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Phytopharmacognostic Investigation and Evaluation of Antioxidant Properties of Leaves and Bark of *Heterophragma Adenophyllum*

Vilas Surana^{1*}, Sri Hari Mishra², Bhavik Satani¹

1. Department of Pharmacognosy, Maliba Pharmacy College, Uka Tarsadia University, Bardoli (Gujarat) India 394350

2. UGC Emeritus Professor, M S University, Baroda (Gujarat) India 390002

ABSTRACT

Heterophragma adenophyllum is a traditional medicinal tree occurring in both tropical and sub tropical regions of the world and is important for the prevention and treatment of various diseases. The physicochemical, phytochemical and antioxidant activity of total methanolic extract of leaves and bark of *Heterophragma adenophyllum* were investigated. The total ash, water soluble, acid insoluble, alcohol soluble extractive, water soluble extractive, moisture content and fluorescence property of *Heterophragma adenophyllum* leaf and bark powder were evaluated. Phytochemical screenings of oven dried extracts were performed to check the presence of various phytoconstituents. The leaf methanolic oven dried extract showed the presence of carbohydrates, phenolic, flavonoids, anthraquinones, alkaloids and amino acids were as bark methanolic oven dried extract shows presence of phenolic, carbohydrates and flavonoids. The capacity of antioxidant property of both the methanolic extract was evaluated by using DPPH and Superoxide anion radical scavenging assay. Total phenolic and total flavonoid contents were estimated to quantify the presence of phenolic content in extracts. The leaf total methanolic oven dried extract showed higher antioxidant activity compared to bark methanolic oven dried extract and thus, the outcome of the present study suggest that the therapeutic activities of leaf methanolic extract of *Heterophragma adenophyllum* can be attributed to its antioxidant property.

Keywords: *Heterophragma adenophyllum*, Physicochemical, Phytochemical, Antioxidant, successive extract.

*Corresponding Author Email: suranavilas@yahoo.com

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INTRODUCTION

Heterophragma adenophyllum, a genus of bignoneace, containing single species from India. It is a large tree with opposite or ternate impairs pinnate leaves and white flower in dense terminal downy panicle. The calyx is campalunated and three lobed; the corolla equally five parted, with the margin of divisions waved; there are for fertile stamens; very is surrounded by a purple disk and surmounted by simple style and two cleft stigma: the capsule is long and pointed; and the seeds have broad wing.¹⁻⁴

In Thai traditional medicine, the leaves are used for external treatment of skin diseases. Fruits of *heterophragma adenophyllum* were cooked such and Flowers were consumed as fresh vegetables. The tree is extensively used in traditional medicine. As an ingredient in message oils, it is supposed to ease muscular tension sparingly cultivated as an ornamental tree. The wood is elastic and is used for making bows in Burma, and also for furniture (katsagon). Folk medicinal uses of *Heterophragma adenophyllum* roots are used in Piles, constipation and also prescribed as drink in viper bite.⁵⁻⁷

α -Lapachone was previously isolated from the wood of the Bignoniaceae tree *Heterophragma adenophyllum* A new symmetric naphthoquinone dimer, dilapachone, and a novel asymmetric naphthoquinone dimer, adenophyllone were isolated from the heartwood of *Heterophragma adenophyllum*. The aim of present study was to evaluate the physiochemical, phytochemical and antioxidant activity of leaves and bark of *Heterophragma adenophyllum*.⁸⁻¹⁰

MATERIALS AND METHOD

Collection of *Heterophragma adenophyllum*

Leaves and bark of *heterophragma adenophyllum* was obtained and collected from Baroda, Gujarat during April-May were voucher specimen authenticated and deposited in herbal drug technology laboratory pharmacy department, The M S University of Baroda, Gujarat, India.

Preparation of extract

Leaves and bark was collected and washed with water to remove soil and straw from base. The leaves and bark was shade dried and coarsely powered for further study. The powdered leaves and bark of *heterophragma adenophyllum* was extracted with methanol by using hot percolation method.¹¹ Both the extracts were oven dried at low temperature and stored in cool and dried place.

Physiochemical Parameters¹²⁻¹³

The leaves and bark powder were subjected to evaluate total ash, water soluble ash, acid insoluble ash, water soluble extractive value, alcohol soluble extractive value, moisture content and fluorescence analysis.

