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Research Article

FREE RADICAL SCAVENGING ACTIVITY AND REDUCING POWER CAPACITY OF METHANOLIC BARK EXTRACT OF *CANTHIUM PARVIFLORUM* LINN

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ABSTRACT

Aim: To carried out the free radical scavenging of CanthiumParviflorum (Rubiaceae) Bark. **Methods:** Methanolic Extract of Canthium Parviflorum Bark powder was investigated for Free radical scavenging activity and Reducing power capacity by using DPPH, ABTS, Reducing Power, Total Antioxidant, Total Flavanoid Contentand Total Phenolic Content. **Results:** Methanolic Extract of Canthium Bark posses Free radical scavenging activity and The Results areDPPH Possess IC_{50} at (508 µg/ml); ABTS Shows IC_{50} at (100µg/ml); Reducing Power capacity at 100 µg/ml showed absorbance value 0.862±0.01 expressed as Ascorbic acid as a standard; Total Anti oxidantcapacity (366.02±0.03 µg/mlexpressed as Ascorbic acid equivalents); Total Phenolic content(222.41±0.03µg/ml expressed as Gallic acid equivalents); Total Flavanoid content (392.98±0.02µg/ml expressed as Quercetin equivalents). **Conclusion:** Results of the present study suggest that the tested Plant material having free radical scavenging activity and reducing power capacity.

Keywords: Canthium parviflorum, DPPH radical scavenging activity, Reducing power activity.

INTRODUCTION

Now a day the fact of harmful effect of reactive oxygen species on human health is well known. The capability of natural defense systems of living organisms against excess production of these species decreases with when influenced with negative environmental factors or aging[1].The traditional medicine all over the world is now a days revalued by an extensive activity of research on different plant species and their therapeutic principles. The increasing cost, non availability of modern drugs, and limited access to adequate health care have compelled about 80% world population to use traditional pharmacopeia for primary health care especially in the tropical and sub tropical regions [2]. As plant produce a lot of antioxidant to control the oxidative stress caused by sunbeams and oxygen, then can represent a source of new compound with antioxidant activity[3]. ROS formed in-vivo such as superoxide anion; hydroxyl radical and hydrogen peroxide are highly reactive and potentially damaging transient chemical species. These are continuously produced in human body, as they are essential for energy supply, detoxification, chemical signaling and immune function. ROS are regulated by endogenous superoxide dismutase, glutathione peroxidase and catalyze due to over production of ROS, induced by exposure to external oxidant substance or a failure in the defense mechanism, damage to cell structure, DNA, lipids and proteins[4] occurs which increases risk of more than 30 different disease process[5].

The most important notorious among them being neurodegenerative conditions like Alzheimer's disease[6-7], mild cognitive impairment (MCI) and Parkinsonism disease.

Other neurodegenerative diseases significantly associated with oxidative stress include multiple sclerosis, creulzfeldt-jocob diseases include highly disabling pathologies like cardiovascular diseases and cardiac failure[8] and alcohol induced liver diseases[9] and ulcerative colitis and cancer caused by complex of different causes of which RNS/ROS is a component.

Various plants have been shown to possess significant antioxidant property[10-12] and different classes of phytochemical have been demonstrated to responsible for the plant antioxidant activity[13-15]. Antioxidant interferes with the oxidation process by reacting with free radicals, chelating with metals, catalytic metals and also by acting as oxygen scavengers[16-17]. Flavonoids and phenolic compounds widely distributed in plants which have been reported to exert multiple biological effect including antioxidant, free radical scavenging abilities, iron chelator, anti-inflammatory, hepato protective and anti-carcinogenic[18] etc Therefore, the evaluation of antioxidant activity of various plant extracts is considered as an important step in the identification of their ability to scavenge the free radicals.

The plant Canthiumparviflorum is the member of Rubiaceae. It is a thorny subscandent shrub with spreading branches distributed throughout India in scrub forests and dry plains. Its leaves are simple, small, obviate, opposite with interpetiolar stipules and auxiliary spines. The leaves and roots are astringent, sweet, thermogenic, diuretic, febrifuge, constipating, anthelmintic, and tonic and these are used in various conditions of kapha, diarrhea, strangury, fever, leucorrhoea, intestinal worms, and general debility[19]. It is traditionally used for snake bites[20]. Leaf paste is externally applied twice a day to treat scabies and the ring worm infection[21]. Decoction of leaves is used for wound healing in animals. Significant antioxidant and diuretic activity was exhibited by extracts of leaves[22].

