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IN VITRO FREE RADICAL SCAVENGING ACTIVITY OF AQUEOUS METHANOLIC EXTRACTOF OROXYLUM INDICUM STEM BARKLINN.

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ABSTRACT

Aim: To carry out the free radical scavenging of oroxylum indicum stem bark**. Methods:** Aqueous methanolic (9:1) Extract of Oroxylum indicum stem bark powder was investigated for free radical scavenging activity by using DPPH and Total Antioxidant capacity, Total flavonoids content and Total phenolic content. **Results:** Aqueous methanolic extract of Oroxylum indicum stem bark possess free radical scavenging activity and the results are DPPH possess IC_{50} at (269.83 ± 0.02µg/ml); Total antioxidant capacity (149.71± 0.03 µg/ml expressed as ascorbic acid equivalents); Total phenolic content (76.576 ± 0.03 µg/ml expressed as Gallic acid equivalents); Total flavonoids content (475.43 ± 0.02 µg/ml expressed as Quercetin equivalents). **Conclusion:** Results of the present study suggest that the tested plant material having free radical scavenging activity.

INTRODUCTION

Medicinal plants play a major role in the rural areas of third world countries as the herbal medicine are used extensively by indigenous people of Asian African and southern American countries. The continued search among the plant secondary metabolites for natural antioxidant has gained importance in recent years because of increasing awareness of herbal remedies as potential source of phenolic antioxidant[1].Biologically active compounds such as phenols and flavonoids found in plants are reported to possess more biological activity including antioxidant activity.The main application of antioxidant wide spread in order to prevent polymers oxidative degradation antioxidation of the synthetic and natural pigment discoloration in most of the natural anti oxidants such as traditional nutrients, polyphenols and flavonoids are obtained from plants.Novel natural antioxidants from some plants have been extensively studied in the past few years for their antioxidant and radical scavenging properties[2].During eighties, the world health organization noticed that, in both developed and poor countries, the population has developed interest in phytotherapy because of the constant failure of modern medicine. 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[3]. Besides this, the fact that a drug has a natural origin did not assure its innocuity. The use of medicinal plant on its

entire form, macerate, infusion, and decoction or on the form of cataplasm, unguent or expression can induce some side effects or allergic reactions of short, middle and long term type. One such plant commonly is ***oroxylum indicum***.

OBJECTIVES

To find out phytochemical screening,IC_{50} value of the antioxidant activity and determination of the total anti oxidant content, total phenolic content and total flavonoids content of aqueous methanolic extract of bark of oroxylum indicum.

MATERIALS AND METHODS

Plant collection and Authentification:

The bark of oroxylum indicum (bignoniaceae) is a short shrubby and woody plant[5] was collected from **Vanantharam herbal PHC centre**, Addathegala mandal, E.G.dist and authentified by T.SRINIVAS, lecturer, Government junior College, Gokavaram, E.G.dist and Andhrapradesh, India. They are chopped into small pieces and they are allowed to air dry at room temperature for 3-4 days and grained into fine powder and further extracted material was stored in an air tight container.

Preparation of the plant extract by using various extraction[4] methods:

a) Maceration Method: In this process, the whole or coarsely powdered crude drug is placed in a stoppered container with the solvent and allowed to stand at room temperature for a period of at least three days with frequent agitation until the soluble matter has dissolved. The mixture then is strained, the marc (the damp solid material) is pressed, and the combined liquids are clarified by filtration or decantation after standing.

b)Soxhlation Method: In this method, the finely ground crude drug is placed "thimble" made of strong filter paper, which is placed in chamber of the Soxhlet apparatus[6]. The extracting solvent in flask is heated, and its vapors condense in condenser. The condensed extractant drips into the thimble containing the crude drug, and extracts it by contact. When the level of liquid in chamber rises to the top of siphon tube, the liquid contents of chamber siphon into flask. This process is continuous and is carried out until a drop of solvent from the siphon tube does not leave residue when evaporated. It is much more economical and viable when converted into a continuous extraction process on medium or large scale.

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 and soxhlation process (Capacity 250ml) with aqueous methanol (9:1). 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^oC in airtight containers until need for further studies.

Qualitative phytochemical Screening:

The bark of aq. methanolic extract was qualitatively 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].

