

Phytochemical Screening and Antioxidant Studies of Different Fractions of *Heterophragma Adenophyllum*

Sahar Khan¹, Muhammad Saleem¹, Afsheen Arshad²

¹Department of Chemistry, Government College University Lahore, 54000, Pakistan.

²Applied Chemistry Research Centre, PCSIR Labs Complex, Ferozpur Road, Lahore, 54600, Pakistan.

*s4sahark4khan@gmail.com <https://orcid.org/0009-0009-2209-841X>

ABSTRACT

Abstract

Heterophragma adenophyllum (maror phalli) belongs to family Bignoniaceae has been used as an ornamental and medicinal plant. Many species of this family exhibit good antioxidant, antibacterial, antifungal, antiulcer, antiinflammatory, antitumor and antiviral activities. Objective of this research was to determine the total phenolic, total flavanoid and condensed tannin contents of different extracts (n-hexane, chloroform, methanol) of the leaves and the bark of *H. adenophyllum* by using Folin-Ciocalteu reagent, Aluminium chloride reagent and Vanilline-HCl assay respectively. Moreover in-vitro antioxidant activity of the extracts was evaluated by using three different assays: 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, total antioxidant activity by phosphomolybdenum method and ferric reducing antioxidant power (FRAP). The results revealed that methanol extract of the leaves have highest total phenolic content (15.139 mg/g $\pm$ 0.012) and total flavanoid content (6.141 mg/g $\pm$ 0.0023) while chloroform extract showed more condensed tannin (1.132 mg/g $\pm$ 0.001) content as compared to other extracts. The antioxidant assays indicated that methanol extract of leaves exhibited promising radical scavenging activity with IC₅₀ value of 0.140 mg/ml $\pm$ 0.003. It also exhibited highest FRAP value (0.667 mg/g $\pm$ 0.017) and highest total antioxidant activity (21.558 mg/g $\pm$ 0.021) among all extracts of leaves and bark. The results revealed that methanolic extract of the leaves of *H. adenophyllum* possesses more potential to be explored as a source of natural antioxidants as compared to bark.

Key Words

Methanolic Extract, IC₅₀ value, FRAP, DPPH, Antioxidant.

Introduction

Herbal Medicine

Herbs are being used as medicines since very beginning of human society. The first recorded use of herbs as medicines is as old as 5000 years from the present [01-02]. Today use of herbs has evolved into a whole new system, and it is largely known as herbal medicine, phytomedicine and botanical medicine. Different parts of a plant are used for medicinal purposes in this system, such as plant seeds, its berries, the roots, and leaves in fresh and dried form, barks and flowers [03-04]. The medicinal secret of these plants is hidden in some chemically active substances. These chemicals produce specific physiological action in human body. Alkaloids, tannins, flavonoids, anthocyanin's, saponins, phenolic compounds and minerals are the most important chemical compounds of plants [05-08].

Bignoniaceae family is known for its multiple uses. Being ethno-botanical, commercial and most importantly as ornamentals, all have species of horticultural significance in tropical areas [09, 10].

Table 01 Folk uses of Bignoniaceae [11-17]

Sr. No	Botanical Names	Family	Local Name	Plant's Part Used	Therapeutic uses
1	<i>Crescentia cujete</i> L	Bignoniaceae	Boan/gota	Whole Plant	Abortifacient, cancer, Snake bite, Itch,

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					Alopecia, Virility, Pneumonia, Hurt.
2	<i>Heterophragma</i> <i>Adenophyllum</i>	Bignoniaceae	Kau-a-turi	Root	Piles and Constipation
3	<i>Oroxylum</i> <i>Indicum(L)</i>	Bignoniaceae	Khonaha, Pahari- jora, Kanai- dingi, Hanghoal Shayonaka	1)...Leaf, Stem, Bark 2)... Leaf, Bark 3)... Fruit	1)...Tonsillitis, Cholera, Spleen enlargement, Indigestion. 2)...
					Tonsillitis, Snake Bite, Rheumatoid, Arthritis, Edema, Gynecological disorders, Colic. 3)...jaundice
4	<i>Stereospermum</i> suaveolens DC	Bignoniaceae	Parul, Nill parul	...Leaf, Bark, Flowers. ... Bark ...Leaf	1)...Mileria, Bronchitis, Heart Deseases, Cancer, Purgative. 2)... Pain 3)...
5	<i>Tabebuiaargentea</i>	Bignoniaceae	Gui-babla	Root	Gonorrhea Worn as a

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						talisman
						around the
						neck to protect
						from evil
						spirits.
6	<i>Tecoma</i>	Bignoniaceae	Sothin	Whole	1)...Infertelity,	
	<i>gaudichaudi</i> DC		Bahar	Plant	Diabetese,	
			Shona	Leaf	Digestive aid	
			Pata		2)... Erectile	
					Disfuntion	
7	<i>Tecoma stans</i> (L).	Bignoniaceae	Sona Pata	Leafe	Pain and Piles.	
	<i>Juss</i>					

Description of Plant

Heterophragma Adenophyllum

Family: Bignoniaceae

Genus: *Heterophragma*

Species: *adenophyllum*

Common Name: Maror phalli, Mustan Phul, Bidri Pata, Sanp Phalli.

Synonym: Fernendoa adenophylla

Category: Trees

Height: 20 to 30 feet (6-9 m) Bottom of Form

30 to 40 feet (9-12 m) Top of Form

Description of *Heterophragma adenophyllum* is a moderate size seasonal tree. Leaves are compound that are 0.3-0.6 m long. It usually contains 5-7 leaflets per leaf.

Its flowers are bright and brownish yellow in colour and their size is approximately 6.35-7.62 cm. In November it flowers. It bears large capsule fruits that are usually 0.3-0.9 m long. These fruits are cylindrical ribbed and twisted and they get mature between first two months of year. [18]

Distribution: It's a native tree of the region that comprises from eastern Himalayas to northern parts of Malaya through a narrow region of Burma.

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In Pakistan this tree has acquired value of being an ornamental plant.

Silvical Characteristics

Habitat and Ecology: *Heterophragma adenophyllum* is an intolerant tree. It requires full sunlight, moist situations and deep soils that are well drained. Precipitation zones having a down pour of at least 800 mm/yr. along with sub-humid, tropical climate with a temperature range of 0-40 °C is considered ideal for the healthy growth of these trees. It has no known insect or pest problems. [19]

Reproduction: It reproduces through its seeds with a condition that seeds should be fresh enough for growth.

Productivity: Its growth rate is approximately 1meter in height every 2 year.

Management Implications: Its large and brightly colored flowers have earned it a position as being an ornamental tree.

Wood Properties

Grain: Straight, fine textured, even grained.

Color: Sapwood is light yellow while the heartwood is orange yellow with occasional dark streaks. [20]

Density: Calorific value of 4800 KCal/Kg.

Strength: Hard, strong resilient.