In view of this screening project, to evaluate antioxidant activity of methanolic Canthium bark extract by using different invitro-antioxidant determination methods including DPPH radical scavenging method, ABTS radical scavenging activity and reducing power assay method were studied in this report. Ascorbic acid, Quercetin, Gallic acid was used as antioxidant standard compounds respectively.

MATERIALS AND METHODS

Chemicals Used For Anti-oxidant activity

Chemicals such as 1,1-Diphenyl-2-picrylhydrazyl(DPPH) and ABTS {2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)} were procured from sigma chemical Co. (INDIA). All remaining chemicals such as (Potassium per sulphate, methanol, Gallic acid, Folin-Ciocalteu reagent, Sodium carbonate, Aluminum chloride, Sodium hydroxide, Quercetin, Ferrous chloride, Ascorbic acid, ferrozine, disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium chloride, sodium nitrite) were obtained from HI-MEDIA laboratories and SISCO research laboratories Pvt.ltd (Mumbai, India).

Spectroscopic measurements

Spectroscopic measurements were performed by UV-Visible double beam spectrophotometer (ELICO SL-210).

Plant collection and authentification

The bark of Canthiumparviflorum (Rubiaceae) is a short shrubby and woody plant. The bark of Canthium was collected from Vanantharam herbal PHC centre, Addathegalamandal, E.G.dist. Plant can be authentified by T.SRINIVAS, J.L, Government junior college, Gokavaram, E.G.dist. Voucher specimen of the collected plant was deposited in the laboratory of phytochemistry and pharmacognosy, *ADARSA COLLEGE OF PHARMACY*, G.Kothapalli, E.G.dist, Andhra Pradesh, India for future reference.

PREPARATION OF EXTRACTS

Collected bark was chopped into small pieces and they are allowed to air dry at room temperature for 4-7 days. The bark was grained into fine powder for extraction process.Dried powder of plant bark (approx. 50gm) was extracted by both maceration method andsoxhlation process (Capacity 250ml) with methanol as well as chloroform. After extraction of the contents were concentrated and maintained at proper condition and dried in desiccators to get corresponding extracts.The extracts were stored at 0^{o}C in airtight containers until need for further studies.

Qualitative phytochemical Screening

The bark of two solvated extracts werequalitatively tested for the presence of various phytochemical constituents(alkaloids, glycosides, reducing sugars, tannins, fixed oils, fats, proteins and free amino acids) using various standard chemical tests[23].

Quantitative analysis of methanolic extract

Determination of total phenolic content

The total phenolic content of the extracts were determined by the Folin-Ciocalteu method with some modifications. 0.5 mL of the sample was added to 2.5 mL of 0.2 N Folin-Ciocalteu

reagents and incubated for 5 minutes at room temperature. 2 mL of 20% of Na_2CO_3 was then added to this and kept on boiling water bath for 2 min. the blue colour formed was read at 650nm. The experiment was performed in triplicate. Then Gallic acid (100 - 500 µg/ml) was used to produce standard calibration curve. The total phenolic content was expressed as (µg/ml or mg/g) of Gallic acid equivalents of extract[24,25].

T= C X V/M

Where **T** is the total phenolic content in $mg \cdot g^{-1}$ of the extract as GAE,
C is the concentration of Gallic acid established from the calibration curve in $mg \cdot ml^{-1}$,
V is the volume of the extract solution in ml
M is the weight of the extract in grams.

Determination of Total flavanoid content
The total flavanoid content was determined using the Dowd method. 5 mL of 2 % aluminium trichloride ($AlCl_3$) in methanol was mixed with the same volume of the extract solution (0.4 mg/mL). Absorption readings at 415 nm using Perkin Elmer UV-VIS spectrophotometer were taken after 10 minutes against a blank sample consisting of a 5 mL extract solution with 5 mL methanol without $AlCl_3$. The total flavanoid content was determined using a standard curve with Quercetin (100-500µg/ml) as the standard. Total flavanoid content is expressed as (µg/ml or mg/g) ofQuercetin equivalents[26].

