IJPSR (2016), Vol. 7, Issue 4 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 06 January, 2016; received in revised form, 18 March, 2016; accepted, 27 March, 2016; published 01 April, 2016

COMPARATIVE *IN-VITRO* BIOLOGICAL STUDY OF AERIAL PARTS OF PLANT *HAMELIA PATENS*

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Key words:

Hamelia patens, standardization, total phenolics, Total flavonoids, DPPH, ABTS, FRAP assay

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ABSTRACT: Background: The plant *Hamelia patens* belong to a family Rubiaceae, which is rich in active phytochemicals like flavonoids and alkaloids. Although Plant has been traditionally used for the treatment of various ailments still no systematic pharmacognostical, phytochemical and pharmacological work has ever been carried out on this potential plant. **Aim:** The present study was carried out for the standardization and determination of *in vitro* antioxidant activity of the aqueous and methanolic extracts of aerial parts of plant *Hamelia patens*. **Method:** After the determination of all the standardization parameter, preliminary phytochemical analysis was done following various qualitative procedures for the determination of alkaloids, flavonoids, etc. Quantitative phytochemical analysis was performed for the determination of total phenolics by Folin-Ciocalteu method, total flavonoid by Aluminium chloride colorimetric assay and *in-vitro* antioxidant properties were evaluated by DPPH, ABTS and FRAP method. **Result:** Phytochemical analysis revealed the presence of various phytoconstituents like alkaloids, flavonoids etc. The highest amount of phenolic was found in the methanolic stem extract i.e. 236.22 mg of GA/g of extract while highest flavonoid content was found in methanolic leaf extract i.e. 331.54mg of RU/g of extract. Methanolic leaf and stem extract and aqueous leaf extract showed antioxidant potential equivalent to standard by DPPH, ABTS and FRAP method respectively. **Conclusion:** The study concludes that plant Hamelia patens is a rich source of phenols and flavonoids, and also showed good *in-vitro* antioxidant activity by all three methods. Thus, the plant *Hamelia patens* can be explored as a potential source of natural antioxidant.

INTRODUCTION: *Hamelia patens* Jacq. commonly known as "redhead," "scarlet," or "firebush" belongs to the Madder family (Rubiaceae). It is a perennial bush, and requires full sun and shade for growth. It grows to about 6 feet. *Hamelia patens* is rich in pentacyclic oxindole alkaloids: isopteropodine, rumberine, palmirine, maruquine and alkaloid A, B and C, other chemical constituents are apigenin, ephedrine, flavanones, isomaruquine, narirutins, pteropodine, rosmarinic acid, seneciophylline, speciophylline and tannin.[1]

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.7(4).1793-08

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.7 (4).1793-08

Therapeutic potential of *Hamelia patens* extracts are directly related to total phenolic and flavonoid content present in plant. The objective of the present study is to determine the total phenolics, flavonoids and antioxidant potential of plant *Hamelia patens.*

Since ancient times, the medicinal properties of plants have been examined due to their impactful pharmacological properties.[2] WHO estimates that 80% of the population living in rural areas are dependent on herbal medicine for their health needs.[3] Crude plant extracts in the form of infusion, decoction, tincture or herbal extracts have been traditionally used by the population for the treatment of various diseases. Although their efficacy and mechanisms of action have not been investigated scientifically, but it is always

considered that these medicinal preparations often exert beneficial responses due to the presence of the active chemical constituents.[4] The organic compounds usually related with physiological actions on the human body include alkaloids, phenols, flavonoids, tannins, terpenoids and steroids.[5] Ayurveda is the most ancient health care system and is practiced widely in India, Srilanka and other countries of the world. In the western world documentation of use of Natural substances for medicinal purposes can be found as far back as 78 A.D., when Dioscorides wrote "De Materia Medica", describing thousands of medicinal plants.[6]

Natural bioactive compounds like phenols and flavonoid compounds are very important plant constituents showing antioxidant activity by preventing the decomposition of hydroperoxides into free radicals or by inactivating lipid free radicals.[7] The continued search on plant secondary metabolites for natural antioxidants has gained importance in recent years because of the increasing awareness of herbal remedies as potential sources of phenolic oxidants. It is well known that phenolic compounds possesses biological properties like antioxidant, anti-aging, protection from cardiovascular diseases, anti-carcinogen, immune/autoimmune diseases and brain dysfunctions viz. Alzheimer's, Parkinson's etc.[8]

Prior to any research on herbal extracts, it is very crucial to estimate and analyze the standardization parameters of any medicinal plant.[9] Standardization of the herbal drug provided the data which will be helpful in the correct identification and authentication of medicinal plant, prevention of its adulteration and helps to utilize this lesser known drug in further research studies to determine various pharmacological activities the plant possess.

MATERIAL AND METHODS:

Collection and Authentication of plant *Hamelia patens:*

The fresh leaves and stems of *Hamelia patens* were collected in August and September 2014 from college campus DIPSAR, Pushp Vihar, New Delhi, India. The crude leaves and stems were authenticated by Dr. Sunita Garg, Chief scientist, Raw Material Herbarium and Museum, Delhi (RHMD), Council of Scientific and Industrial Research (CSIR), National Institute of Science Communication and Information Resources (NISCAIR), Pusa Campus, New Delhi under Ref. No. NICCAIR/RHMD/consult/ 2015/ 2873/66, Dated 13/08/2015.

Macroscopy and microscopy study: [10, 11, 12]

Morphological characters of leaves and stems like shape, size, color, odor and texture were examined by following standard procedures. For microscopical study, a thin transverse section (T.S.) of leaf and stem of plant *Hamelia patens* were taken on a clean glass slide, decolorized using chloral hydrate and was moistened with phloroglucinol solution, then few drops of conc. HCl were added and allowed to stand for 5 minutes, followed by addition of 2 drops of glycerine then covered with a cover slip and observed under motic microscope using 10X and 40X lens.

Fluorescence analysis: [13]

Fluorescence characteristics of the powdered drug (leaves and stem) were observed in day light (254nm) and ultraviolet light (366 nm) in UV chamber by treating with different chemicals.

Preparation of plant extract: [14]

Air dried coarsely powdered leaves (400g) and stems (400g) of plant *Hamelia patens* were extracted with methanol and doubled distilled water separately by continuous hot percolation using soxhlet apparatus for 48 hours. Methanolic and aqueous extracts were lyophilized, covered with paraffin foil, stored in vacuum desiccator at room temperature for further study of different in-vitro biological activities.