Total Ash value:

About 2gm of both the leaves and bark powdered drug was weighed accurately into a tarred crucible and spread in an even layer. Incinerated at 560°C in a muffle furnace until free from carbon. The crucible was cooled in a desiccator and weighed. Total ash content was calculated in mg per g of air-dried material.

Water soluble Ash:

Ash obtained from the total ash was boiled with 25 ml of distilled water and insoluble matter was filtered through an ash less filter paper. The filter paper was transferred into a tarred silica crucible and was incinerated at 560°C in a muffle furnace until free from carbon, cooled and weighed. Percentage of water-soluble ash was calculated with reference to air-dried substance.

Acid insoluble ash:

Ash obtained from the total ash was boiled with 25ml of 2N HCl and the insoluble matter was filtered through an ash less filter paper. The filter paper was transferred into a tarred silica crucible. It was washed with hot water, ignited in tarred crucible, cooled and the residue obtained was weighed. Percentage of acid insoluble ash was calculated with reference to air-dried substance.

Alcohol soluble extractive value:

About 5gms of air dried coarse powdered drug of both leaves and bark was weighed and macerated alcohol in a closed flask for 24 hours, shaking frequently during the first 6 hrs & these allowed standing for 18 hrs. Thereafter, it was filtered taking precautions against loss of the solvent. 25 ml of the filtrate was evaporated to dryness, dried at 105°C & weighed. The % of the alcohol soluble extractive values was calculated with reference to the air-dried drug.

Water soluble extractive value:

Coarsely powdered drug of leaves and bark was macerated with 100 ml of chloroform water in a closed flask for 24 hrs shaking frequently during the first 6 hrs and then allowed to stand for 18 hrs. Thereafter, it was filtered taking precautions against loss of the solvent. 25 ml of the filtrate was evaporated to dryness, dried at 105°C & weighed. The percentage of the water soluble extractive value was calculated with reference to the air-dried drug.

Moisture content:

Leaves and bark Weigh separately 2 gm of each powdered drug and dry it in the oven at 105°C for about 30 min, cooled and loss in weight is usually recorded as moisture.

Fluorescence analysis:

Fluorescence study is an essential parameter for first line standardization of crude drugs. The powder drug of leaves and bark was treated separately with different reagents and exposed to visible and ultraviolet light to study their fluorescence behaviour.

PHYTOCHEMICAL AND CHROMATOGRAPHICAL ENVESTIGATION

Both the extracts were screened for the presence or absence of secondary metabolites such as alkaloids, steroidal compounds, glycosides, flavonoids, tannins, saponins, carbohydrates, amino acid and protein using standard procedures.¹⁴⁻¹⁵

Chromatographic studies included generation of HPTLC fingerprinting of extracts of powdered leaves and bark using Camag system equipped with Linomat V sample applicator, Camag TLC scanner 3 and CATS 4 software for interpretation of data. An aluminium plate (10X10 cm) precoated with silica gel 60 F254 (E Merck) was used as an adsorbent. The plates were developed using toluene: ethyl acetate (9:1) as mobile phase in previously saturated twin trough chamber (CAMAG). Before derivatization plate was scanned at the wavelength 254 and 360 nm. The plate was derivatized by spraying with anisaldehyde-sulphuric acid (AS) reagent and after heating the plate at 110 °C for 10 min scanned at the wavelength 540 nm.

Test for carbohydrate**Molisch test:**

A small quantity of the extracts were dissolved separately in 4 ml of distilled water and filtered. The filtrate was then subjected to Molisch's reagent and formation of brick red colour confirmed the presence of reducing sugar.

Fehling's test:

Equal volume of Fehling A (copper sulphate solution) and Fehling B (potassium tartrate and sodium hydroxide in distilled water) reagents were mixed with few drops of extract and boiled, a brick red precipitate of cuprous oxide forms, reducing sugar are present.

Test for glycosides**Borntrager's test:**

200 mg crude extract was mixed with 2 ml of dilute sulphuric acid and 2 ml of 5 % aqueous ferric chloride solution, boiled for 5 minutes which lead to oxidation to anthroquinones, indicating the presence of glycosides.