Uses

1. It is used as ornamental, making furniture and used in burning fuel. [21]
2. Its wood is used for preparing fishing rods. [22]





Fig 1-5 *Heterophragma Adenophyllum*

Antioxidants

Definition

“An antioxidant may be defined as ‘any substance that when present at low concentrations, compared with those of the oxidisable substrate, significantly delays or inhibits oxidation of that substrate’ [23].

A substance that reduces damage due to oxygen, caused by its free radicals is called antioxidant. It helps in prevention of oxidative stress. Cell damages can be delayed or may get prevented by the use of antioxidants. Antioxidants can be manmade as well as naturally occurring. Naturally occurring antioxidants are found in different parts of herbs and plants and trees e.g. flowers, fruits, barks and seeds.

Examples of antioxidants include [24]

- Beta-carotene
- Lutein
- Lycopene
- Selenium
- Vitamin A
- Vitamin C
- Vitamin E
- Enzymes

Chemistry of Antioxidants

When in result of some chemical reaction an electron is transferred from electron rich entity to electron deficient entity, the process is called oxidation. Antioxidants are substances that are capable of counter acting the damage to animal tissue in result of oxidation. Hence they play substantial role in preventing the development of cancer, heart strokes, Alzheimer, Rheumatoid arthritis, and cataracts of eyes due to aging.

Oxidative Stress

Oxidative stress occurs due to the production of harmful molecules called **free radicals** is beyond the protective capability of the antioxidant defenses.

Free Radical

Free radicals are chemically active atoms or molecular fragments with unpaired electrons that form due to the reaction of oxygen molecules with different molecules in their entity.

Free radicals containing oxygen, known as **reactive oxygen species (ROS)**, are the most biologically significant free radicals. ROS include

Examples of free radicals and ROS

- Superoxide Anion, $O_2^\bullet$
- Hydroxyl Radical, $OH^\bullet$
- Peroxyl Radicals $ROO^\bullet$
- Alkoxyl Radicals $RO^\bullet$
- Hydrogen Peroxide H_2O_2
- Singlet Oxygen O_2^1
- Transition metals such as iron and copper, nitric acid
- Ozone
- Hypochlorous acid

Free radicals have one or more unpaired electrons, these are highly unstable. They scavenge animal body to grab or donate electrons, thereby damaging cells, proteins, and DNA (genetic material).

The same oxidative process also causes

- Oils to become rancid,

- Peeled apples to turn brown,
- Iron to rust.

Materials and Methods

Standards

1. BHT (Butylated hydroxytoluene)
2. Quercetin
3. Catechin

Chemicals Used

1. Aluminium Chloride ($\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$)
2. Follin Ciocalteu (FC) Reagent (2N)
3. Sodium Carbonate (Na_2CO_3)
4. Vanillin
5. Hydrochloric Acid (HCl)
6. Sodium Phosphate (Na_3PO_4)
7. Ammonium Molybdate ($(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$)
8. Sulphuric Acid (H_2SO_4)
9. DPPH $\cdot$ (1,1-Diphenyl-2-picrylhydrazyl) radical
10. Sodium Acetate ($\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$)
11. Acetic Acid (CH_3COOH)
12. TPTZ (2,4,6-Tripyridyl-s-triazine)
13. Ferric Chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$)

Solvents

Used for extraction of *Heterophragma adenophyllum* leaves and stem.

1. n-Hexane (C_6H_{12})
2. Chloroform (CHCl_3)
3. Methanol (CH_3OH)
4. Distilled water

Collection of Plant Material

Plant material including (leaves and stem) of *Heterophragma adenophyllum* was collected from new block, Government College University Lahore on 20.09.2013. A taxonomist Prof. Dr. Muhammad Ajaib, from Department of Botany, Government

College University Lahore, identified it. The voucher specimen with number G.C. Herb. Bot. 2412 has been deposited in the Herbarium of the Department of Botany, Government College University Lahore.

Extraction of Plant Material with Solvents

Collected plant material (leaves and barks) weighed 1800.15g was dried under shade at room temperature for one month and finely grounded shown in **Figure (3 and 4)**. The grounded material was first extracted twice with n-Hexane by (vigorous shaking) at room temperature for each time taking 48 hours stay. The extract was evaporated on rotary evaporator at 40 °C. Plant material was then extracted twice with chloroform by vigorous shaking after the interval of 8 hours. The extract was evaporated on rotary evaporator at 40 °C. Methanolic extract of plant material was also collected by vigorous shaking twice after the interval of 8 hours at room temperature, methanol extract was evaporated on rotary evaporator at 40 °C.

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Fig 6 and Fig 7 Actual and Dried Form of Plant Leaves



Table 02 % Yield of Different leave Extracts

Sr. No.	Weight of the Dried Plant	Name of Plant Extracts	Quantity of Solvent used	% Yield
1	928.5g of leaves	n-hexane extract of leaves	2 L	1.01 %
2	//	Chloroform extract of leaves	2 L	01 %
3	//	Methane extract of leaves	2.5 L	2.56 %

Table 03 % Yield of Different Bark Extracts

Sr. No.	Weight of the Dried Plant	Name of Plant Extracts	Quantity of Solvent used	% Yield
1	234g of Bark	n-hexane extract of bark	1 L	2.35 %
2	//	Chloroform extract of bark	1 L	5.47 %
3	//	Methane extract of bark	1 L	2.78 %

Physical Appearance of Extracts

Table 04. Physical appearance of leaves extracts

Sr.No.	Extracts with different Solvents	Physical Appearance
1	n-Hexane extract of leaves	Waxy
2	Chloroform extract of leaves	Waxy
3	Methanolic extract of leaves	Solid residue

Table 05. Physical appearance of bark extracts s

Sr.No.	Extracts with different Solvents	Physical Appearance
1	n-Hexane extract of bark	Oily residue
2	Chloroform extract of bark	Oily residue
3	Methanolic extract of bark	Semisolid residue

Phytochemical Analysis

The extracts obtained from n-hexane, chloroform and methanol of *Heterophragma adenophyllum* were screened to check the presence of flavonoid contents, total phenolic contents and condensed tannin contents in the leaves and bark by using standard methods.

Determination of Total Flavonoid Content

- Total flavanoid contents of *Heterophragma adenophyllum* (leaves and bark)

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extracts were estimated by method explained by ((Djeridane et al)) [59].

Preparation of Reagents:

- **2 % $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$** was prepared by dissolving 2 g of $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ in methanol and make it upto 100 mL in a volumetric flask.
- **Methanolic extracts** of *Heterophragma adenophyllum* (leaves and bark) were prepared in following concentrations from different fractions.