Phyto-chemical screening: [13, 15, 16, 17]

Aqueous and methanolic extracts of the leaf and stem of plant *Hamelia patens* were tested for the presence of different phytochemical constituents by performing the standard tests such as Mayer's test, Dragendorff's test, Wagner's test and Hager's test for Alkaloids, Libermann-Burchard test and H_2SO_4 test for sterols, Shinoda's test, H_2SO_4 and alkaline reagent test for Flavonoids, Fehling's test and

Benedict's test for reducing sugars, potassium dichromate test and lead acetate test for tannins, foam test for saponins, Salkowski test for terpenoids, modified Borntrager's test for anthraquinones, Raymond test and Keller Kiliani tests for cardiac glycosides and the ferric chloride ($FeCl_3$) test for phenols.

Determination of the percentage yield of extract: The percentage yield of extracts was calculated from the following equation:

$$\% \text{ yield} = W_1 \times 100/W_2,$$

Where,

W_1 = Weight of the extract after the solvent evaporation and,

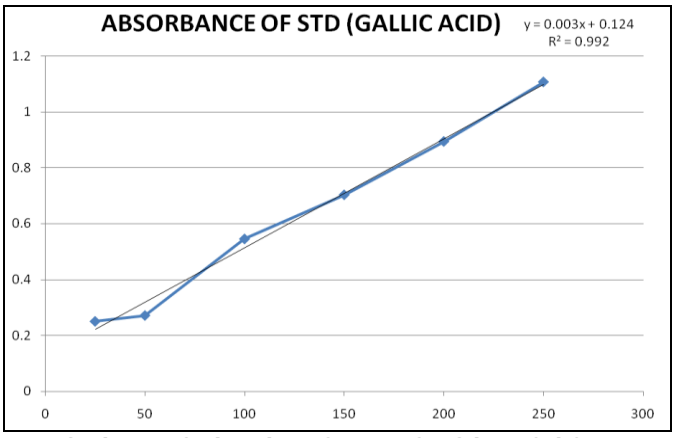
W_2 = Weight of powdered drug taken

Standardization Parameter: [18, 19, 20, 21, 22, 23]

All the standardization parameter was conducted following standard procedures.

Determination of total Phenolic Content: [24, 25, 26, 27, 28]

The amount of phenolics in the plant extracts of *Hamelia patens* was determined with Folin-Ciocalteu method using Gallic acid as a reference standard. The total phenolic content was calculated from the calibration curve, and the results were expressed as mg of gallic acid equivalent per g dry weight.



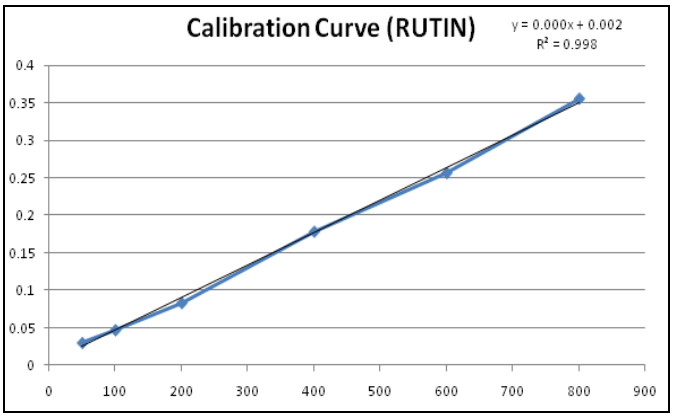
GRAPH 1: STANDARD CURVE OF GALLIC ACID

Procedure: 100 mg of Gallic acid and extracts were dissolved in 100 ml of methanol (1mg/ml) and then further diluted to 50, 100, 150, 200, 250 and 300µg/ml. 10 ml of distilled water, 1.5 ml of 2% F.C. reagent and 4 ml of 1M Na_2CO_3 solution were added to 1.5 ml of each dilution and final volume was made-up to 25ml using methanol. This mixture was incubated for 30 minutes at room temperature. After incubation absorbance was taken at 765 nm using UV/VIS spectrophotometer.

Blank Solution: 10 ml of distilled water, 1.5 ml of F.C. reagent and 4 ml of Na_2CO_3 solution were added to 1.5 ml of methanol and volume was made-up to 25 ml with methanol. This mixture was incubated for 30 minutes at room temperature. After incubation absorbance was taken at 765 nm using UV/VIS spectrophotometer.

Quantification of Flavonoid content: [24, 27, 29, 30]

Flavonoid Content was determined by the Aluminum Chloride Colorimetric Assay Method. The total flavonoid content was calculated from a calibration curve and the result was expressed as mg rutin equivalent per g dry weight.



GRAPH 2: STANDARD CURVE OF RUTIN

Procedure: To 1ml of each dilution of standard (Rutin) and extracts (50µg/ml, 100µg/ml, 200µg/ml, 400 µg/ml, 600 µg/ml and 800 µg/ml), 4 ml of water and 0.3ml of 5% $NaNO_2$ were added. After 5 minutes, 0.3ml of 10% $AlCl_3$ was added. After 6 min, 2ml of 1 M NaOH was added and the total volume was made up to 10 ml with distilled water. Then the solution was mixed well and the absorbance was measured against a freshly prepared blank at 510 nm.

Blank Solution: 4 ml water, 0.3 ml $NaNO_2$ was mixed, after 5 min 0.3ml of 10% $AlCl_3$ was added. After 6 min 2 ml of 1M NaOH was added and total volume was made up to 10 ml with distilled water.

Quantification of antioxidant activity

2, 2-Diphenyl-1-picryl-hydrazyl (DPPH) radical-scavenging assay: [31, 32, 33]

Procedure: 20 mg/L DPPH solution in methanol was prepared and 1.5 ml of this solution was added to 0.75 ml of the sample (extracts) and Ascorbic acid (standard) (20-100 µg/ml). The mixture was shaken vigorously and kept at room temperature for 30 minutes. Then the absorbance of the mixtures was measured at 517 nm. Water (0.75 ml) in place of the plant extract was used as control. The decrease in the absorbance indicated an increase in free radical scavenging activity.

This activity was calculated by the equation given below:

$$\text{DPPH Scavenging Effect (\%)} = \frac{Abs_0 - Abs_1}{Abs_0} X\ 100$$

A_0: Absorbance of the control reaction.
A_1: Absorbance in the presence of the extracts or standard.