T = C X V/M

Where **T** is the total flavanoid content in $mg \cdot g^{-1}$ of the extract as QE,
C is the concentration of Quercetin established from the calibration curve in $mg \cdot ml^{-1}$,
V is the volume of the extract solution in ml
M is the weight of the extract in grams.

Evaluation of free radical scavenging activity and reducing capacity by in-vitro methods
Further methanolic extract was screened forfree radical scavenging activity by In-vitro methods are Total anti-oxidant capacity, DPPH radical scavenging activity method, ABTS radical scavenging activity method and total reductive capacity.

Total antioxidant capacity
The total antioxidant capacity was eluted by using the method described by Prieto et al (1999). Plant extracts were dissolved in methanol to obtain a concentration of 500 µg/ml. 3 ml of extract was placed in a test tube, 0.3 ml of reagent solution (0.6 M Sulphuric Acid, 28 mM Sodium Phosphate, 4 mM Ammonium molybdate) was then added and the resulting mixture was incubated at 95^0C for 90 minutes. After the mixture was cooled to room temperature, the absorbance of the each solution was measured by using UV-Visible spectrophotometer at 695 nm against blank. The experiment was performed in triplicate. A calibration curve was constructed, using ascorbic acid (100-500 µg/ml) as standard and total antioxidant activity of extract (µg/ml or mg/g) expressed as ascorbic acid equivalents[27].

DPPH radical[28] scavenging activity
The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant component. This method is based on the reduction of DPPH in methanol solution in presence of a hydrogen donating antioxidant due to the formation of the non radical form DPPH-H[28].
The free radical scavenging activity of the extract was evaluated by1,1-diphenyl-2-picryl-hydrazyl (DPPH) in methanol was prepared and 1ml of this solution is added to 3ml of the solution of methanolic extract at different concentrations (100-800µg/ml). The solution was incubated for 30 min and then absorbance was measured at 517nm using UV-VIS spectrophotometer (Elico-210) along with reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability to scavenging the DPPH radical was calculated by using the following formula.

Percentage of inhibition = ([(A control – A test/standard) /A control]) × 100

Where ***A control*** is the absorbance of the control and ***A test*** is the absorbance of the test sample/standard. Test was performed in triplicate, and the results were averaged. A percent inhibition Vs Concentration curve was plotted and the concentration of sample required for 50% inhibition and expressed as IC_{50} value.

ABTS radical[29] scavenging activity
In order to produce $ABTS^{\cdot+}$, prepare 2mM ABTS (0.0548gm in 50ml) in distilled water and treated with 70mM of potassium per sulphate (0.0189gm in 1ml). The mixture is allowed to stand at room temperature in the dark for 12-16 h until it turns dark blue. The solution is diluted with phosphate buffer (PH-7.4) before use until the absorbance at 734 nm

reaches ca. 0.73±0.02 and equilibrated at 30°C. The ABTS·+ radical formed shows absorption maxima at various wavelengths: 415 nm, 645 nm, 734 nm and 815 nm. The reaction with the antioxidant solution is monitored at 734 nm, this being the most stable absorption maximum over time. When the antioxidant compound is added in ratio of 1:3 to the reacting mixture (1ml of sample/standard and 3ml of $ABTS_{.+}$), the radical is reduced in proportion to the compound concentration causing a colour variation, which is spectrophotometrically determined. The variation is calculated with respect to the ascorbic acid (Vitamin-C) and expressed as ascorbic acid equivalents.Reaction mixture can without sample/standard acts as a positive control[29].

Percentage of inhibition = ([(A control – A test) /A control]) × 100

Where *A control* is the absorbance of the control sample and *A test* is the absorbance of the test sample. Test was performed in triplicate, and the results were averaged. A percent inhibition Vs Concentration curve was plotted and the concentration of sample required for 50% inhibition and expressed as IC_{50} value.