Table 06. Concentration of Leaves Extract of *Heterophragma Adenophyllum*

Sr.No.	Leave extracts of <i>Heterophragma adenophyllum</i>	Concentrations of methanolic extracts in ppm
01	n-hexane extract	1000 ppm
02	Chloroform extract	500 ppm
03	Methanolic extract	250 ppm

Table 07. Concentration of Bark Extract of *Heterophragma Adenophyllum*

Sr.No.	Bark extracts of <i>Heterophragma adenophyllum</i>	Concentrations of methanolic extracts in ppm
01	n-hexane extract	1000 ppm
02	Chloroform extract	1000 ppm
03	Methanolic extract	1000 ppm

- 2.5 mL of 2 % methanolic AlCl_3 solution was mixed with 2.5 mL of each extracts of leave and bark in amber glass bottles.
- **Standard:** 2.5 mL of 2 % methanolic AlCl_3 solution was mixed with each 2.5 mL of (2.5 ppm, 5 ppm, 10 ppm, 15 ppm, 20 ppm, 30 ppm) of quercetin standard in amber glass bottles.
- **Blank Solution:** 2.5 mL of 2 % methanolic AlCl_3 solution was mixed with 2.5 mL of methanol in amber bottles.
- All above samples, standards and blank were incubated at room temperature for 10 minutes.
- After 10 minutes absorbance was measured by UV-Visible Spectrophotometer at 415nm.

Determination of Total Phenolic Content

- Total phenolic content of *Heterophragma adenophyllum* (leaves and bark) extracts were estimated by method explained by (Savitree et al) [60].
- 10 % Na_2CO_3 was prepared by dissolving 10 g Na_2CO_3 in distilled water and make it up to 100 mL in volumetric flask.
- **Methanolic extracts** of *Heterophragma adenophyllum* (leaves and bark)
All extracts were prepared in the concentrations of 1000 ppm.
- 0.2 mL of each extracts of (leave and bark) was mixed with 5.6 mL of 10 % Na_2CO_3 solution and then added 0.2 mL of 2N Folin-Ciocalteu reagent in each amber glass bottles.
- **Standard:** 0.2 mL of Quercetin standard concentrations (2.5 ppm, 5 ppm, 10 ppm, 15 ppm, 20 ppm, 30 ppm) was mixed with 5.6 mL of 10 % Na_2CO_3 solution and then added 0.2 mL of 2N Folin-Ciocalteu reagent in each amber

glass bottles.

- **Blank Solution:** 0.2 mL of methanol was mixed with 5.6 mL of 10 % Na_2CO_3 solution and then added 0.2 mL of 2N Folin-Ciocalteu reagent in amber glass bottles.
- All the above samples, standards and blanks were kept incubated at room temperature for 40 minutes.
- After 40 minutes absorbance was measured by UV-Visible Spectrophotometer at **725nm**.

Determination of Total Condensed Tannin Content:

- Total condensed tannin content of *Heterophragma adenophyllum* (leaves and bark) extract was estimated by method explained by (H.P.S. Makkar et al) [61].
- **Vanillin Reagent** was prepared by dissolving 50 mL of 4% vanillin in methanol and 50 mL of 8% concentrated HCl in methanol.
- All the extracts were dissolved in methanol at the concentration of 1000 ppm.
- 1 mL of each extract (leave and bark) was mixed with 5 mL of vanillin reagent (freshly prepared) in amber glass bottles.
- **Standard:** 1 mL of catechin standard concentrations (2.5 ppm, 5 ppm, 10 ppm, 15 ppm, 20 ppm, 30 ppm) was mixed with 5 mL of vanillin reagent (freshly prepared) in amber glass bottles.
- **Blank Solution:** 1 mL of distilled methanol was mixed with 5 mL of vanillin reagent (freshly prepared) in amber glass bottles.
- All above samples, standards and blank were kept incubated at room temperature for 20 minutes.
- After 20 minutes absorbance was measured by UV-Visible Spectrophotometer at **500nm**.

Antioxidant Assays

Antioxidant activity of *Heterophragma adenophyllum* (leave and bark) extracts was determined by using following antioxidant assays.

1. Total antioxidant activity by phosphomolybdenum method explained by (K.

Lee et al) [62]

2. DPPH radical scavenging activity explained by (P. Prieto et al) [63]
3. Ferric reducing antioxidant power (FRAP) explained by (I. E. F. Benzie et al) [64]

1. Total Antioxidant Activity by Phosphomolybdenum Method

1. Different dilutions (500, 250, 125, 50 µg/mL) of each extract of *Heterophragma adenophyllum* (leave and bark) were in methanol and 0.4 mL from each sample was taken in amber glass bottles.
2. **Phosphomolybdenum Reagent Solution:** 2.66 g of sodium phosphate, 2.47 g of ammonium molybdate and 8.35 mL of concentrated sulphuric acid was dissolved in 250 mL distilled water and it was filtered off until clear phosphomolybdenum reagent was obtained.
3. 4 mL of phosphomolybdenum reagent was added in all.
4. **Standard:** Antioxidant activity of plant extracts was evaluated by using butylated hydroxytoluene (BHT) standard.
5. **Blank solution:** 0.4 mL of distilled water was added in amber glass bottle and mixed with 4 mL of phosphomolybdenum reagent.
6. The samples, standard and blank solutions were capped and incubated in water bath at 95°C for 90 minutes.
7. Absorbance of the samples and standard solutions were measured against blank at **695nm** by UV-Visible Spectrophotometer.

2. DPPH radical scavenging activity:

1. Different dilutions of extract of *Heterophragma adenophyllum* (leaves and bark) obtained from n-hexane and chloroform were prepared as under.
 - 2000µg/mL
 - 1500 µg/mL
 - 1000 µg/mL
 - 500 µg/mL
 - 250 µg/mL
 - 125 µg/mL
 - 50 µg/mL

Whereas different dilutions of *Heterophragma adenophyllum* (leave and bark) extracts were dissolved in methanol.

- 500 µg/mL
 - 250 µg/mL
 - 125 µg/mL
 - 50 µg/mL
2. **DPPH radical solution** was prepared by dissolving 40 mg of DPPH solid in 1000 mL of methanol and kept for 24 hours to stabilize it.
 3. 2 mL was withdrawn from each sample dilution and mixed with 2.5 mL of DPPH radical solution and vigorously shaken.
 4. **Standard:** Antioxidant activity was compared by using butylated hydroxytoluene (BHT) and catechin standards.
 5. **Blank solution:** 2 mL methanol was mixed with 2.5 mL of DPPH radical solution in amber glass bottle and vigorously shaken.
 6. Sample solution, standards dilutions and blank solution were capped and kept at room temperature for 30 minutes.
 7. Absorbance of the samples and standard solution were measured against blank at **517nm** by UV-Visible Spectrophotometer.
3. **Ferric reducing antioxidant power (FRAP) assay:**
1. **Acetate buffer solution** was prepared first by mixing 0.31g of sodium acetate ($\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$) and 1.6 g of concentrated acetic acid (CH_3COOH) make it up to 100 mL with distilled water and its pH was adjusted at 3.7.
 2. **40 mM HCl Solution:** prepared by dissolving 0.36 mL of concentrated HCl in distilled water and make it up to 100 mL in a volumetric flask.
 3. **TPTZ (2,4,6-Tripyridyl-s-triazine) Solution** was prepared by dissolving 0.312 g of TPTZ in 40 mM HCl solution and make it up to 100 mL in a volumetric flask.
 4. **Ferric Chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) Solution** was prepared by dissolving 0.54 g of Ferric Chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) in distilled water and make it up to 100 mL in a volumetric flask.
 5. **FRAP Reagent** was prepared by mixing 25 mL of acetate buffer solution

with 2.5 mL of TPTZ solution and 2.5 mL of ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) solution. The solution was warmed at 37°C before using it.