The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage plotted against extract concentration.

FRAP Assay: [31, 34, 35, 36, 37, 38]

The method described measures the ferric reducing anti-oxidant power (FRAP) of the extracts. (Benzie &Strain et al. 1996)

Preparation of Reagents:

1. **300mmol/l acetate buffer**: 3.1g of sodium acetate.$3H_2O$ was mixed with 16 ml of glacial acetic acid and volume was making up to 1L with distilled water.

2. **10mmol/l TPTZ:** 0.031g of TPTZ was dissolved in 10ml 40mMHCl.

3. **40mMHCl**: 1.46 ml of conc. HCl was mixed with 1L of distilled water.

4. **20mmol/l Ferric Chloride**: 0.054g of $FeCl_3.6H_2O$ was dissolved in 10 ml of distilled water.

5. **1mmol/l Ferrous sulphate**: 0.278g of $FeSO_4.7H_2O$ was dissolved in 1L of distilled water.

Preparation of FRAP reagent:

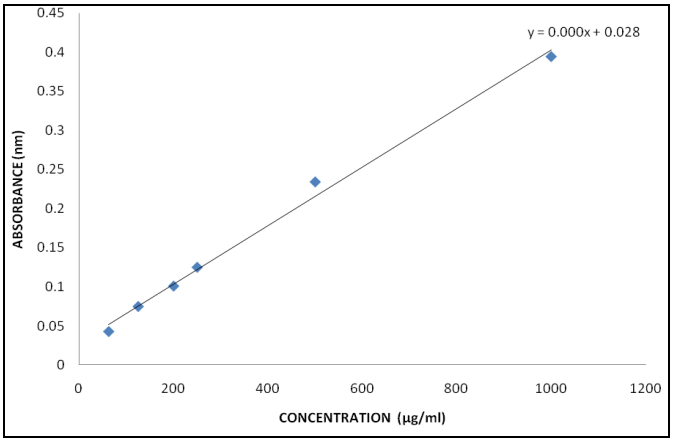
FRAP reagent was prepared by mixing 300 mmol/l of acetate buffer, 10 mmol/l of TPTZ and 20 mmol/l of Ferric Chloride in a ratio of 10:1:1.

The reagent was warmed at 37ºC for 10 minutes before use.

Blank: The FRAP reagent was used as blank.

Preparation of standard curve:

From the stock solution of 1mM (1000µM) of ferrous sulphate, following five dilutions were prepared 62.5, 125, 250, 500, 1000µM. The 0.2ml sample of each dilution ,was diluted with 0.6ml of distilled water and mixed with 6ml of FRAP reagent. After the reaction time of 4 to 8 Minutes, absorbance was determined at 593nm. The standard curve was obtained by plotting absorbance (on Y axis) vs. concentration (on X axis).



GRAPH 3: STANDARD CURVE OF FERROUS SULPHATE

Similarly, the absorbance for 1000µg/ml of each extracts and standard was determined at 593nm using UV/VIS Spectrophotometer.

The results were expressed as the FRAP value i.e. the FRAP value in µmol/l. This was calculated as follows:

$$\text{FRAP value} = \frac{A_{593nm} \text{ of test sample (conc. 1000µg/ml) X } FeCl_3 \text{ concentration (µmmol/l)}}{4 \text{ min } A_{593nm} \text{ of } FeCl_3 \text{ (at conc. 1000µg/ml)}}$$

ABTS radical scavenging assay: [31, 34, 39, 40, 41]

For ABTS assay, the stock solutions included 7mM $ABTS^+$ solution and 2.4mM potassium per sulphate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 hour at room temperature in the dark. The solution was then diluted by mixing 1ml $ABTS^+$. solution with 60 ml methanol to obtained an absorbance of 0.706±0.001 units at 734 nm using the spectrophotometer. Fresh $ABTS^+$ solution was then prepared for each assay.

Plant extracts/standard (Trolox) (1ml) of different concentration was allowed to react with 1ml of the $ABTS^+$ solution freshly prepared solution and the absorbance was taken at 734 nm after 7 min using the spectrophotometer.

The $ABTS^+$ scavenging capacity of the extracts was compared with that of standard (Trolox) and percentage inhibition calculated as:

$$\text{ABTS radical scavenging activity (\%)} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} X\ 100$$

$Abs_{control}$: The absorbance of ABTS radical + methanol.

Abs_{sample}: The absorbance of ABTS radical + extracts/standard.

Statistical analysis:

Data are expressed as mean ± SD from three separate observations. For assays one way ANOVA test followed by Tukey's test ($P < 0.05$) was used to analyze the differences among IC_{50} values of various fractions. A probability of $P < 0.05$ was considered as significant.

RESULTS:

TABLE 1: PERCENTAGE (%) YIELD DETERMINATION

S. No.	Part of Plant used	Solvent used for extraction	Weight of drug taken (gm)	Color of Extract	%Yield
1.	Leaf	Methanol	450	Dark green	11.37%
2.	stem	Methanol	300	Brown	7.71%
3.	Leaf	Distilled water	350	Dark Brown	12.47%
4.	stem	Distilled water	300	Brown	10.45%

TABLE 2: PRELIMINARY PHYTOCHEMICAL ANALYSIS OF EXTRACTS

S. No.	Chemical test	M. Leaf Ext.	M. Stem Ext.	Aq. Leaf Ext.	Aq. Stem Ext.
1.	**Test for Alkaloids**				
	Dragendorff's	+	+	+	++
	Hager's	++	++	++	++
	Wagner's	++	++	++	++
	Mayer's	++	++	++	++
2.	**Test for Glycosides**				
	Legal	++	++	++	++
	Keller-kiliani	++	++	++	+
	Borntrager's test	-	+	-	-
	Modified Borntrager's test	+	+	+	+
3.	**Test for Tannins & Phenolics**				
	5% $FeCl_3$	++	++	++	++
	Lead acetate	++	++	++	++
	Pot. Dichromate	-	+	+	+
	$KMnO_4$	+	+	+	+
4.	**Test for Proteins and Amino Acids**				
	Millon's	+	+	+	+
	5% $HgCl_2$	-	+	+	+

	5% $CuSO_4$	-	+	+	+
	Ninhydrin test	+	+	+	+
6.	**Test for Steroids**				
	Salkowski reaction	++	++	++	++
	Liebermann–Burchard	++	++	++	++
	Liebermann's reaction	++	++	++	++
7.	**Test for Flavonoids**				
	Shinoda	-	+	+	+
	Lead acetate	++	++	++	++
	$FeCl_3$ reagent	++	++	++	++
	Alkaline reagent	+	+	+	+
8.	**Test for Carbohydrate**				
	Molisch's test	+	+	+	+
9.	Test for Saponins				
	Foam test	+	+	-	

(+) Present, (-) Absent.