Reducing power[30] activity

2.5 ml of different concentration of extract/standard was mixed with phosphate buffer (2.5 ml,0.2M P^H 6.6) and potassium ferric cyanide (2.5ml, 1%). This was incubated at 50°C for 20min.After the incubation, 2.5 ml of 10%trichloro acetic acid was added. 2.5ml of the reaction mixture was mixed with distilled water (2.5ml) and ferric chloride (0.5ml 0.1%).The solution absorbance was measured 700 nm. The experiment was performed in triplicate. Vitamin-c was used as positive control. Increase in absorbance of the reaction mixture indicated the increased reducing power of the sample[30].

STATISTICAL ANALYSIS

The results were expressed as means ± SD. linear regression analysis was used to calculate IC_{50} values. Correlation analysis of total antioxidant capacity versus TPC (Total phenolic content) and TFC (total flavanoid content) were carried out using the correlation programmed in Microsoft EXCEL.

RESULTS AND DISCUSSION

Phytochemical screening

In the past few decades, secondary metabolite production from plant tissue culture has been identified as a tremendous resource for new drug development and clinical research in the fields of pharmacology and medicine. The bark extract of *Canthiumparviflorum*contains flavonoids, phenols, terpenoids and saponins in methanolic extract **(Table-1)**. The phlobatannins, alkaloids, steroids and tannins were not detected but anthraquinones cannot detect in both extracts which was prepared from maceration process and detected in methanolic extract which was prepared by soxhlation process only. Phytochemical analyses revealed the presence of secondary metabolites like alkaloids, flavonoids, tannins, steroids, saponins, terpenoids, and cardiac glycosides in *Canthium* leaf extractsSignificant antioxidant and diuretic activity was exhibited by extracts of leaves. For the first time, we report the phytochemical analysis of *CanthiumParviflorum* of bark extract **(Table-1)**. Based on results we concluded that the bark extract will be shown antioxidant activity due to presence of flavonoids and phenols present.

Total phenolic content

Methanolic extracts from the bark was standardized for their contents of phenolic compounds. The calibration curve showed linearity for Gallic acid in the range of (100-500µg/ml) with a correlation coefficient (R_2) of 0.972 (**Figure:1**). It Illustrate that total phenol content of the selected bark extract of Canthiumparviflorum increased with increased concentration. Total phenolic content of Canthiumparviflorum extract found to be at (222.41±0.03µg/ml or 0.222mg/g) calculated as Gallic acid equivalent of phenols was detected.

Total flavanoid content

Flavonoids comprise the most widespread and diverse group of poly phenolic plant secondary metabolites. These compounds play an important role in biological and chemical activities including free radical scavenging properties. Flavonoids are potent antioxidants in-vitro, and therefore one of the main interests in the compounds has involved in the protection against cardiovascular disease. Anti-oxidation is however, one of the many mechanisms through which flavonoids could exert their actions. Methanolic extracts from the bark was standardized for their contents of flavonoids compounds. The calibration curve showed linearity for Quercetin in the range of (100-500µg/ml**)** with a correlation coefficient (R_2) of 0.997 **(Figure:2)**.

It illustrate total flavonoids content of the selected plant increased with increased concentration, The extract of Canthium shows maximum total flavonoids content and compared it was to be (392.98±0.02µg/ml or

0.393mg/g) calculated as Quercetin equivalent of flavonoids was detected.

Total antioxidant capacity

Methanolic extract from the bark was standardized for their content of antioxidant containing compounds. The calibration curve showed linearity for ascorbic acid as a standard in the range of (100-500µg/ml) with a correlation coefficient (R_2) of 0.992 (**Figure:3**). It illustrate that total antioxidant capacity of the selected extract increased with increased concentration. Total antioxidant content of Canthiumparviflorum extract found to be at (366.02±0.03µg/ml or 0.367mg/g) calculated as ascorbic acid equivalent of antioxidant content was detected.

The phosphor molybdenum methodwas based on antioxidant compound and the formation of a green Mo (V) complex with a maximal absorption at 695nm.