Kedde's test:

Crude extract was mixed with chloroform, one drop of 90% alcohol and 2 drops of 2% 3, 5 dinitrobenzoic acid in 90% alcohol and made alkaline with 20% sodium hydroxide. A purple color so produced, suggested the presence of glycosides.

Test for Alkaloids

Dragondroff's test:

Crude extract were mixed with Dragondroff's reagent (potassium bismuth iodide solution). Reddish brown precipitate was formed which suggested the presence of alkaloids.

Mayer's test:

Crude extracts were mixed with Mayer's reagent (potassium mercuric iodide solution). Cream colour precipitate was formed, indicating the presence of alkaloids.

Test for Steroids

Libermann-Buchard test:

Crude extracts were mixed with few drops of acetic anhydride, boiled and cooled, conc. H₂SO₄ was then added from the sides of the test tube. A brown ring at the junction of two layers was formed. The upper layer turned green which showed the presence of steroids.

Salkowski test:

Crude extracts were mixed with chloroform and a few drops of conc. H₂SO₄, shaken well and allowed to stand for some time. Red color appeared at the lower layer indicated the presence of steroids.

Test for Flavonoids

Alkaline reagent test:

Crude extracts were mixed with few drops of sodium hydroxide solution. An intense yellow colour was formed and turned to colorless on addition of few drops of diluted acid, which marked the presence of flavonoids.

Lead acetate test:

1ml of 10% lead acetate solution was added to 0.5 ml of extracts, yellow precipitate is formed, indicated the presence of flavonoid.

Test for Saponins

Froth test:

0.5g extracts were dissolved in 10ml of distilled water for about 30 seconds. The test tube was stoppered and shaken vigorously for about 30 seconds and allowed to stand and observed after 30 min. If a "honey comb" froth above the surface of liquid persists after 30 minutes the sample is suspected to contain saponin.

Test for Tannins

Ferric chloride test:

Crude extract was mixed with ferric chloride. Blue green colour appeared, suggested the presence of tannins.

Test for Amino acids and proteins

Millons test:

Crude extract was mixed with 2 ml of Millon's reagent (mercuric nitrate in nitric acid containing traces of nitrous acid), white precipitate appeared, which turned red upon gentle heating.

Ninhydrin test:

Crude extract when boiled with 0.2 % solution of ninhydrin (Indane 1, 2, 3, trione hydrate), violet color appeared which suggest the presence of amino acids and protein.

ANTIOXIDANT ACTIVITIES¹⁶⁻¹⁷

Total Phenolic content:

Total phenolic content were determined by Folin-Ciocalteu colorimetric method. 200 µl of each leaves and bark methanolic extract solution (100µg/ml) was mixed with 2000 µl of distilled water and 0.2ml of Folin-Ciocalteu reagent was added. After 2 hours at room temperature, 2ml of 15% sodium carbonate was mixed and shaken. Thereafter, the reaction mixture was allowed to stand for 10 minutes at 50°C and the absorbance at 760 nm was taken. Results were expressed as gallic acid equivalents per gram dry extract weight, with the use of the standard curve.

Total Flavonoid content:

Total flavonoids were determined by Aluminium chloride colorimetric method. Briefly, 1 ml of each leaves and bark methanolic extract solution (100µg/ml) was mixed with 4 ml of distilled water and 300 µl of 5% sodium nitrite. After 5 min, add 300 µl of 10% aluminium chloride, allowed it to stand for 6 min and then add 2 ml of 1 M NaOH. Absorbance was taken at 415 nm and the results were expressed as mg quercetin equivalent per gram dry extract weight with the use of standard curve.

DPPH Assay (1,1-diphenyl-2-picryl-hydrazyl):

1.2 ml of the both extracts was added to 0.1 ml of 1 M tris-HCl buffer (pH 7.9) and mixed with 1.2 ml of 5 mM DPPH in methanol. The reaction mixture was allowed to stand for 30 min in dark and the absorbance was measured at 517 nm. Radical scavenging assay is expressed as (%) = [(A0-AS)/A0×100] Where AO=Absorbance of control, AS= Absorbance of Sample. Gallic acid was taken as standard solution. DPPH radical scavenging activity was expressed as mg gallic acid equivalent per 1 gm of sample.