TABLE 3: FLUORESCENCE ANALYSIS OF THE POWDERED LEAVES

S. No.	Reagent	Day light	Short Wavelength (254nm)	Long Wavelength (366nm)
1.	Acetic acid	Brown	Dark green	Orange
2.	Conc. HCl	Green	Dark Green	Black
3.	Conc. H_2SO_4	Brown	Dark Green	Light Green
4.	5% $FeCl_3$	Black	Black	Black
5.	Conc. HNO_3	Brown	Dark Green	Black
6.	10% Picric acid	Yellow	Light Green	Black
7.	15% Aq. KOH	Black	Black	Black
8.	15% Alc. KOH	Brown	Green	Green
9.	I_2 solution	Brown	Dark Green	Black
10.	25% NH_3 solution	Dark Brown	Brown	Yellowish orange

TABLE 4: FLUORESCENCE ANALYSIS OF THE POWDERED STEM

S. No.	Reagent	Day light	Short Wavelength (254nm)	Long Wavelength (366nm)
1.	Acetic acid	Light brown	Dark green	Yellow
2.	Conc. HCl	Brown	Dark green	Black
3.	Conc. H_2SO_4	Brown	Dark green	Dark green
4.	5% $FeCl_3$	Black	Black	Black
5.	Conc. HNO_3	Brown	Dark green	Black
6.	10% Picric acid	Yellow	Light green	Black
7.	15% Aq. KOH	Brown	Dark green	Greenish yellow
8.	15% Alc. KOH	Brown	Green	Greenish orange
9.	I_2 solution	Brown	Dark green	Black
10.	25% NH_3 solution	Light brown	Light green	Yellowish green

TABLE 5 MACROSCOPIC STUDY OF *HAMELIA PATENS* LEAVES

S. No.	Character	Observations
1.	Color	Dark green
2	Odor	Pleasant and characteristic odor
3.	Shape	Elliptic to oval, Entire, short pointed at the apex, oblique at the base, wavy margin and in autumn the leaves turn deep red.
4.	Texture	Feathery on a stalk
5.	Size	1/4 inch long and 1/16 inch wide
6.	Surface	Glabrous surface having dense villous hair above and beneath.

TABLE 6: MACROSCOPIC STUDY OF *HAMELIA PATENS* STEM

S. No.	Character	Observations
1.	Color	Brown
2.	Odor	Odorless
3.	Shape	Cylindrical
4.	Texture	Soft hairy

Transverse Section (T.S.) of Leaf:

Transverse section of leaf showing typical unicellular non-lignified trichomes, paracytic stomata, vascular bundle in the lower bulge covered with pericyclic fibers towards the lower surface. The laminar region showed single layer of palisade cells and raphides in the mesophyll cells.

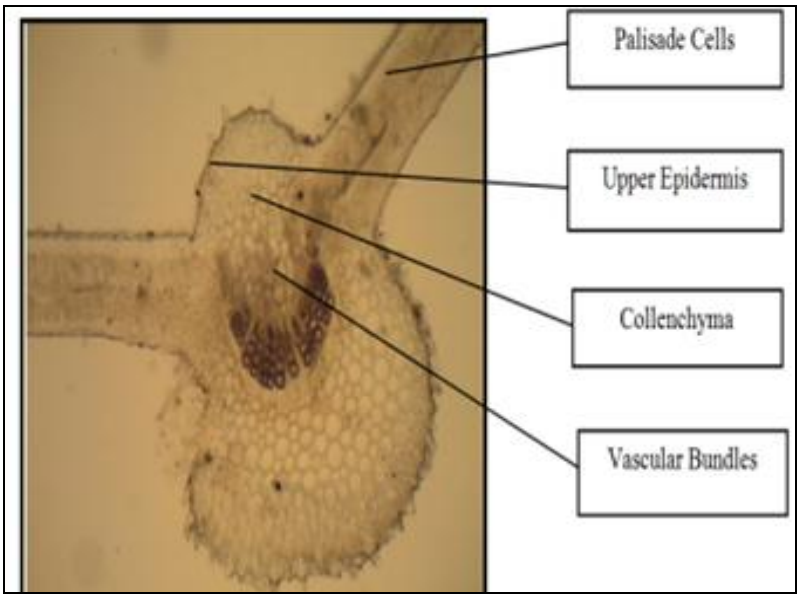


FIG.1: T.S. OF LEAF (20X)

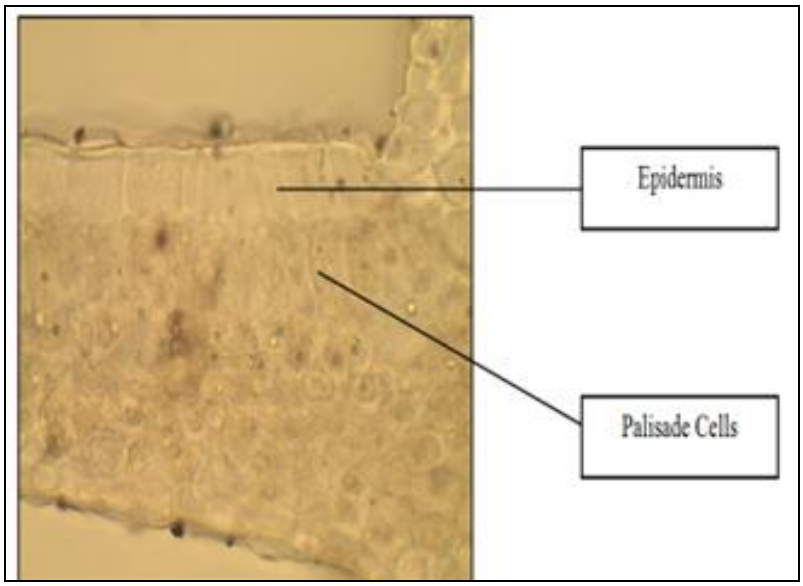


FIG.2: T.S. OF LEAF SHOWING PALISADE CELLS (40X)