4. Qualitative analysis

4.1. Test for Alkaloids

The extracts were tested for presence of alkaloids by treating with Dragondroff's, Wagner's, Mayer's and Hager's reagents and observed for the color change and precipitation.

4.2. Test fort Flavonoids

4.2.1. Shinoda test: The extracts are treated with 95% ethanol, few drops of HCl, 0.5 gm of magnesium turnings and observed for the pink/magenta color.

4.3Test for Saponins

4.3.1. Foam test: The extracts are mixed with $NaHCO_3$ and distilled water, upon vigorous shaking and observed for the formation of froth.

4.5. Test for Glycoside

4.5.1. Killer – Killiani Test: The extracts are treated with glacial acetic acid, few drops of $FeCl_3$, H_2SO_4 and observed for

color at the junction of two layers for the presence of glycoside.

4.5. Test for Tannin

4.5.1. Gelatin solution test: The extracts are treated with 1% w/v gelatin solution contains 10% NaCl and observed for the white color.

4.6. Test for Anthraquinones

4.6. 1. Borntranger's test: The extracts are boiled with H_2SO_4, filtered and then cooled. The filtrate was treated with $CHCl_3$ and dil. ammonia, the ammonical layer colour was observed.

4.6.2. Modified Anthraquinones test: The extracts are treated with $FeCl_3$, HCl, then boiled and cooled. The cooled solutions treated with benzene and dilute ammonia followed byammonical layer and observed for pinkish red color.

4.7. Test for amino acids and Proteins

The presence of amino acids and proteins wereobserved using reagents ninhydrine and millon's reagents. Xanthoprotein reaction is used for further confirmation of any specific amino acids and proteins.

4.13. Test for Phenols

4.13.1. Ferric chloride Test: The extracts are treated with $Fecl_3$ and observed for blue/green color.

Quantitative analysis of aqueous 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 [6]

$$T = C \ \mathbf{X} \ V/M$$

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 and **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 % aluminiumtrichloride ($AlCl_3$) 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) of Quercetin equivalents [7].

$$T = C \ \mathbf{X} \ V/M$$

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 and **M** is the weight of the extract in grams.

Biological evaluation:

Further aq. methanolic extract was screened for biological activity such as invitro-antioxidant activity by using Total anti-oxidant capacity and DPPH radical scavenging activity

In-vitro Anti-oxidant activities:

Total antioxidant capacity:

The total antioxidant capacity was eluted by using the method described by Prieto et al (1999). Plant extract was 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[8,9].

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 aq. 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.

RESULTS AND DISCUSSION:

The bark extract of *Oroxylum indicum* contains flavonoids, phenols, terpenoids and saponins in aqueous methanolic extract and gums are not aqueous methanolic extract **(Table-1)**. The alkaloids, anthraquinones and tannins were detected in both extracts which was prepared from maceration process and soxhlation process only.For the first time, we report the phytochemical analysis of *Oroxylum indicum* of stem 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.

DPPH: The extract was screened for free radical. It is widely used relatively quick and precise method for DPPH as by using antioxidant capacity by invitro method. Aqueous methanolic bark extract exhibited promising scavenging activity comparing with standard ascorbic acid in dose dependent manner (Table:-2). In the tested conditions (Figure:-1) Oroxylum indicum showed a 50% inhibitory concentration of IC_{50}=269.83 µg/ml [50%] but the standard drug as ascorbic acid showed a 50% inhibitory concentration of IC_{50} =15 µg/ml [49.5= 50%] only.

TABLE-1: PHYTO CHEMICAL SCREENING OF *OROXYLUM INDICUM* BARK

S.No.	PHYTO- CHEMICAL CONSTIUENTS SCREENING	AQUEOUS METHANOLIC EXTRACT	
		Maceration	Soxhlation
1.	Alkaloids	+ve	+ve
2.	Flavonoids	+ve	+ve
3.	Saponins	+ve	+ve

4.	**Terpenoids**	+ve	+ve
5.	**Tannins**	+ve	+ve
6.	**Anthraquinones**	+ve	+ve
7.	**Proteins**	+ve	+ve
8.	**Gums**	-ve	-ve
9.	**Volatile oils**	+ve	+ve
10.	**Fixed oils**	-ve	-ve
11.	**Steroids**	+ve	+ve
12	**Amino acids**	+ve	+ve
13	**Phenols**	+ve	+ve

Note: +ve is present & -ve is absent

Table-2: FREE RADICAL SCAVENGING ACTIVITY BY USING ***DPPH***' METHOD (Control = 3.002).