DPPH radical scavenging activity

The extract was screened for free radical scavenging activity by using DPPH as a free radical. It is widely used relatively quick and precise method for the evaluation of free radical scavenging activity. DPPH is a stable free radical and accepts an electron or hydrogen to become unionized form. Plant extract interact with DPPH both transfer electron or hydrogen atom to DPPH and thus neutralizing its free radical and converts it to reduced form. This reducing capacity of DPPH radical is determine by the decrease in absorbance of DPPH radical caused by antioxidants because of the reaction between antioxidant molecules and radical progress which results in scavenging of the radical by hydrogen donation. It is visually noticeable as a change in colour from purple to yellow. Methanolic bark extract exhibited promising scavenging activity comparing with standard ascorbic acid in dose dependent manner (**Table:2**).

In the tested conditions (**Figure: 4**) *Canthium Parviflorum* showed a 50% inhibitory concentration of IC_{50} = 508 µg/mL (50%) but the standard drug as ascorbic acidshowed a 50% inhibitory concentration of IC_{50}=6.2µg/mL (50%) only.

ABTS radical scavenging activity

Antioxidant property of Canthiumparviflorum bark extract can be checked by in-vitro method. The ABTS•+ assay has several advantages: it is simple, colorimetric, does not require sophisticated analytical equipment and provides a good estimate of the antioxidant activity of pure compounds and complex matrices. The rate of the decrease in absorbance at 734 nm depends on the type and amount of antioxidants and the results are thus expressed as antioxidant standard equivalents. Thus, initially we used the ABTS•+ assay to estimate the total antioxidant activity of the extracts. In Both (**Table:3)** the suppression of the absorbance of ABTS•+ in a concentration-dependent manner is typically shown. In the tested conditions (**Figure:5**) *Canthium Parviflorum* showed a 50% inhibitory concentration of IC_{50} = 108µg/mL (50%) but the standard drug as ascorbic acid showed a 50% inhibitory concentration of IC_{50}=15µg/mL (49.9%) only.

Reducing power activity

For the capacity of the reductive capability it has been investigated from Fe^{3+} to Fe^{2+} transformation in the presence of plant extract sample using the method described by Oyaizu. It **(Figure:6)** shows reducing power of methanolic bark extract and standard compound ascorbic acid **(Figure:7)**exhibited the following way:

The reducing properties are commonly associated with the presence of reductones, which have been shown to apply antioxidant action by breaking the free radical chain or by donating a hydrogen atoms[30].

CONCLUSION

In conclusion, the results of the present study suggest that tested plant bark material having antioxidant activity/free radical scavenging activity. More detailed of in vivo assay are essential to characterizes them as biological antioxidant which is beyond the scope of the study. It should also be kept in mind that antioxidant activity measured in in-vitro method may or may not reflect in vivo effects of antioxidants. Many other factors such as absorption and metabolism are also important. The finding of the study support that some medicinal plants are promising source of antioxidant and may be efficient as preventive agents in some major diseases like cardiovascular diseases, cancer etc… the providing data can just enrich the existing comprehensive data of antioxidant activity of plant material. Finally this observation suggested that the selected methanolic bark extract of Canthiumparviflorum possess free radical scavenging activity, reducing power activity and a suitable formulation of the isolated compound can be designed.

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pharmacy for their help in conducting the research work.

Table 1:

S.NO	PHYTO- CHEMICAL CONSTIUENTS SCREENING	Methanolic Extract		Chloroform Extract	
		Maceration	Soxhlation	Maceration	Soxhlation
1.	Alkaloids	-ve	-ve	-ve	-ve
2.	Flavonoids	+ve	+ve	+ve	+ve
3.	Saponins	+ve	+ve	+ve	+ve
4.	Terpenoids	+ve	+ve	+ve	+ve
5.	Tannins	-ve	-ve	-ve	-ve
6.	Anthraquinones	-ve	+ve	-ve	-ve
7.	Quinones	+ve	+ve	+ve	+ve
8.	Gums	-ve	-ve	-ve	-ve
9.	Oils	+ve	+ve	+ve	+ve
10.	Phenols	+ve	+ve	+ve	+ve
11.	Phlobatannins	-ve	-ve	-ve	-ve
12	Carbohydrates	+ve	+ve	+ve	+ve