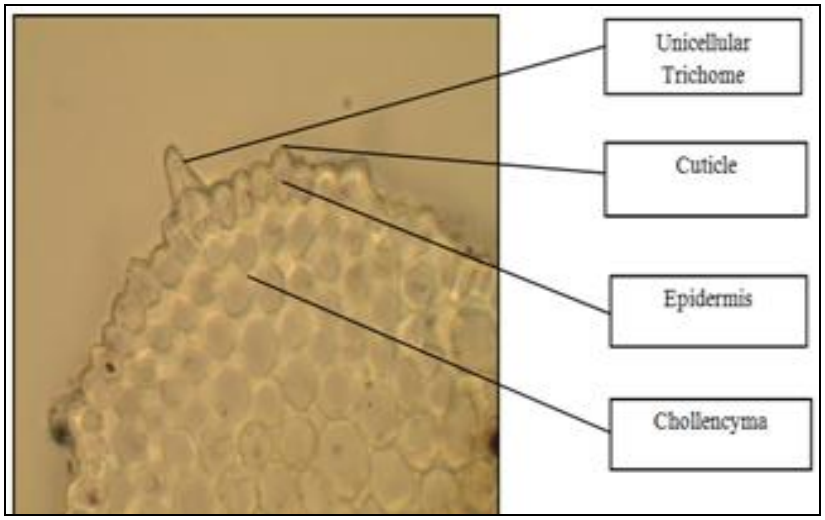


FIG.3: MAGNIFIED VIEW OF LEAF SHOWING CUTICLE AND UPPER EPIDERMIS (40X)

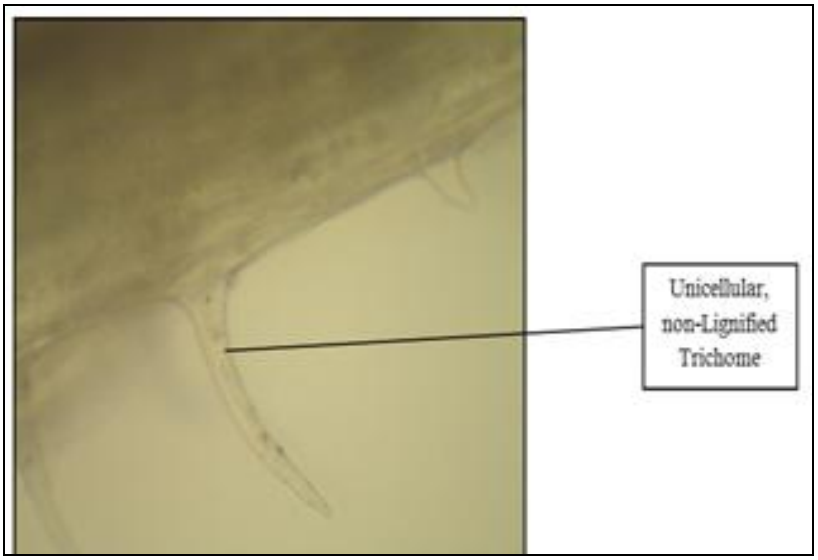


FIG.4: T.S. OF SHOWING MAGNIFIED VIEW OF TRICHOMES (40X)

Transverse Section of Stem:

Transverse section of stem showed single layer of epidermis, parenchymatous cortex, endodermis, pericyclic fibers, phloem, xylem and medullary rays in the continuous ring and parenchymatous pith.

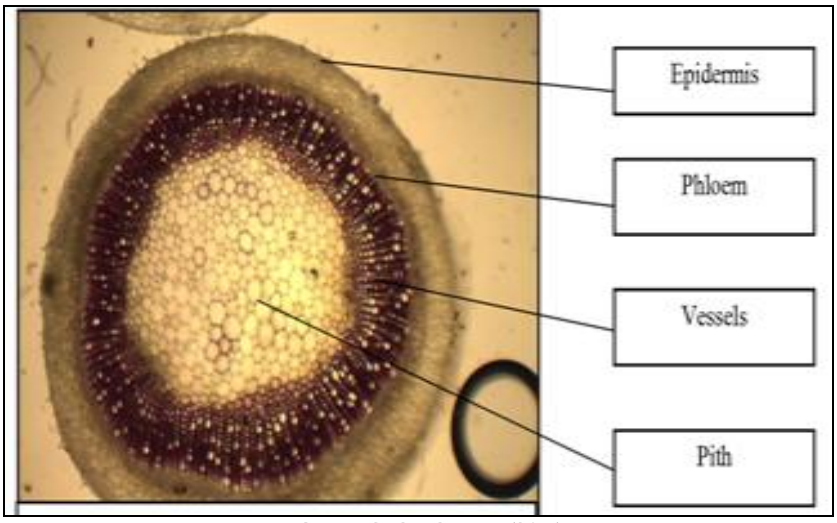


FIG.5: T.S. OF STEM (20X)

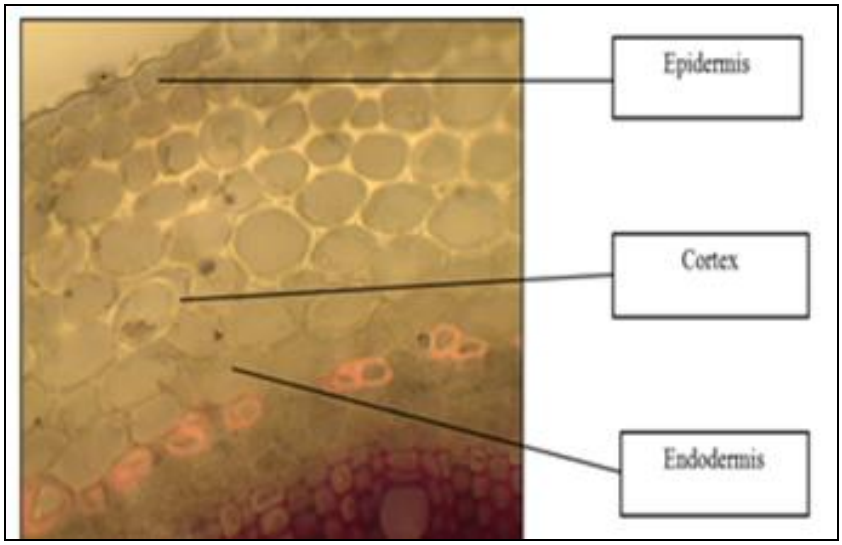


FIG.6 T.S. OF STEM SHOWING MAGNIFIED VIEW OF UPPER PORTION (40X)