Superoxide anion scavenging assay:¹⁸⁻¹⁹

After certain modification the activity was performed from the literature given in Lacin Aksoy *et al*, 2013 and Malaya gupta *et al* 2004. The reaction mixture containing Phenazine methosulphate (0.1 mmol/L), Nicotinamide adenine dinucleotide reduced (1 mmol/L), Nitroblue tetrazolium (1 mmol/L) in phosphate buffer (0.1 mol/L, pH 7.4) with different concentrations of the extract were incubated at room temperature for 5 min and the colour was read at 560 nm against a blank. The scavenging effect was calculated using the following equation: Effect of scavenging (%) = [(1-AS/A0)×100] Where, AO=Absorbance of control, AS= Absorbance of Sample.

RESULTS AND DISCUSSION**Physicochemical and chromatographical investigation**

Percent weight loss on drying or moisture content was found to be 08.6±1.8 for leaves and 07.6±1.3 for bark of *Heterophragma adenophyllum*. During storage the less moisture content of drugs prevents from fungal, bacterial or yeast growth. The ash values, acid insoluble ash and water soluble ash were found to be 10.0±0.19, 02.03±0.26 and 07.10±0.22 for leaves and 12.0±0.18, 10.41±0.28 and 01.21±0.14 for bark respectively. Ash values are used to find out the authenticity, quality and purity of crude drug. Through acid insoluble ash impurities of silicates and other earthy materials are indicated. Water soluble ash value indicates the presence of inorganic elements present in drugs. Furthermore, the extractive values are important to estimate the chemical constituents present in the drug. The observational values are given in Table 1. Fluorescence studies were also performed and summarized in Table 2 and Table 3 shows behavior of leaf and bark powder with different chemical reagents.

Table 1: Physicochemical characterization of *Hetrtophragmaadenophyllum*.

Parameters	Leaves	Bark
Loss on drying	8.6±1.8	7.6±1.3
Ash Value (% w/w)	10.0±0.19	12.0±0.18
Acid insoluble ash (% w/w)	2.03±0.26	1.21±0.14
Water soluble ash (% w/w)	7.10 ± 0.22	10.41±0.28
Water soluble extractive value (% w/w)	9.50 ± 1.6	4.43±0.28
Alcohol soluble extractive value (% w/w)	15.82 ± 1.3	11.63±0.24

Values are expressed in mean± standard deviation

Table 2: Fluorescence Value of *Heterophragma adenophyllum* leaves and bark under visible and ultraviolet light

Powder and Reagent	Leaves		Bark	
	visible light	UV light (365 nm)	Visible light	UV light (365 nm)
Powder as such	Green	green	Brown	Brown
Powder + 1N NaOH in methanol	Green	green	Brown	Brown
Powder + 1N NaOH in water	Yellowish green	Bluish green	Greenish brown	Greenish brown
Powder + 1N HCl	Yellow Green	green	Brownish green	Brownish green
Powder + 50% HNO ₃	Yellowish brown	Blackish brown	Dark brown	Dark brown
Powder + 50% H ₂ SO ₄	Greenish brown	brown	Dark brown	Dark brown
Powder + methanolic NaOH + nitrocellulose in amyl acetate	Yellow Green	Blackish green	Grayish brown	Grayish brown
Powder + 1N NaOH + nitrocellulose in amyl acetate	Yellow Green	Bluish green	brown	brown
Powder + 1N HCl + nitrocellulose in amyl acetate	Yellowish green	green	brown	brown

Table 3: Behavior of leaf and bark powder with different chemical reagent

Chemical reagent	Leaves		Bark	
	Color/ppt	Constituents	Color/ppt	Constituents
Powder + conc. H ₂ SO ₄	Reddish brown	Steroids present	Reddish brown	Steroids present
Powder + aqueous FeCl ₂	No change	Tannin absent	Bluish black	Tannin present
Powder + Iodine	No change	Starch absent	Blue	Starch present
Powder + aqueous HgCl ₂	Brown	Alkaloid present	Brown	Alkaloid present
Powder + picric acid	Yellow	Alkaloid present	Yellow	Alkaloid present
Powder + magnesium+HCl	Pink	Flavonoid present	Pink	Flavonoid present
Powder + aqueous AgNO ₃	White precipitate	Protein present	No change	Protein absent
Powder + ammonia solution	Pink colour	Anthraquinone glycoside present	No change	Anthraquinone glycoside absent
Powder + aqueous KOH	Decolouration of KOH	Anthraquinone glycoside present	No change	Anthraquinone glycoside absent

