was expressed as mcg equivalent of ascorbic acid.

Reducing Power Assay[16]

The principle of this assay is higher the absorbance represents the stronger the reducing power.

Preparation of test and standard solutions

Weighed accurately 2 mg of each extracts and the standard, ascorbic acid and dissolved in 2 ml of DMSO. Then take 0.5 ml of above solution then make up to 2.5 ml with phosphate buffer (0.2 M, pH 6.6). The lower dilutions were made serially with DMSO.

Procedure

The above sample were spiked with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was then kept in a 50°C water-bath for 20 min.

The resulting solution was then cooled rapidly, spiked with 2.5 mL of 10% trichloroacetic acid, and centrifuged at 3000 rpm for 10 min. The supernatant (5 mL) was then mixed with 5 mL of distilled water and 1 mL of 0.1% ferric chloride. The absorbance at 700 nm was then detected after reaction for 10 min. The higher the absorbance represents the stronger the reducing power.

Determination of Total Phenolic Content[17]

Total phenolic in methanol extracts was determined by the method of Singleton[17]. 20 µl of extract (5 mg/ ml) was mixed with 0.75 ml of 20% sodium carbonate solution and 0.25 ml of Folin-Ciocalteau reagent.

The reaction mixture was allowed to stand in light for 3 min and incubated for 2 h in dark. The absorbance was measured at 765 nm using UV-Visible Spectrophotometer.

Total phenolics were quantified by calibration curve obtained from measuring the absorbance of known concentrations of Gallic acid standard (0-100µg/ ml).

The concentrations were expressed as µg of Gallic acid equivalents per ml and all the determinations were performed in triplicates.

RESULTS AND DISCUSSION

The antioxidant potential of various leaf extracts of *Gardenia gummifera* Linn. were summarized in the Table 1. All the data were reported as mean ± standard deviation of 3 replicates.

Among the three extracts, ethanolic extract have presented a remarkable radical scavenging activity.

Recently we reported that the phytochemical analysis of *Gardenia gummifera* Linn. revealed the presence of phytochemicals such as phenols, tannins, terpenoids in all the extracts.

Some extracts showed the presence of resins, flavonoids, glycosides and steroids. Alkaloids, carbohydrates, saponins, protein and amino acid were absent in all the plant extracts.[18] These bioactive compounds are responsible for antioxidant activity.

Table 1: DPPH radical scavenging assay, Superoxide scavenging assay, Cerium (IV) amaranth dye assay, FRAP assay, Total antioxidant capacity and Total phenol content of different extracts of *Gardenia gummifera* Linn.

Sample	DPPH radical scavenging assay (IC_{50} µg/ml)	Superoxide scavenging assay (IC_{50} µg/ml)	Cerium (IV) amaranth dye assay (µM equivalent of gallic acid)	FRAP assay (µM equivalent of gallic acid)	Total antioxidant capacity (µg equivalent of ascorbic acid)	Total phenol content (µg equivalent of gallic acid)
Petroleum ether	291.58 ± 5.85	831.33 ± 3.21	4.045 ± 0.032	3.901 ± 0.035	48.122 ± 0.722	19.813 ± 0.104
Ethyl acetate	53.14 ± 0.70	493.22 ± 1.31	6.093 ± 0.037	7.152 ± 0.192	74.957 ± 0.551	32.385 ± 0.09
Ethanol	48.33 ± 0.58	276.6 ± 6.42	9.683 ± 0.034	11.679 ± 0.254	91.804 ± 0.478	39.735 ± 0.052
Rutin	31.61 ± 0.98	-	-	-	-	-
Ascorbic acid	-	17.44 ± 0.39	-	-	-	-

DPPH Radical Scavenging Assay

DPPH radical scavenging is considered to be good *in vitro* model widely used to assess antioxidant efficacy of single compound as well as for different plant extracts within a very short period of time.[19]

In radical form, DPPH disappears on reduction by an antioxidant compound or a radical species to become a stable diamagnetic molecule resulting in the colour change from purple to yellow, due to the formation of diphenyl picryl hydrazine. It could be taken as an indication of the hydrogen donating ability of the extracts.[20]

In the present study, the petroleum ether, ethyl acetate and ethanol extracts of *Gardenia gummifera* Linn. were evaluated for their free radical scavenging activity with rutin as standard compound is shown in Figure 1.