Table 2:

S.No	Concentration (µg/ml) of Methanolic Plant Extract	Percentage of inhibition (%)
1.	100	06.86
2.	200	23.72
3.	300	29.25
4.	400	36.08
5.	500	59.84
6.	600	61.87
7.	700	67.84
8.	800	72.74

Table 3:

S.No	Concentration (µg/ml) of Methanolic Plant Extract	Percentage of Inhibition (%)
1.	20	10.41
2.	40	17.91
3.	60	26.98
4.	80	34.93
5.	100	46.71
6.	120	70.27
7.	140	74.52
8.	160	83.83
9.	180	86.84
10.	200	94.52

Table 4:

S.No	Concentration of ascorbic acid (µg/ml)	Absorbance
1	100	0.183
2	200	0.395
3	300	0.522
4	400	0.621
5	500	0.884

Table 5:

S.No	Concentration of plant extract(µg/ml)	Absorbance of plant extract
1	20	0.337
2	40	0.456
3	60	0.574
4	80	0.796
5	100	0.865

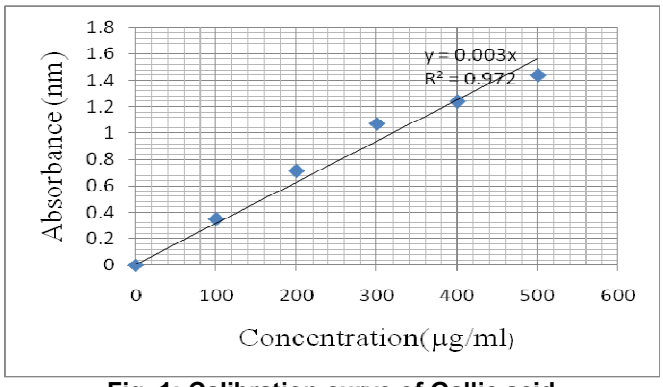

Fig. 1: Calibration curve of Gallic acid

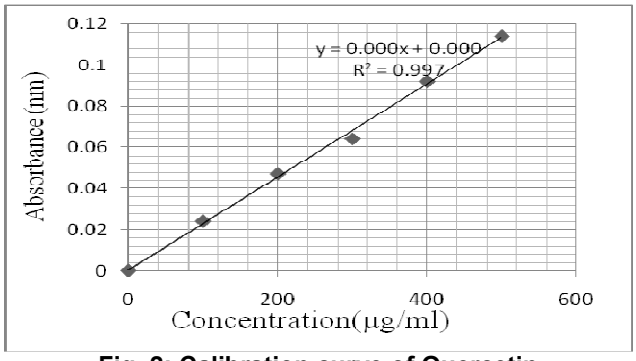

Fig. 2: Calibration curve of Quercetin

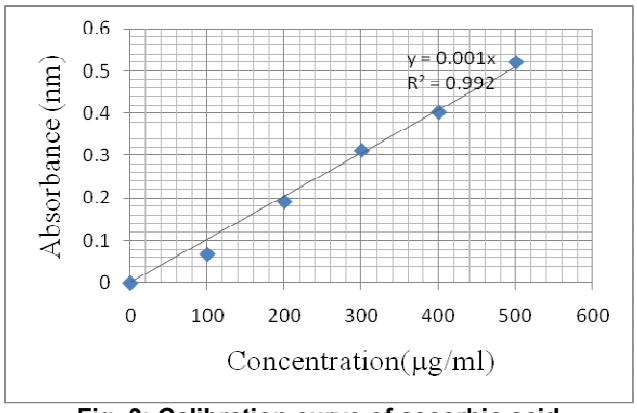

Fig. 3: Calibration curve of ascorbic acid

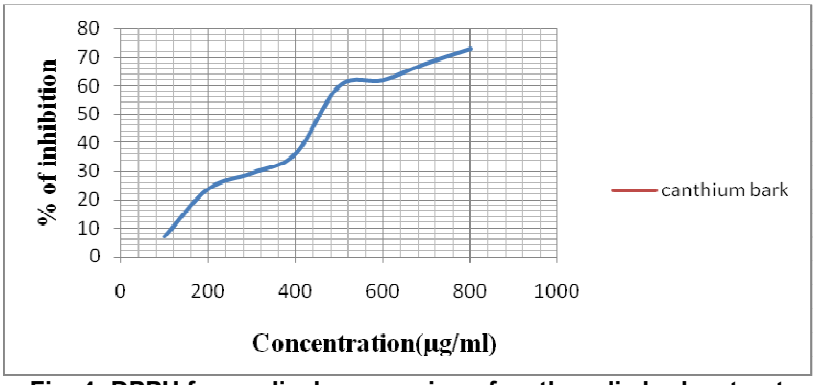

Fig. 4: DPPH free radical scavenging of methanolic bark extract

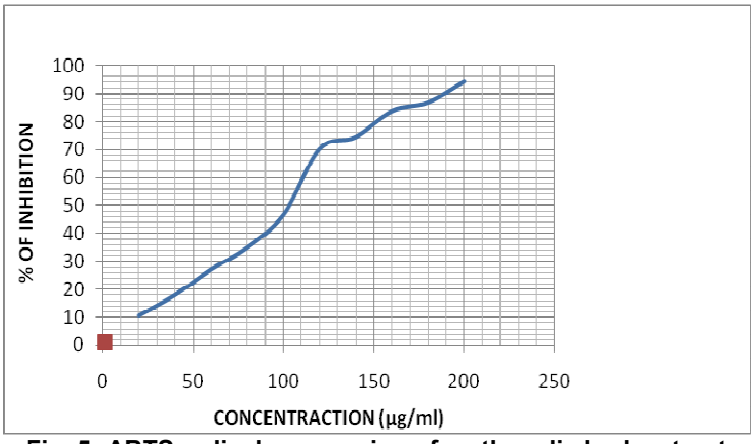

Fig. 5: ABTS radical scavenging of methanolic bark extract

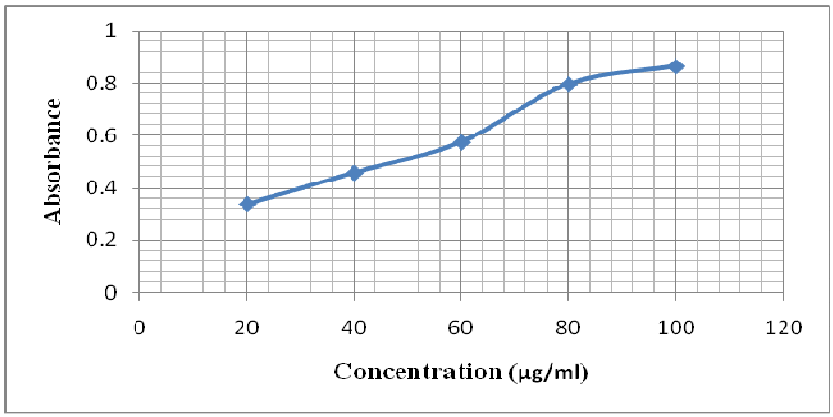

Fig. 6: Reducing power of methanolic bark extract

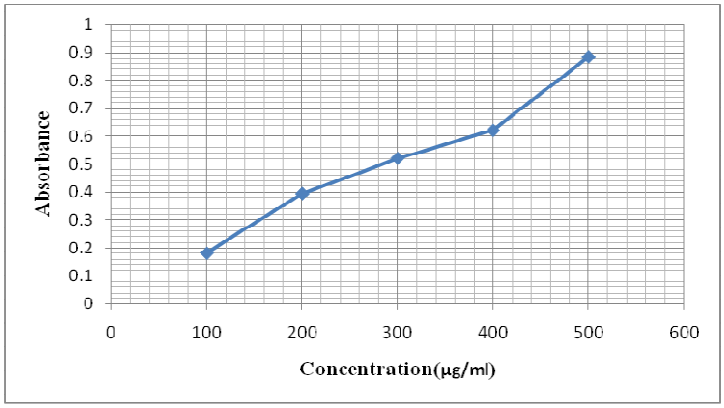

Fig. 7: Reducing power of ascorbic acid as a standard

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