S.No	**CONCENTRATION (µg/ml) of Aqueous methanolic Plant Extract**	**SAMPLE ABSORBANCE**	**PERCENTAGE OF INHIBITION (%)**
1.	100	2.627	12.49
2.	150	2.115	29.54
3.	200	2.028	32.44
4.	250	1.841	38.70
5.	300	1.333	55.59
6.	350	1.192	60.29
7.	400	0.993	66.92
8.	450	0.694	76.88

Table-3:FREE RADICAL SCAVENGING ACTIVITY BY USING***DPPH***' METHOD (Control = 3.002).

	STANDARD *(Ascorbic acid)*		**PERCENTAGE OF INHIBITION (%)**
	CONCENTRATION (µg/ml)	**ABSORBANCE**	
1.	5	2.3416	22
2.	10	2.0774	30.8
3.	15	1.5161	49.5
4.	20	1.2489	58.4
5.	25	0.9277	69.1
6.	30	0.5584	81.4

TABLE:4-DETERMINATION OF TOTAL PHENOLIC CONTENT IN PLANT EXTRACT

S.NO	**concentration (µg/ml)**	**gallic acid absorbance**
1.	100	0.752±0.002
2.	200	0.716±0.003
3.	300	1.073±0.005
4.	400	1.243±0.001
5.	500	1.443±0.004

TABLE:5-DETERMINATION OF TOTAL ANTIOXIDANTCONTENT IN PLANT EXTRACT:

S. No	CONCENTRATION(µg/ ml)	ASCORBIC ACID ABSORBANCE
1.	100	0.068±0.002
2.	200	0.192±0.001
3.	300	0.313±0.005
4.	400	0.403±0.004
5.	500	0.521±0.003

TABLE:6-DETERMINATION OF TOTAL FLAVANIODSCONTENT IN PLANT EXTRACT

S.NO	CONCENTRATION (µg/ml)	QUERCETIN ABSORBANCE
1.	100	0.024±0.006
2.	200	0.047±0.002
3.	300	0.064±0.003
4.	400	0.092±0.009
5.	500	0.114±0.011

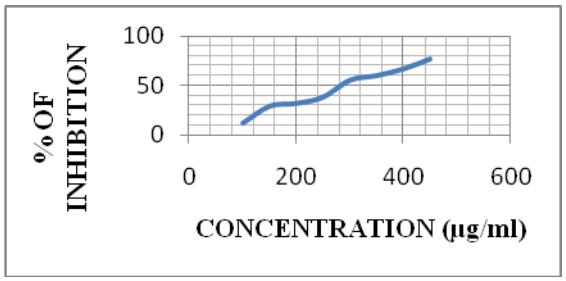

FIGURE-1: Diagrammatic representation of various concentrations of ***plant extract*** axis and its percentage of inhibition on y-axis, for DPPH free radical scavenging activity.

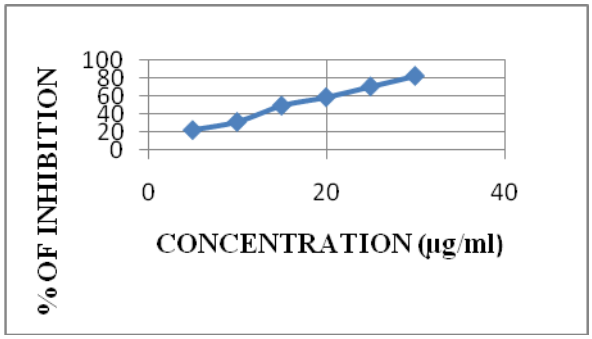

FIGURE- 2: Diagrammatic representation of ***Standard (Ascorbic acid)*** on x-axis and its percentage of inhibition on y-axis for **DPPH** free radical scavenging activity.