This activity was increased by increasing the concentration of the extracts. This result indicated that the ethanolic extract showed significant antioxidant activity with the IC_{50} value of 48.33±0.58 µg/ml followed by its ethyl acetate extract with 53.14±0.70 µg/ml and petroleum ether extract with 291.58±5.85 µg/ml.

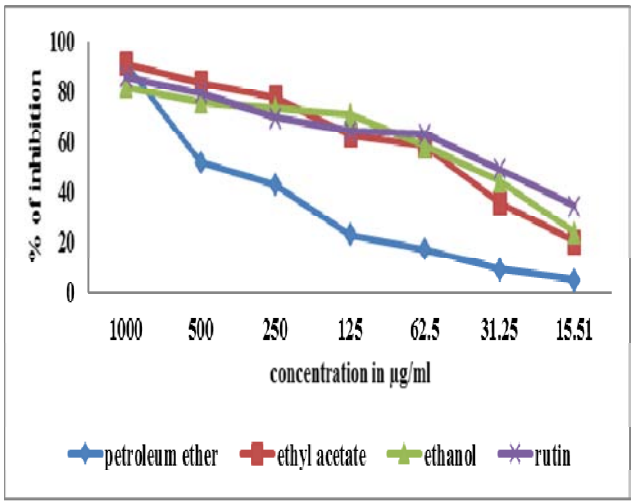

Figure 1: DPPH radical scavenging assay of *Gardenia gummifera* Linn.

Superoxide Scavenging Assay

Superoxide anions are formed in the normal physiological reactions taking place mainly in the mitochondria. Oxidation of the glucose contributes the major percentage of total superoxide anions formed.

These superoxide anion radicals are converted to hydrogen peroxide which in turn results in the production of highly reactive hydroxyl radicals.[21]

The results of superoxide anion radical scavenging activity is shown in Figure-2, the ethanol fraction had significantly higher superoxide scavenging activity with IC_{50} 276.6±6.42 µg/ml followed by its ethyl acetate with 493.22±1.31 µg/ml and petroleum ether extract with 831.33±3.21 µg/ml. This indicated that the active compounds were polar since ethanol extract was more polar compared to ethyl acetate and petroleum ether.

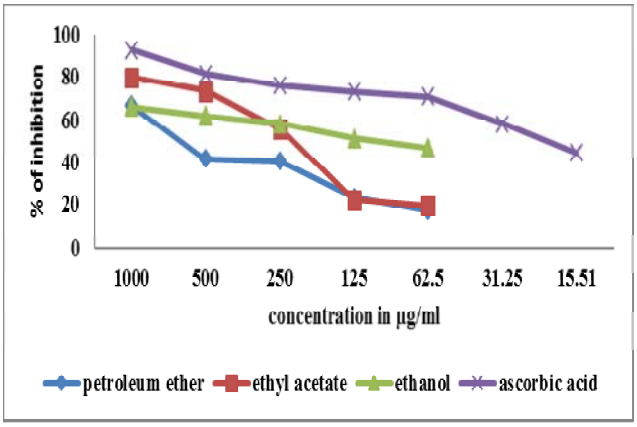

Figure 2: Superoxide scavenging assay of *Gardenia gummifera* Linn.

Cerium (IV)-Amaranth Dye Assay

Cerium (IV) amaranth dye assay of *Gardenia gummifera* Linn. extracts was calculated using the standard curve of gallic acid (y = 0.0430x - 0.1172; R2 = 0.9969) (Figure 3). Among the three extracts, the ethanolic extract of *Gardenia gummifera Linn.* was found to possess the highest antioxidant activity.