Different dilutions of each extract of *Heterophragma adenophyllum* (leave and bark) were prepared in amber bottles are as under.

- 250 $\mu\text{g/mL}$
- 500 $\mu\text{g/mL}$
- 1000 $\mu\text{g/mL}$
- 1500 $\mu\text{g/mL}$
- 2000 $\mu\text{g/mL}$

0.1mL=100 $\mu\text{g/mL}$ of each sample was mixed with 3 mL of FRAP reagent.

6. **Standard:** Antioxidant activity of plant extracts was evaluated by using catechin standard dilutions (5 ppm,10 ppm,15 ppm,20 ppm,25 ppm).
7. **Blank solution:** 0.1 mL of distilled water was added in amber bottle and mixed with 3 mL of FRAP reagent.
8. Sample solution, standards dilutions and blank solution was capped and incubated at room temperature for 30 minutes.
9. Absorbance values of the samples and standard solution were measured against blank at **593nm** by UV-Visible Spectrophotometer.

Results and Discussion

Phytochemical Analysis Of *Heterophragma Adenophyllum*

Total Phenolic Contents Of *Heterophragma Adenophyllum*

The phenolic compounds have been recognized as antioxidant agents and show medicinal activities and have many physiological functions [65].

1. Standard calibration curve of Quercetin prepared in different concentrations was used for the determination of total phenolic contents in the leaves and bark of *Heterophragma adenophyllum* as shown in **Figure 08**.

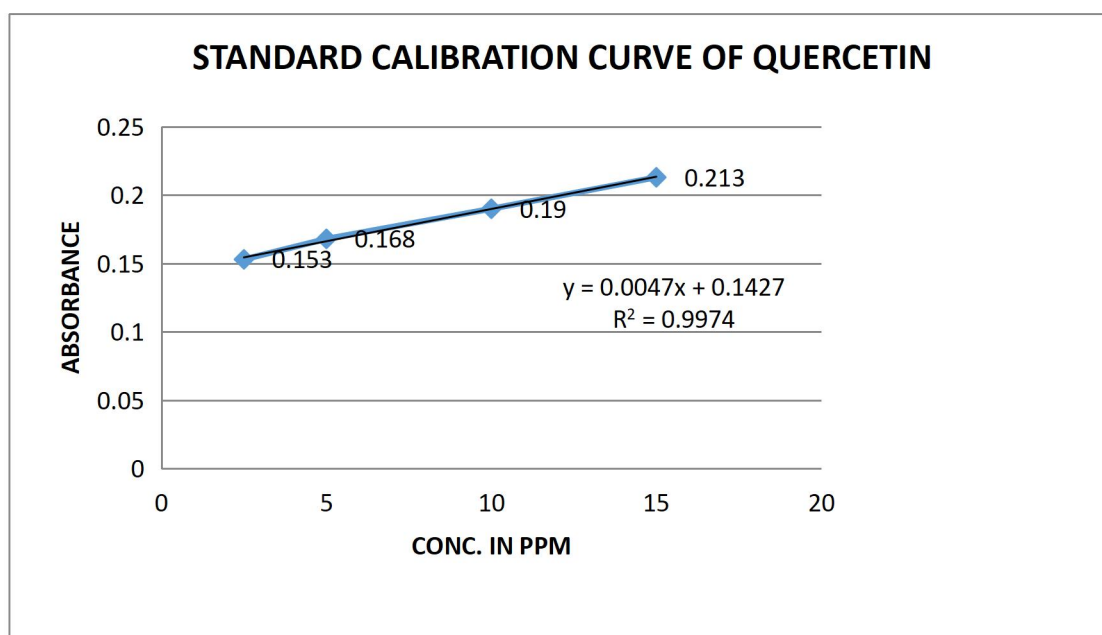


Figure 08, Standard calibration curve of different concentrations of Quercetin at 725nm.

2. Total phenolic contents were significantly greater in amount in the methanolic extracts of leaves and bark as compared to chloroform and n- hexane extracts

Table 08. Total phenolic contents studied in leaves and bark of *Heterophragma adenophyllum*.

Sr. No.	Name of extracts	Symbols used for Samples	Total Phenolic Content Quercetin Equivalent (mg/g)
01	leave n-hexane	HLHE	1.428
02	leave choloroform	HLCE	5.369
03	leave methanol	HLME	15.139
04	bark n-hexane	HBHE	1.354
05	bark chloroform	HBCE	1.95
06	bark methanol	HBME	3.364

3. n-Hexane being a non-polar solvent extracted minimum quantity of total phenolic contents both from leaves and bark of plant.

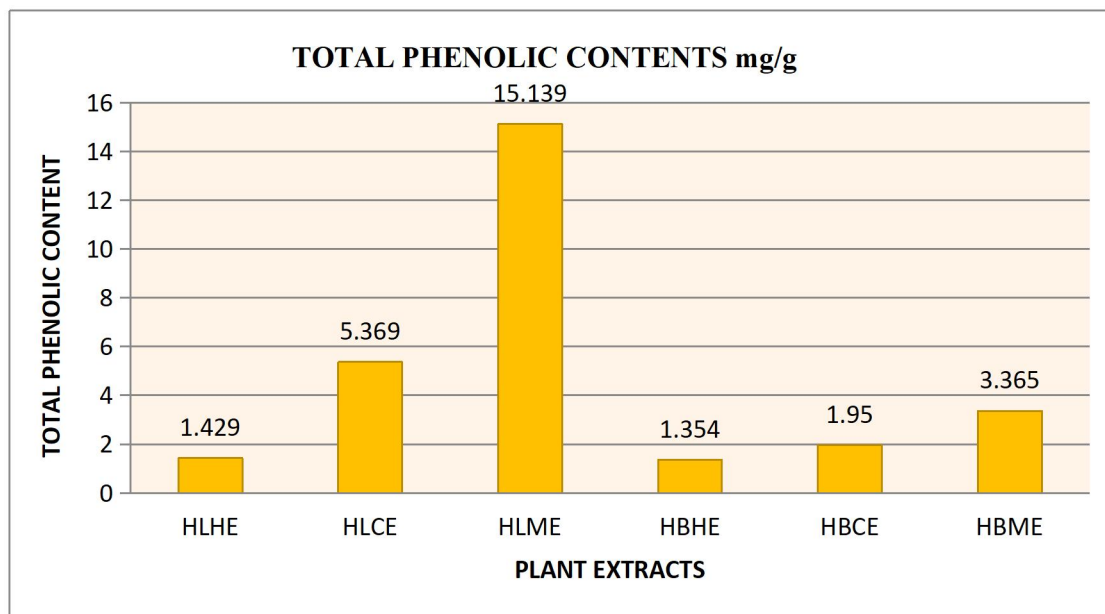


Figure 09, Bar graph showing comparison of total phenolic contents in leaves and bark extracts of *Heterophragma adenophyllum*

Phenolic and flavonoids have been reported to have good antioxidant action in human beings, as they act as a scavenging agent or chelating agent [66, 67].