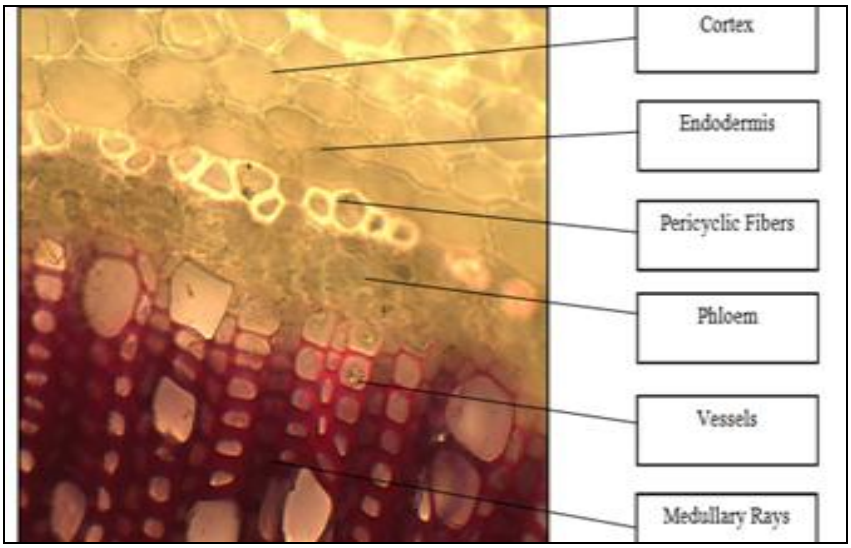


FIG.7: MAGNIFIED VIEW OF MIDDLE PORTION OF STEM (40X)

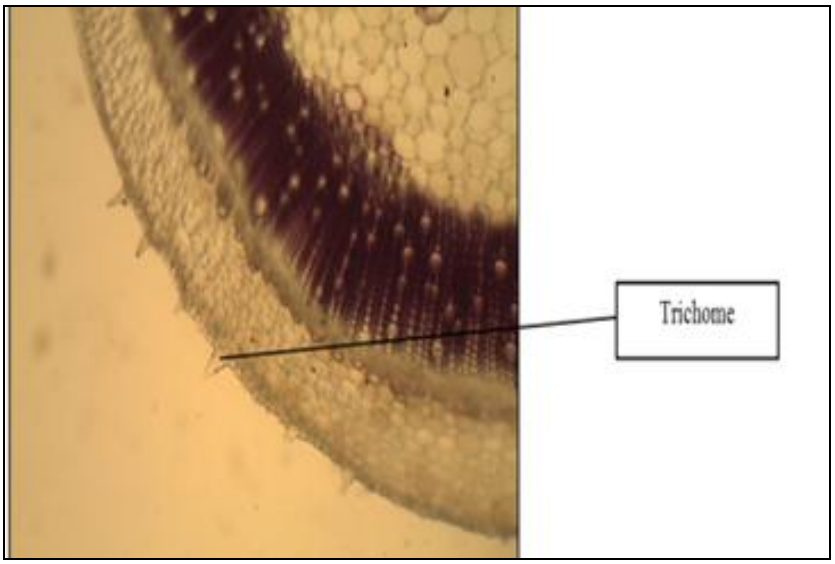


FIG.8: T.S. OF STEM SHOWING MAGNIFIED VIEW OF TRICHOMES (40X)

TABLE 7: STANDARDIZATION PARAMETER

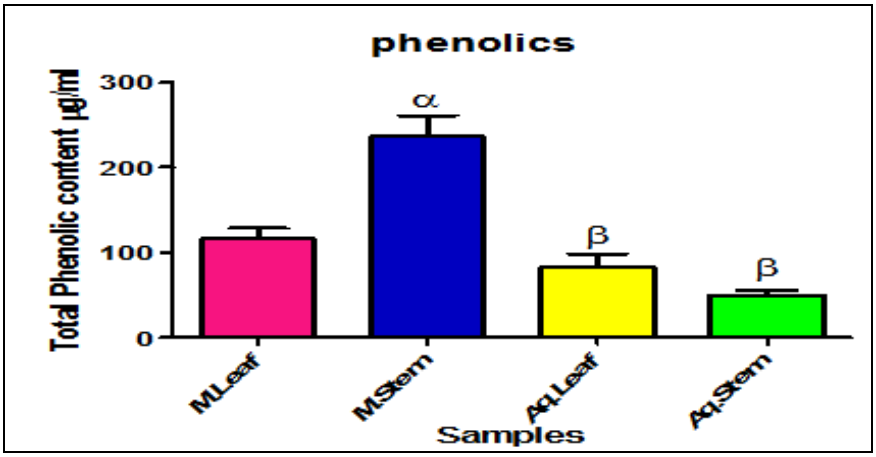
Standardization parameter	Result	
	Leaf	Stem
Total Ash value	6.617±0.295	5.427±0.089
Acid-insoluble Ash Value	0.988±0.05	2.182±0.025
Water-soluble Ash Value	3.285±0.299	1.788±0.127
Cold Water Soluble Extractive	12.57±1.29	5.71±0.02
Hot Water Soluble Extractive	14.77±1.68	7.30±1.24
Alcohol Soluble Extractive	2.64±0.73	1.80±0.52
Loss on Drying	0.296±0.09	0.259±0.07
Swelling Index	Nil	Nil
Foaming index	100	Less than 100
Aflatoxin B1, B2, G1, G2	Nil	Nil
Total Bacterial count (cfu/g)	Less than 10	235
Enterobacteriaceae/g	205	Less than 10
Heavy metal detection (Arsenic, Mercury, Cadium, Lead)	Not detected	Not detected

TABLE 8: ELEMENTAL ANALYSIS

Element	Iron	Zinc	Potassium	Calcium
Leaf	460ppm	44ppm	6898ppm	63696ppm
Stem	188ppm	27ppm	7838ppm	5907ppm

TABLE 9: TOTAL PHENOLIC CONTENT OF DIFFERENT PLANT EXTRACT

S. No.	Plant Extract	Total Phenolic Content (mg of GA/g of extract)
1.	Methanolic leaf	113.33±21.63
2.	Methanolic stem	$236.22±43.11^{\alpha}$
3.	Aqueous leaf	$82.5±28.16^{\beta}$
4.	Aqueous stem	$49.83±10.53^{\beta}$