A study of theoretical redox chemistry of Ce (IV) shows that Ce (IV) sulfate solutions are remarkably stable over prolonged periods. Formal potential measurements show that the redox potential of the Ce (IV)–Ce (III) system is greatly dependent upon the nature and concentration of mineral acid present, and takes the smallest value in sulfuric acid due to the complexation effect of so_4^{2-} on Ce (IV).[22]

Also the presence of complexing inorganic anions like sulfate may reduce the complexing ability of Ce (IV) with organic substrates, and may therefore retard the oxidation of organic compounds by Ce (IV), since Ce (IV)–organic substrate complexation is a prerequisite of fast inner-sphere electron transfer.[23]

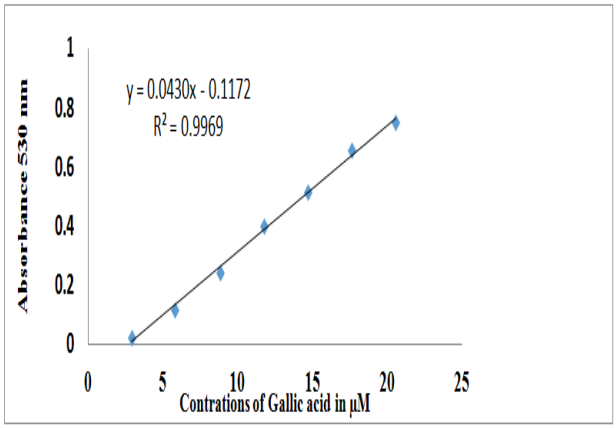

Figure 3: The calibration curve of standard Gallic acid of various concentrations using the Ce(IV)-amaranth dye method.

FRAP Assay

The FRAP assay is a simple, convenient and reproducible method that was initially developed to measure the plasma antioxidant capacity, but is now widely employed in the antioxidant studies of other biological samples, such as food, plant extracts, juices and beverages, etc.[24]

FRAP assay of *Gardenia gummifera* Linn. extracts was calculated using the standard curve of gallic acid (y = 0.0329x - 0.0307; R2 = 0.9613) (Figure-4).

The results (Table 1) showed that FRAP values were higher in ethanol extracted sample compared to other extraction. This showed that ethanol extraction was more efficient in extracting antioxidants in plant material compared to other extraction.

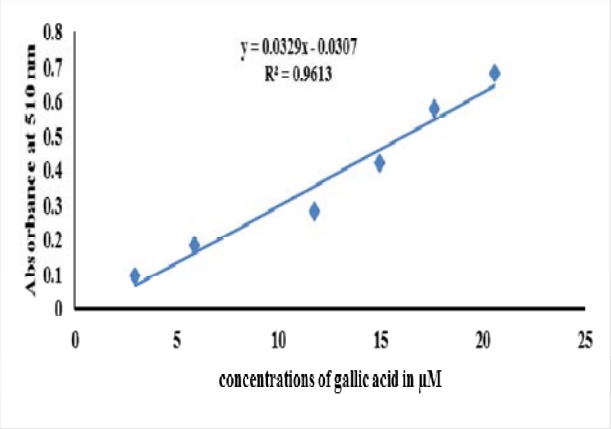

Figure 4: The calibration curve of Gallic acid solution of various concentrations using the FRAP method.

Total Antioxidant Capacity

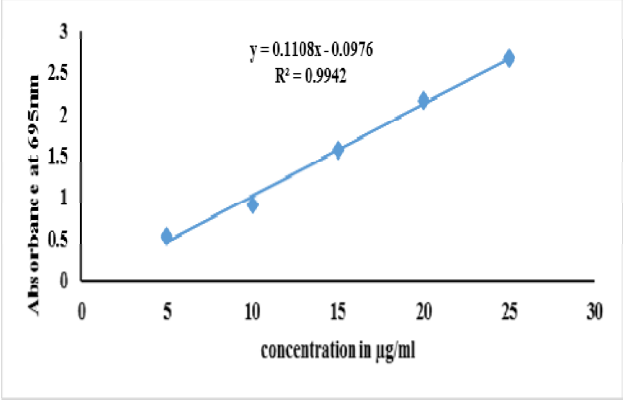

Figure 5: The calibration curve of ascorbic acid solution of various concentrations using the total antioxidant capacity method.

Total antioxidant capacity of all the 3 extracts of *Gardenia gummifera* Linn. was evaluated by the phosphomolybdenum method and was expressed as ascorbic acid equivalents (AAE) per gram of plant extract.