Total Flavonoid Contents Of *Heterophragma Adenophyllum*

Flavonoids belong to a group of polyphenolic compounds having the known properties of free radical scavenging activity, anti-inflammatory action and are oxidative enzymes [68].

Quercetin, the most abundant dietary flavonol, is a potent antioxidant because it has all the right structural features for free radical scavenging activity [69].

1. Standard calibration curve of quercetin preparing in different concentrations was used for the determination of total flavanoid content in the leaves and bark of *Heterophragma adenophyllum* as shown in **Figure 10**.

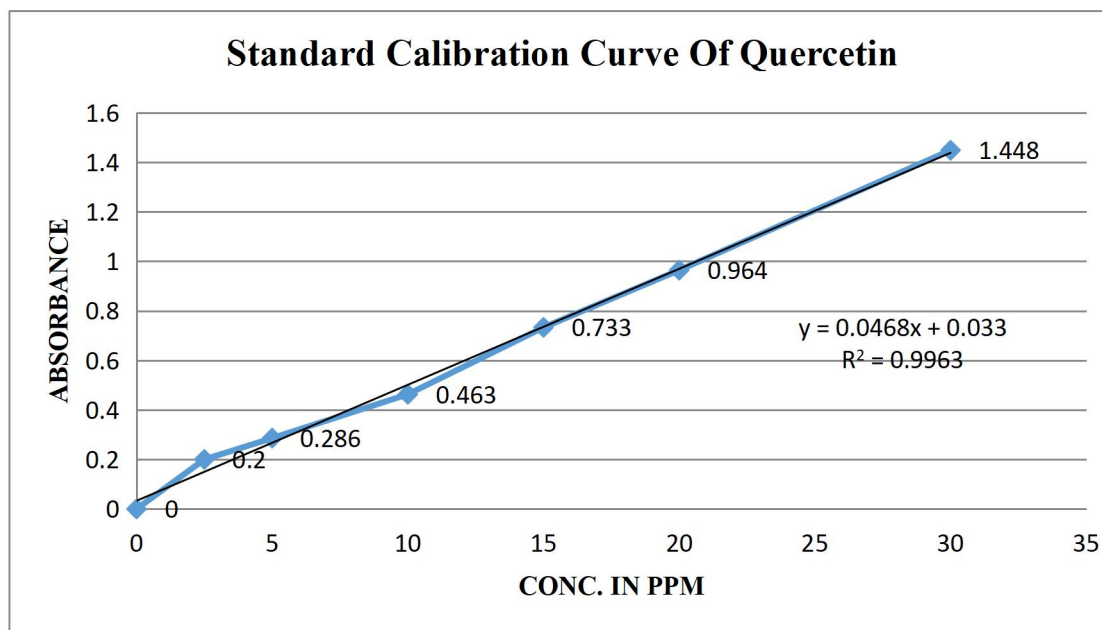


Figure 10, Standard calibration curve of different concentrations of quercetin at 415nm.

2. Total flavonoid content were significantly greater in the methanolic leaves extract as compared to chloroform and n- hexane extracts.
3. Methanol and chloroform extracts of the leaves showed high total flavanoid contents.
4. n- Hexane being a non-polar solvent extracted minimum quantity of total phenolic contents both from leaves and bark extract of the plant.

Table 09. Total Flavonoid contents studied in leaves and bark of *Heterophragma adenophyllum*.

Sr. No.	Name of extracts	Symbols used for		Total Flavonoid Content
		Samples	Quercetin Equivalent (mg/g)	
01	leave n-hexane	HLHE	0.705	
02	leave chloroform	HLCE	3.162	
03	leave methanol	HLME	6.141	
04	bark n-hexane	HBHE	0.093	
05	bark chloroform	HBCE	0.11	
06	bark methanol	HBME	0.131	

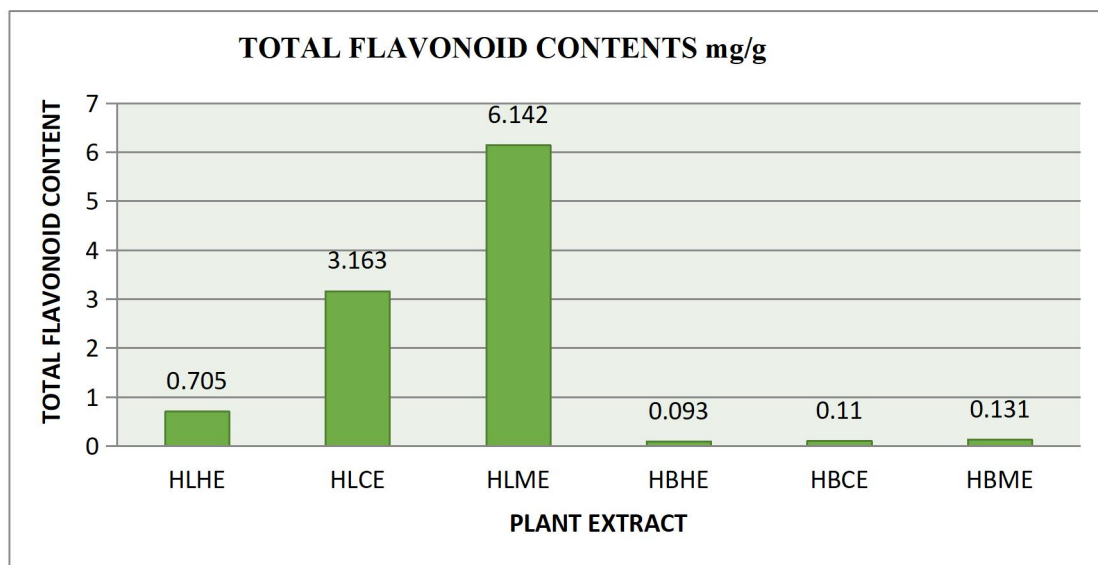


Figure 11, Bar graph showing comparison of total flavonoid contents in leaves and bark extracts of *Heterophragma adenophyllum*

Total Condensed Tannin Contents In *Heterophragma Adenophyllum*

Standard calibration curve of catechin was used for the determination of condensed tannin contents in the leaves and bark extracts of *Heterophragma adenophyllum* as shown in Figure 12.