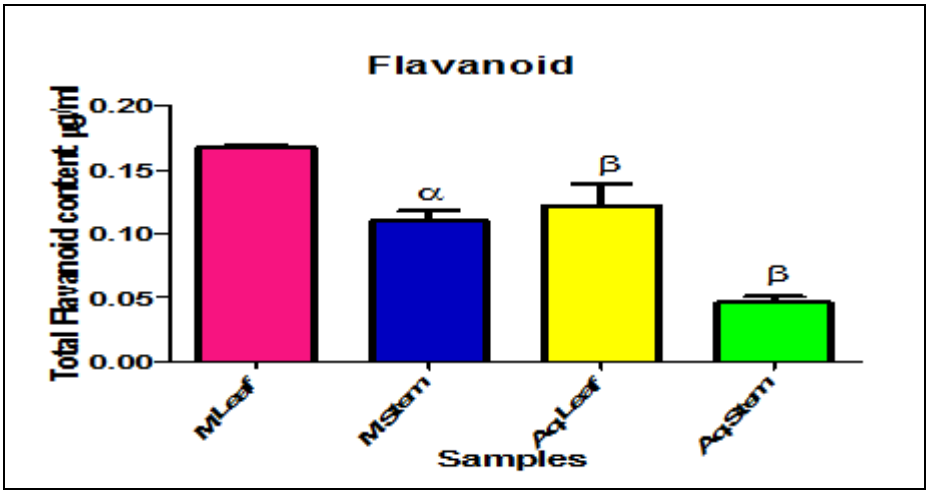


GRAPH 4: TOTAL PHENOLIC CONTENT OF DIFFERENT EXTRACTS
All values are Mean ± SD, $^{\alpha}p<0.05$ with STD, $^{\beta}p<0.05$ with M. stem and Aq. leaf.

The total content of phenolic compounds varied between 80 to 260 mg of GA/g of extract among the different extracts of leaf and stem of *Hamelia patens* (**Table 9**). The highest amount of phenolic was found in the methanolic stem extract i.e. 236.22 mg of GA/g of extract while the aqueous stem extract had the least 49.83 mg of GA/g of extract phenolic content.

TABLE 10: TOTAL FLAVONOID CONTENT OF DIFFERENT PLANT EXTRACTS

S. No.	Plant Extract	Total Flavonoid Content (mg of RU/g of extract)
1.	Methanolic leaf	331.54±95.18
2.	Methanolic stem	$212.80±35.87^{\alpha}$
3.	Aqueous leaf	$245.73±95.34^{\beta}$
4.	Aqueous stem	$89.02±20.37^{\beta}$



GRAPH 5: TOTAL FLAVONOID CONTENT OF DIFFERENT EXTRACTS.

All values are Mean ± SD, $^{\alpha}p<0.05$ with STD, $^{\beta}p<0.05$ with M. stem and Aq. leaf.

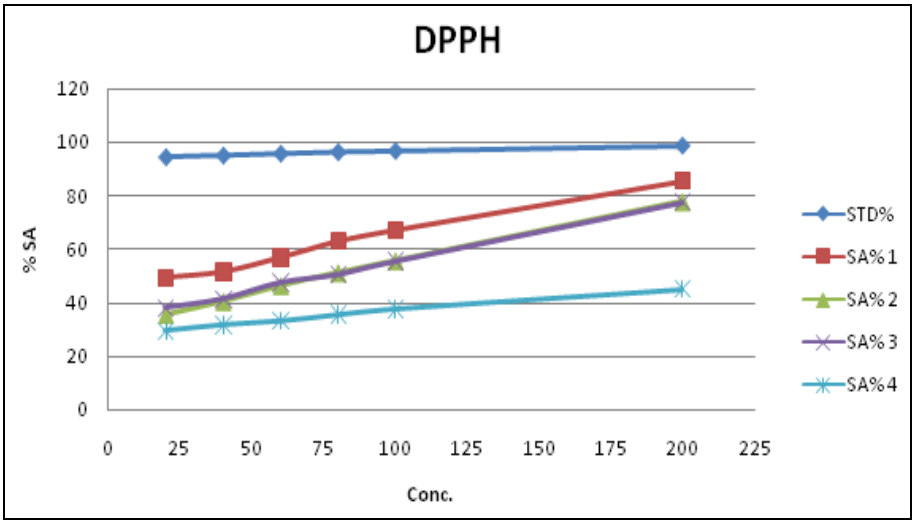
Methanolic leaf extract showed the highest flavonoid content of 331.54±95.18 mg of RU/g of extract and Aqueous stem extract showed the lowest flavonoid content of 89.02±20.37 mg of RU/g of extract.

Antioxidant activity:

DPPH Assay

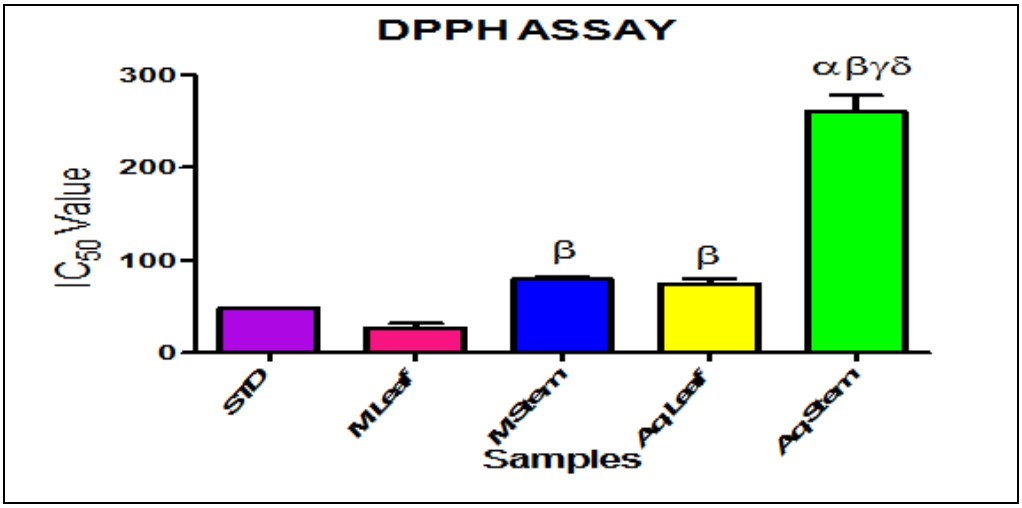
TABLE 11: IC_{50} VALUE OF STANDARD AND EXTRACTS (GRAPH 6)