Phytochemical Screening:

Table 4 indicates the phytochemical constituents of methanolic extracts of *Heterophragmaadenophyllum* leaves and bark. The oven dried extracts of leaves and bark showed the presence of various secondary metabolites like, Carbohydrates, Glycosides, Alkaloids, Flavonoids, tannins, amino acids, anthraquinones, steroids and terpenoids.

Table 4: Preliminary Phytochemical screening of dried methanolic extract of leaves and bark of *Heterophragma adenophyllum*.

Class	Tests	Leaves	Bark
Fats	Iodine absorption test	-	-
	Spot test	-	-
Carbohydrates	Molisch's test	+	+
	Fehling's test	+	+
	Benedict's test	+	+
Tannins	Ferric chloride test	-	+
	Lead acetate test	-	+
Alkaloid	Mayer's test	+	+
	Dragendorff's test	+	+
	Hager's test	+	+
Flavonoid	Sodium hydroxide test	+	-
	Mineral acid test	+	-
	Zinc- hydrochloric reduction test	+	-
Phytosterols	Salkowski test	+	+
	Liebermann-Burchard Test	+	+
Saponins	Foam test	-	-
Glycoside	Legal's test	+	-
	Fehling's test	+	-
Proteins	Biuret test	-	-
	Xanthoproteic test	-	-
Tri-terpenes	Hirschorn test	+	-
	Tschugajew's test	+	-

+: to be present

-: to be absent

Total phenolic content:

It was reasonable to investigate the total phenolic content of natural extracts because plant phenol content constitutes one of the major groups of compounds acting as free radical terminators or primary antioxidants. The total phenol content in methanolic leaves and bark oven dried extracts of *Heterophragma adenophyllum* were found to be 0.49 ± 0.62 and 0.11 ± 0.34 mg GAE/gm dry extracts respectively. These phenolic compounds possess redox properties, which allow them to act as antioxidants and their free radical scavenging ability is due to the presence of hydroxyl groups, thus determination of phenolic concentration in plant extracts could be used as rapid screening for their

antioxidant activity.²⁰ The extracts which have higher content of phenol also indicated that they have better antioxidant activities. So, antioxidative activity may be correlated with total phenolic contents of the extracts.

Total Flavonoid content:

Total flavonoid content of methanolic leaves and bark oven dried extracts of *heterophragma adenophyllum* were found to be 0.52 ± 0.24 and 0.34 ± 0.31 mg QE/gm dry extracts respectively. Flavonoids are one of the major and widespread groups of natural compounds and are one of the most important natural phenols.²¹ They also possess several biological and chemical activities and are having free radical scavenging properties too. Its antioxidant property depends primarily on their hydroxyl group position in the molecule and their ability as electron donor to a free radical. Flavonoids are plant secondary metabolites and their antioxidant activity depends on the presence of free OH groups, especially 3-OH.

DPPH Assay:

The antioxidant activities of the prepared extracts were determined by using DPPH radical scavenging assay and Superoxide anion scavenging assay.²² DPPH radical scavenging assay is a sensitive method to determine the antioxidant activity of the extracts. Hydrogen donating ability of the antioxidant molecule contributes to its free radical scavenging nature and was measured from the bleaching of violet colored DPPH solution at 517nm. The DPPH assay method is a sensitive method based on the reduction of DPPH, a stable free radical. When plant extracts having antioxidants react with DPPH, DPPH becomes paired off in the presence of a hydrogen donor (e.g., a free radical scavenging antioxidant) and is reduced to the DPPH-H and as a result, the absorbance gets decreased from the DPPH. Radical to the DPPH-H form, results in decolorization (yellow colour) with respect to the number of electrons captured. More is the decolorization, more is the reducing ability. DPPH assay has now been the most accepted model for evaluating the free radical scavenging activity of any new drug. The concentration of extracts required to inhibit 50% DPPH free radicals are shown in Table 6. Among the lyophilized and oven dried of both extracts, the higher scavenging activity was shown by lyophilized extracts.