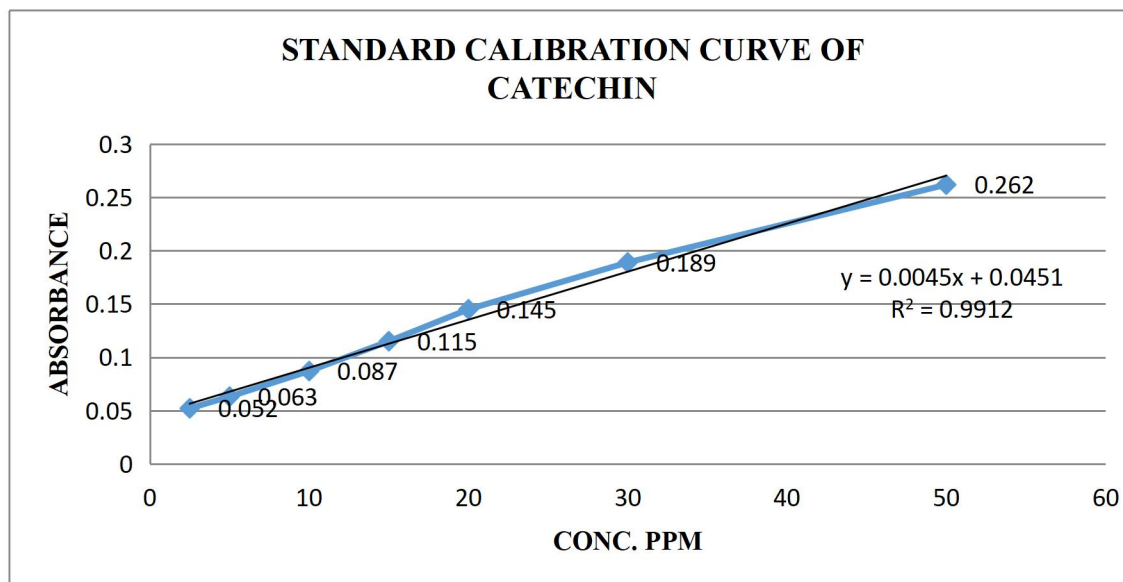


Figure 12, Standard calibration curve of different concentrations of catechin at 500nm.

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Table 10. Total condensed tannin contents studied in leaves and bark of *Heterophragma adenophyllum*.

Sr. No.	Name of Samples	Symbols used for Samples	Condensed Tannin Content Equivalent (mg/g)
01	leave n-hexane	HLHE	-
02	leave chloroform	HLCE	1.132
03	leave methanol	HLME	0.999
04	bark n-hexane	HBHE	0.61
05	bark chloroform	HBCE	0.843
06	bark methanol	HBME	0.421

1. Condensed tannin contents were significantly greater in amount in the chloroform leaves extract as compared to methanolic and n- hexane extracts.
2. n- Hexane being a non-polar solvent extracted minimum quantity of condensed tannin contents both from leaves and bark of plant.

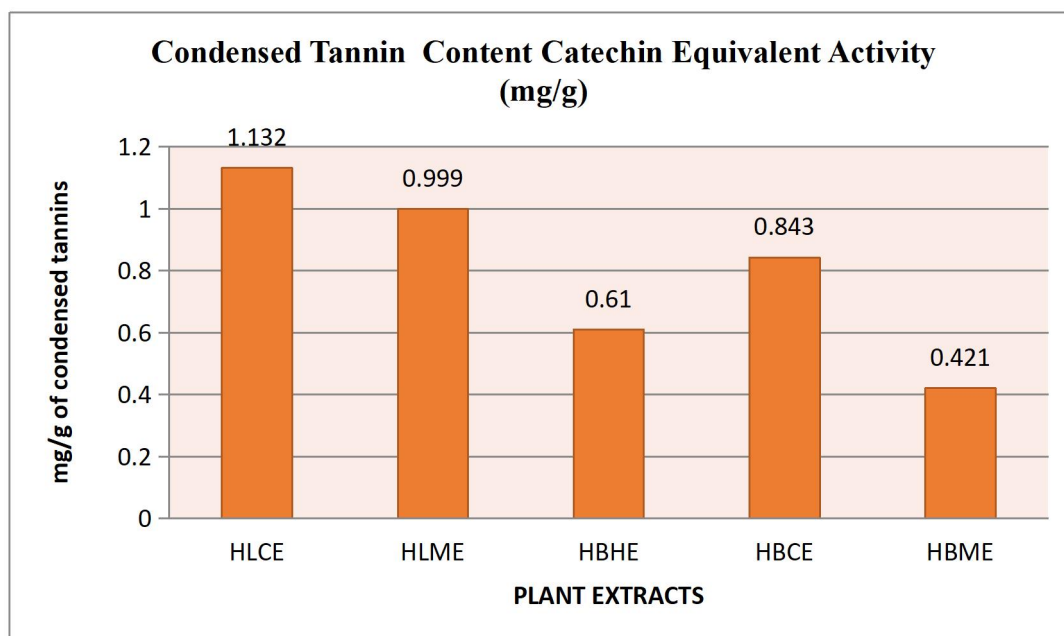


Figure 13, Bar graph showing comparison of total condensed tannin contents in leaves and bark extracts of *Heterophragma adenophyllum*

Anti-Oxidant Assays

Different anti-oxidant assays were used to evaluate the anti-oxidant activities of the

different extracts of *Heterophragma adenophyllum*

Dpph Radical Scavenging Activity

DPPH assay has been widely used to provide basic information on the antioxidant ability of Different extracts from plants, food material or on single compounds, because this method has shown to be rapid and simple available. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability and is a useful reagent for investigating the free radical scavenging activities of compounds. DPPH radical is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radical was determined by the decrease in its absorbance at 517 nm induced by antioxidants. The decrease in absorbance of DPPH radical is caused by antioxidants, because of the reaction between antioxidant molecules and the radical, progresses, which results in the scavenging of the radical by hydrogen donation [70]. After reaction absorbance at 517 nm of DPPH radical decrease due to change of color from purple to yellow [71].

$$\text{DPPH Scavenged (\%)} = [(A_0 - A_1) / A_0] \times 100 \quad [72]$$

Whereas

A_0 = Absorbance of blank,

A_1 = Absorbance of extract

1,1-Diphenyl-2-Picryl Hydrazyl (DPPH) radical scavenging activity of the various Extracts of *Heterophragma adenophyllum* is shown in the **Figure 14**.