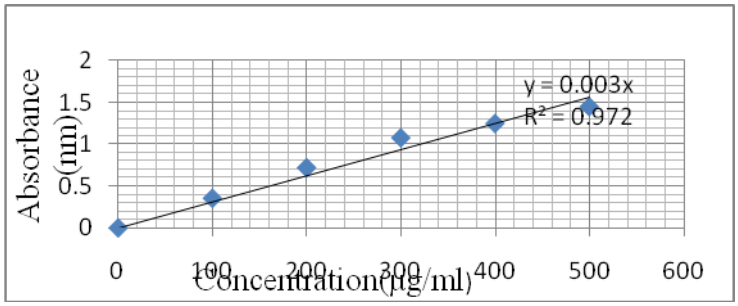

FIGURE-3: Diagrammatic representation explains that various concentrations of ***Gallic Acid*** on X-axis and its absorbance on Y-axis, determining total phenolic content in plant extract.

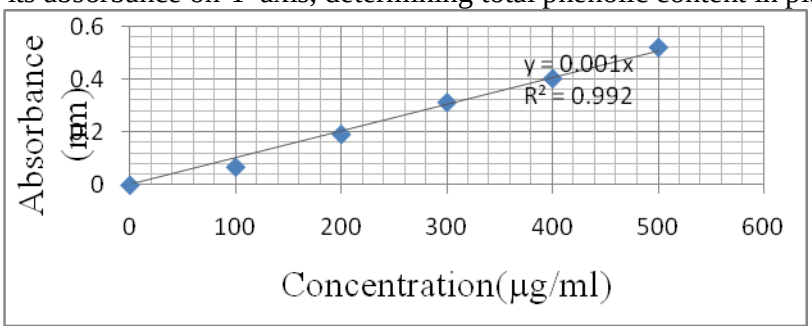

FIGURE-4: Diagrammatic representation explains that various concentrations of ***Ascorbic acid*** on X-axis and its absorbance on Y-axis, determining Total antioxidant content in plant extract.

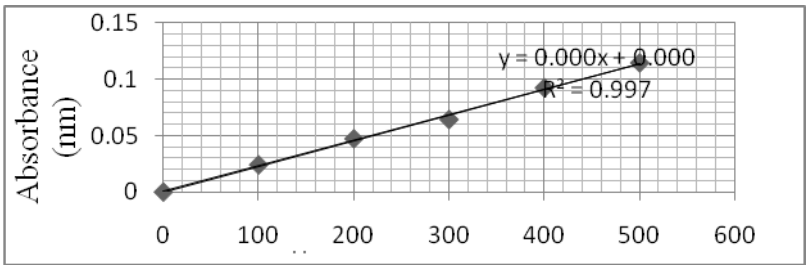

FIGURE-5: Diagrammatic representation explains that various concentrations of ***Quercetin*** on X-axis and its absorbance on Y-axis, determining Total flavonoids content in plant extract.

Aqueous methanolic bark extract exhibited promising scavenging activity comparing with standard ascorbic acid in dose dependent manner (Table:-2). In the tested conditions (Figure:-1) Oroxylum indicum showed a 50% inhibitory concentration of IC_{50}=269.83 µg/ml [50%] but the standard drug as ascorbic acid showed a 50% inhibitory concentration of IC_{50} =15 µg/ml [49.5= 50%] only.Aqueous Methanolic extracts from the bark was standardized for their contents of phenolic compounds, antioxidant content compounds,flavonoids content compounds. The calibration curve showed linearity for Gallic acid, Ascorbic acid, Quercetin in the range of 100-500µg/ml (**Table-4,5,6**) with a correlation coefficients (R_2) are 0.972,0.992,0.997 (**Figure:-3,4,5**) respectively. Total phenolic content of *Oroxylum indicum* extract found to be at (76.576±0.03µg/ml or 0.076 mg/kg) calculated as Gallic acid equivalent of phenols was detected. Total antioxidant content of *Oroxylum indicum* extract found to be at (149.71±0.03µg/ml or 0.149 mg/g) calculated as ascorbic acid equivalent of antioxidant content was detected. Total flavonoids content and compared it was to be (475.43±0.02µg/ml or 0.475 mg/g)

calculated as Quercetin equivalent of flavonoids was detected.

CONCLUSION:

In conclusion, the results of the present study suggest that tested plant 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 aqueous methanolic bark extract of oroxylum indicum possess free radical scavenging activity and a suitable formulation of the isolated compound can be designed.

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