Table 5: Determination of total phenolic and total flavonoid content of methanolic extract

Extract	Total phenol content (%)	Total flavonoid content (%)
Leaf methanolic extract	0.11 ± 0.34	0.52 ± 0.24
Bark methanolic extract	0.49 ± 0.62	0.34 ± 0.31

Table 6: Free radical scavenging capacity of leaves and bark methanolic extract of *Heterophragma adenophyllum*.

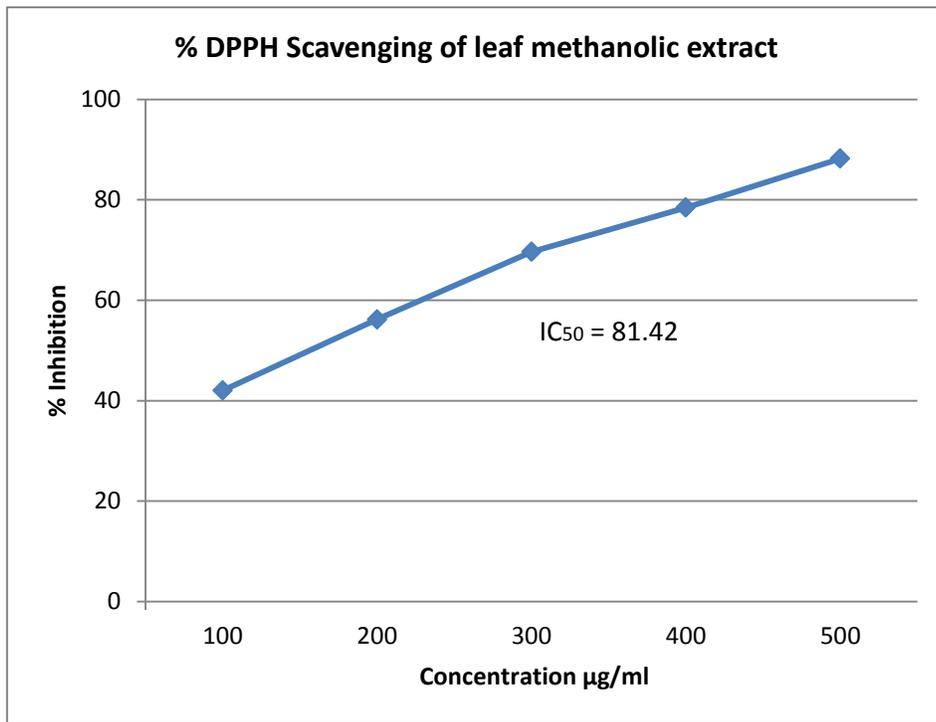
Concentration $\mu\text{g/ml}$	DPPH Scavenging %	
	Leaves methanolic extract	Bark methanolic extract
100	42.03 \pm 0.23	34.06 \pm 0.43
200	56.17 \pm 0.35	47.17 \pm 0.44
300	69.63 \pm 0.53	58.20 \pm 0.81
400	78.46 \pm .42	66.79 \pm 0.34
500	88.22 \pm 0.11	79.24 \pm 0.49
IC ₅₀	81.42 \pm 0.32	72.24 \pm 0.14
Ascorbic acid	92.55 \pm 0.22	

Superoxide anion radical scavenging assay:

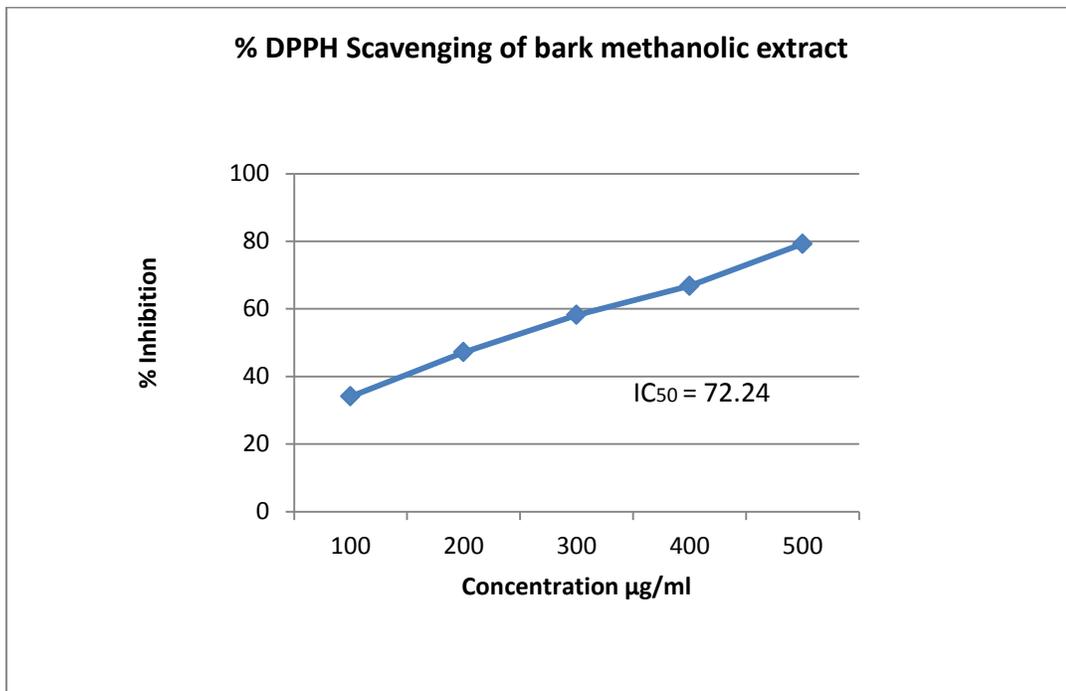
This is quite a weak oxidant but it results to the generation of powerful and dangerous hydroxyl radical as well as singlet oxygen both of which contribute to oxidative stress. Superoxide anion radicals are produced endogenously by flavoenzymes like Xanthine oxidase which converts hypoxanthine and subsequently to uric acid. Significant decrease in absorbance at 560nm with extracts indicates the consumption of superoxide anion in the reaction mixture and thereby exhibiting a dose dependent increase in superoxide scavenging activity. The results are summarized in Table 7

Table 7: superoxide scavenging capacity of leaves and bark methanolic extract of *Heterophragma adenophyllum*.

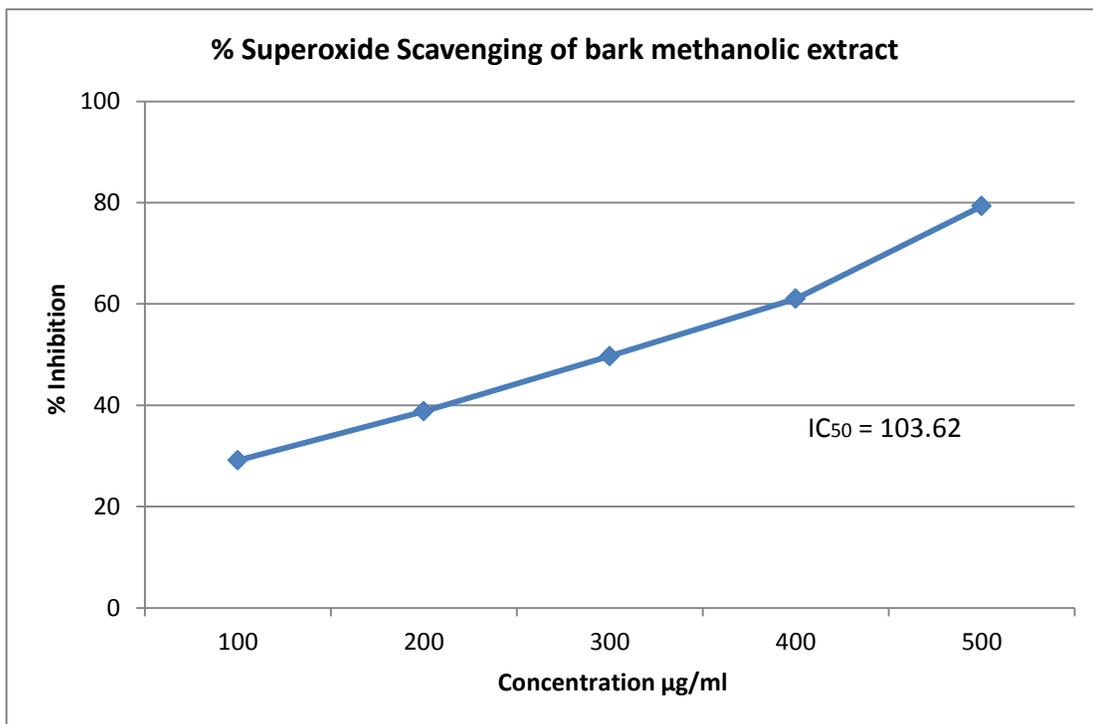
Concentration $\mu\text{g/ml}$	Superoxide Scavenging %	
	Leaves methanolic extract	Bark methanolic extract
100	31.05 \pm 0.11	29.14 \pm 0.32
200	46.08 \pm 0.43	38.82 \pm 0.43
300	59.45 \pm 0.23	49.72 \pm 0.41
400	66.73 \pm 0.83	61.06 \pm 0.71
500	82.34 \pm 0.52	79.33 \pm 0.63
IC ₅₀	116.42 \pm 0.35	103.62 \pm 0.42
Ascorbic acid	87.48 \pm 0.32	