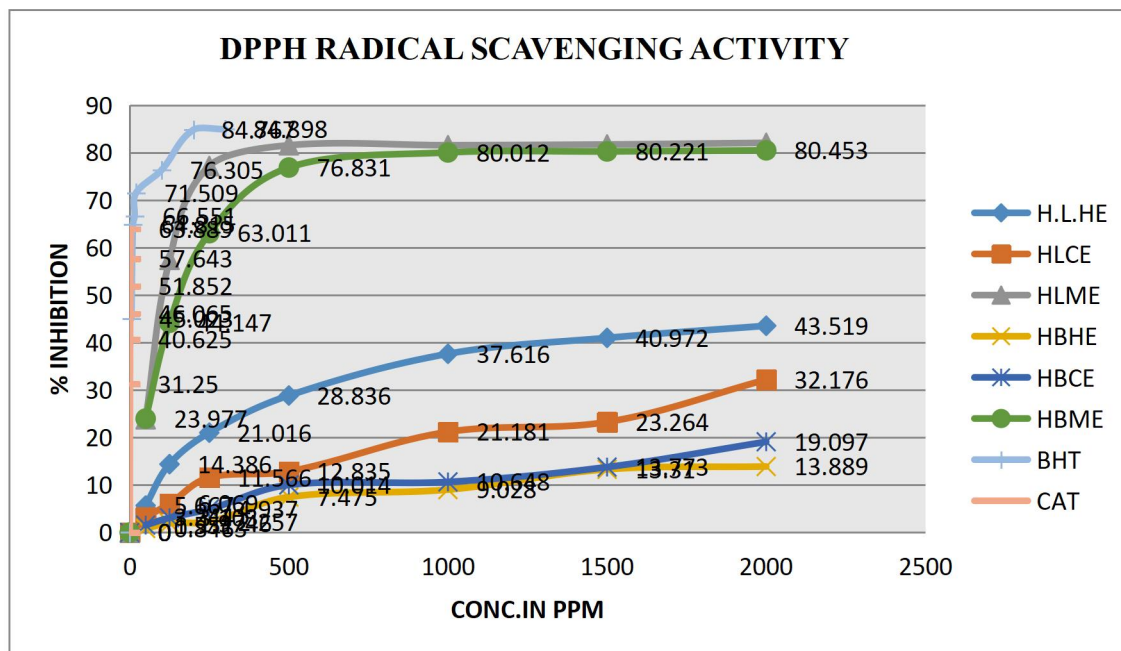


Figure 14, DPPH radical scavenging activity of the different extracts of

Heterophragma adenophyllum

Antioxidant activities of different extracts tested by DPPH radical scavenging assay showed that methanol extract of both leaves and bark at concentration of 500 ppm have given highest % inhibition of DPPH radical i.e. 82% and 80 % respectively. Chloroform extract of the leaves also exhibited antioxidant activity to a little extent.

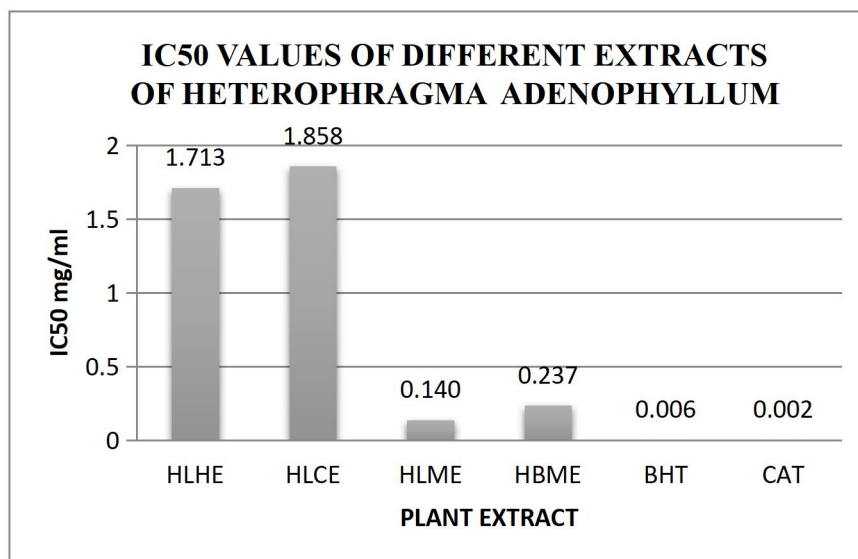


Figure 15, IC₅₀ values of different extracts of *Heterophragma adenophyllum*

IC₅₀ values of different fractions were calculated. IC₅₀ value is the concentration of

substrate which inhibits 50% of DPPH radical [73]. Calculated IC_{50} values of all the fractions have been shown in **Figure 15**. Lower value of IC_{50} means that the studied extract can exhibit the DPPH radical at low concentration. It was observed that methanol leaves extract showed lowest IC_{50} value 0.1403 mg/ml. So we can say methanol leave extract has high potential to inhibit DPPH radical. Methanol extract of bark showed IC_{50} value 0.2373 mg/ml. So we can say that methanol leaves extract has high percent inhibition of DPPH radical. Whereas chloroform extracts of leaves and bark had higher IC_{50} values and indicated that these fractions did not exhibit good antioxidant activity.

Phosphomolybdenum Assay

In phosphomolybdenum method, plant extract reduced Mo (VI) to Mo (V) which can be detected by formation of green phosphate Mo (V) measured at 695nm by using UV/VIS spectrophotometer. The assay basically deals with electron transfer which depends upon structure of antioxidant [44].

Table 11. Total antioxidant activity by phosphomolybdenum assay studied in leaves and bark of *Heterophragma adenophyllum*.

Sr. No.	Name of samples	Symbol of sample	of Total antioxidant assay BHT equivalent mg/g
01	leave n-hexane	HLHE	5.788
02	leave chloroform	HLCE	8.865
03	leave methanol	HLME	21.558
04	bark n-hexane	HBHE	7.327
05	bark chloroform	HBCE	2.327
06	bark methanol	HBME	3.096

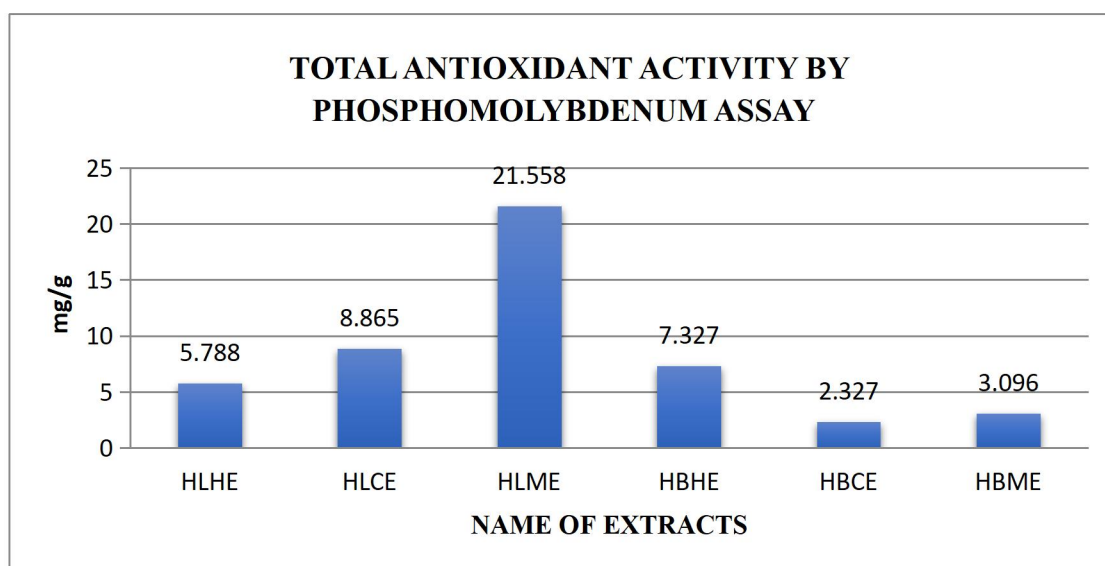


Figure 16, Total antioxidant activity of different fractions of *Heterophragma adenophyllum* by phosphomolybdenum assay

The total antioxidant activity of all the fractions of *Heterophragma adenophyllum* was measured and the results were shown in **Figure 16**. Methanol leaves extract showed highest total antioxidant activity 21.558 ± 0.113 mg/g, chloroform extract of leaves also showed good total antioxidant activity 8.865 ± 0.03 mg/g and the lowest activity was shown by chloroform extract of bark that was 2.327 ± 0.0487 mg/g.