Total antioxidant capacity of *Gardenia gummifera* Linn. extracts were calculated using the standard curve of ascorbic acid (y = 0.1108x - 0.09761; R2 = 0.9942) (Figure 5). Among the three extracts, the ethanolic extract of *Gardenia gummifera* Linn. was found to possess the highest total antioxidant capacity (Table 1).

The results indicate a concentration dependent total antioxidant capacity. It means that the ethanolic extract of *Gardenia gummifera* Linn. will have to contain as much quantity of antioxidants compounds as equivalents of ascorbic acid to effectively reduce the oxidant in the reaction matrix.

Antioxidant capacity of ascorbic acid has been used as a reference standard from which plant extracts with potential antioxidant activity are compared.[25]

Reducing Power Assay

The reducing properties of antioxidants are generally associated with the presence of reductones, such as ascorbic acid and other secondary metabolites. Such reductones exert antioxidant action by breaking the free radical chain by donating hydrogen atoms. Reductones have also been reported to react with certain precursors of peroxide, thus preventing peroxide formation.[26]

Reducing power of different extracts of *Gardenia gummifera* Linn. was compared with ascorbic acid (Figure 6). Among the extracts the ethanolic extract exhibited the most reducing power. This result indicates that the extracts may consist of polyphenolic compounds that usually show great reducing power. This has been justified by ethanolic extract being the most reducing agent with highest phenolic content (Table 1).

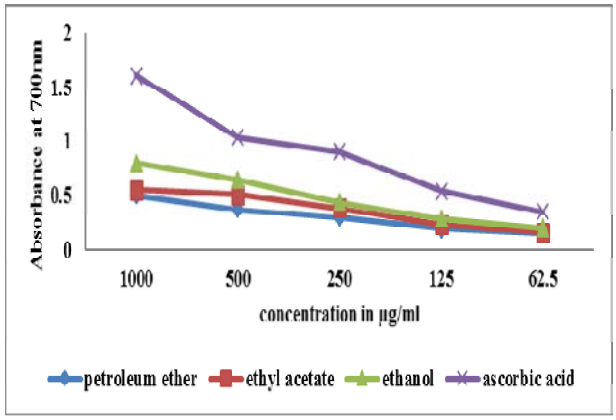

Figure 6: Reducing power assay of *Gardenia gummifera* Linn.

Determination of Total Phenolics

Phenols are important constituent because of their scavenging ability due to their hydroxyl groups.[27] In addition, it was reported that phenolic compounds were associated with antioxidant activity and play an important role in stabilizing lipid peroxidation.[28]

Total phenolic content of the different extracts of *Gardenia gummifera* Linn. was determined by using the Folin-Ciocalteu reagent and were expressed as gallic acid equivalents per micro gram of plant extract. The total phenolic contents of the test fractions were calculated using the standard curve of gallic acid (y = 0.111x-0.112;

R^2 = 0.994) (Figure 7). Ethanolic extract of *Gardenia gummifera* Linn. was found to contain the highest amount of phenols.

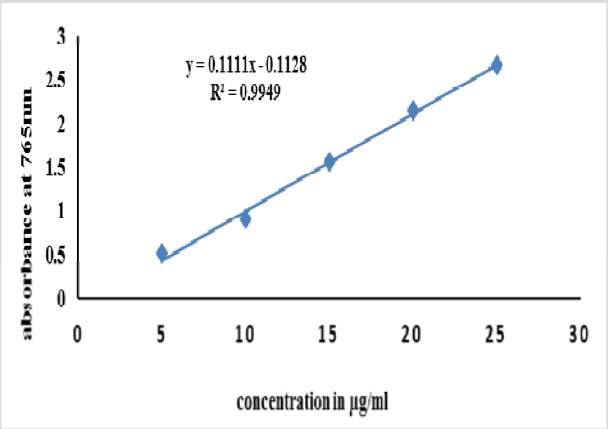

Figure 7: The calibration curve of Gallic acid solution of various concentrations using the total phenolic content method.

CONCLUSION

In this study, the leaf extracts of *Gardenia gummifera* Linn. found to posses antioxidant activity. In all the assays, among the three extracts, ethanolic extracts showed significant activity compared to ethyl acetate and petroleum ether.

However, further investigations are to be carried out to isolate and characterize the specific bioactive principles.

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