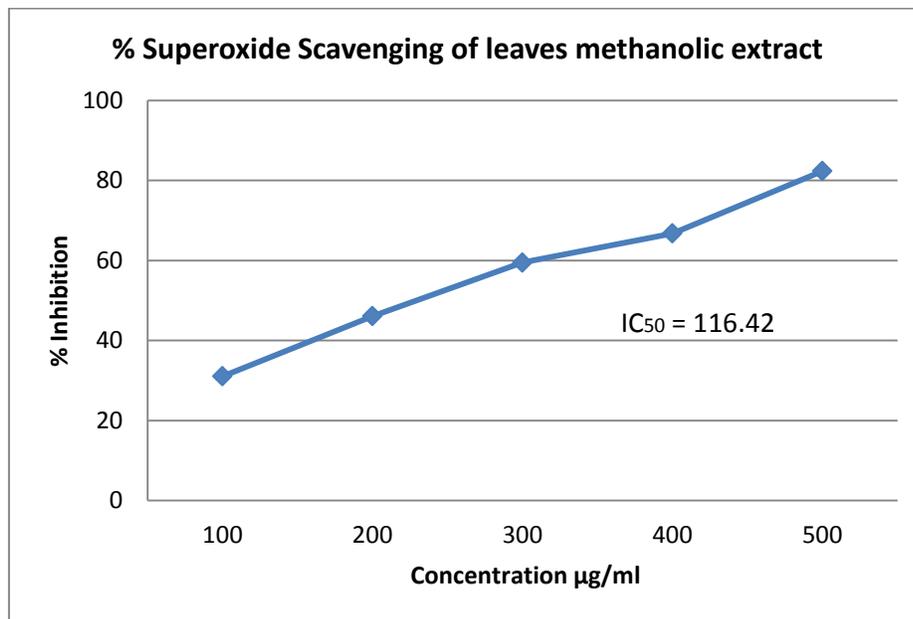
Graph 1: DPPH scavenging of Lyophilized *V. volvacea* extract



Graph 2: % DPPH Scavenging activity of oven dried *V. volvaceae* extract



Graph 3: % Superoxide scavenging activity of Lyophilized *V. volvaceae* extract



Graph 4: % Superoxide scavenging activity of oven dried *V. volvaceae* extract

CONCLUSION

In the present study, phytochemical, physiochemical and antioxidant activities of *heterophragma adenophyllum* extracts were evaluated. The leaves methanolic extract of *Heterophragma adenophyllum* showed maximum antioxidant activity; and their total phenolic and flavonoid content are also high. The phytochemical screening of *Heterophragma adenophyllum* extracts demonstrated

presence of Carbohydrates, Glycosides, Alkaloids, Flavonoids, saponins, tannins and steroids. Likewise the physiochemical properties were also demonstrated. Thus, from the data obtained it can be concluded that the methanolic extract of leaves will be the best for quantitative phytochemical analysis and further pharmacological evaluation.

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