Ferric Reducing Antioxidant Assay (FRAP)

FRAP test is a simple method to measure antioxidant contents. FRAP test was used to measure antioxidant capacity of plasma, The ferric reducing antioxidant power (FRAP) method [74,75] is based on the reduction of a ferroin analog, the Fe^{3+} complex of tripyridyltriazine $\text{Fe}(\text{TPTZ})^{3+}$, to the intensely blue coloured Fe^{2+} complex $\text{Fe}(\text{TPTZ})^{2+}$ by antioxidants in acidic medium. Results are obtained as absorbance increases at 593 nm and can be expressed as micromolar Fe^{2+} equivalents or relative to an antioxidant standard. The reducing capacity of any compound is the significant indicator of antioxidant activity, so FRAP assay was used to check reducing ability of different fraction of plant extract to measure antioxidant activity against reactive O_2 (oxygen) species.

In FRAP test we measure the ability of antioxidants to reduce Fe^{3+} into Fe^{2+} . Complex of Fe^{2+} and tripyridyltriazine give intense blue colour that has

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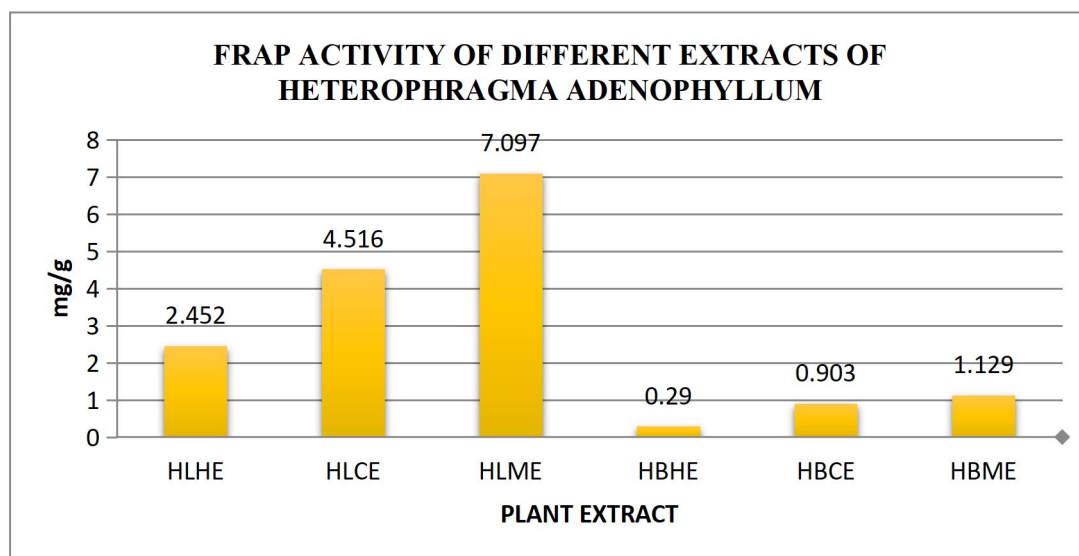
maximum absorbance at 593nm.

Table 12. Antioxidant activity by ferric reducing antioxidant assay studied in leaves and bark of *Heterophragma adenophyllum*.

Sr. No.	Name of Samples	Symbol of sample	FRAP assay mg/g
01	leave n-hexane	HLHE	2.452
02	leave chloroform	HLCE	4.516
03	leave methanol	HLME	7.097
04	bark n-hexane	HBHE	0.290
05	bark chloroform	HBCE	0.903
06	bark methanol	HBME	1.129

Figure 17, FRAP assay of different extracts of *Heterophragma adenophyllum*

The FRAP antioxidant activity of all the extracts of *Heterophragma adenophyllum* was measured and the results are shown in **Figure 17**. Methanol leaves extract showed highest FRAP antioxidant activity 7.097 ± 0.0347 mg/g, and the lowest activity was shown by n-hexane extract of bark that was 0.290 ± 0.01 mg/g.



Conclusion

Based on the study about the results revealed that methanol extract of the leaves of *Heterophragma adenophyllum* show highest total phenolic content and total flavonoid contents ($6.141 \text{ mg/g} \pm 0.002$) while chloroform extract of leaves showed higher condensed tannin ($1.132 \text{ mg/g} \pm 0.001$) contents as compared to other extracts.

Whereas, the chloroform and n-hexane leaves and bark extracts showed minimum concentration of these contents.

The antioxidant assays indicated that methanol extract of *Heterophragma adenophyllum* leaves exhibited promising radical scavenging activity with IC₅₀ value of (0.140 mg/mL $\pm$ 0.003). It also exhibited highest FRAP value (7.097 $\pm$ 0.034 mg/g) and highest total antioxidant activity (21.558 mg/g $\pm$ 0.021) amongst all the extracts of leaves and bark. Moreover, chloroform extracts of leaves and bark fractions showed moderate antioxidant activity and n-hexane extracts of leaves and bark fractions exhibited lowest activity. The results revealed that methanolic extract of the leaves of *Heterophragma adenophyllum* possessed more potential to be explored as a source of natural antioxidants as compared to the bark extract.

The conclusion according to quantitative findings of this study highlighted important points that the compounds showing antioxidant activity might be mostly polar and moderately polar. The present study on the different parts of this plant suggests that the plant has a potential source of natural antioxidants. Further experimental work is required to be performed on the isolation of the active constituents of *Heterophragma adenophyllum* to describe its antioxidant potential and to find its pharmacological, medicinal and molecular aspects for the betterment of human health.

Purpose of Research

According to us, little information is available about *Heterophragma adenophyllum* for its application as a natural source of antioxidants. Purpose of this research is to measure the phytochemical and antioxidant activity of different fraction of *Heterophragma adenophyllum*. This plant has been least experimented among herbal plants till now.

Future Aspects of Research

Compounds with antioxidant activity possess ability to decrease or prevent cell degeneration and decay caused by free radicals. This quality has increased the demand for such compounds at both traditional and industrial level. Search for new herbal plants with potential amount of antioxidants is increasing along with research

to develop antioxidants to meet the boosting requirements of pharmaceutical industry.

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