S. No.	Standard/Extracts	IC_{50} Value
1.	Ascorbic acid (Standard)	22.09333±6.42
2.	Methanolic leaf	27.283±4.96
3.	Methanolic stem	$79.60±3.04^{\beta}$
4.	Aqueous leaf	$74.86±7.98^{\beta}$
5.	Aqueous stem	$263.74±30.28^{\alpha\beta\gamma\delta}$



GRAPH 6: SCAVENGING ACTIVITIES OF STANDARD AND DIFFERENT EXTRACTS

Where,
SA% 1 - Methanolic leaf extract
SA% 2 - Methanolic stem extract
SA% 3 - Aqueous leaf extract
SA% 4 - Aqueous stem extract
STD% - Standard (Ascorbic acid)



GRAPH 7: IC $_{50}$ VALUE OF STANDARD AND DIFFERENT EXTRACTS

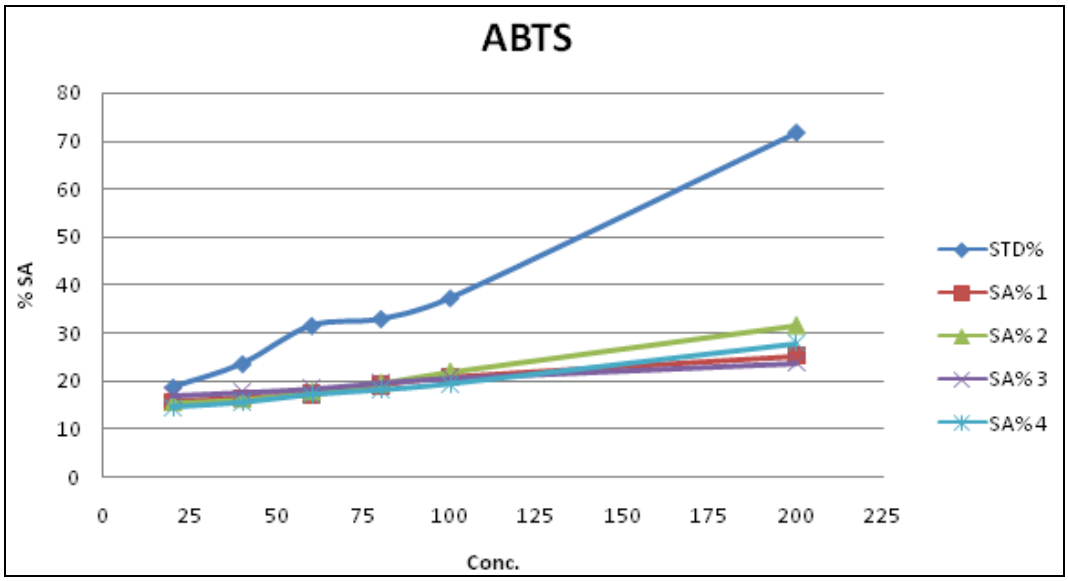
All values are Mean ± SD, $^{\alpha}p<0.05$ with STD, $^{\beta}p<0.05$ with M. leaf, $^{\gamma}p<0.05$ with M. stem, $^{\delta}p<0.05$ with Aq. leaf.

Methanolic and aqueous extract of leaf and stem exhibited varying degrees of antioxidant activity (**Table 11).** The methanolic leaf extract of the plant exhibited higher value in total antioxidant activity expressed as the lowest of the amount of sample (µg/ml), needed for 50 % decrease of the initial DPPH concentration (IC_{50}) were 27.28µg/ml, which had significantly greater antioxidant effect than aqueous solvents. The difference was probably due to the characteristics of the solvent.

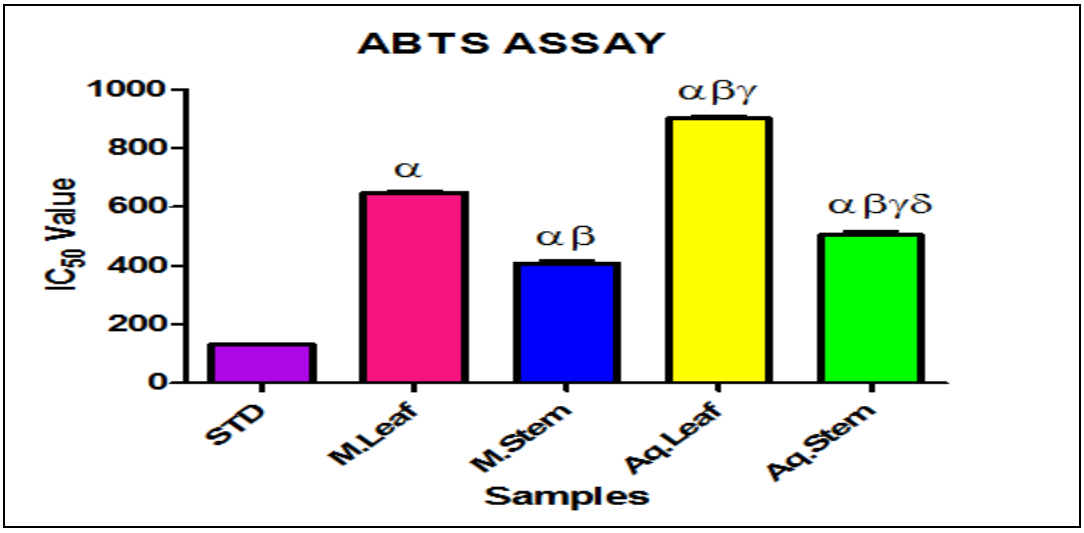
ABTS Assay:

TABLE 13: IC$_{50}$ VALUE OF STANDARD AND EXTRACTS (GRAPH 8)

S. No.	Standard/Extracts	IC$_{50}$ Value (µg/ml)
1.	Trolox (Standard)	131.906±0.598
2.	Methanolic leaf	$648.953\pm6.271^{\alpha}$
3.	Methanolic stem	$407.33\pm18.27^{\alpha\beta}$
4.	Aqueous leaf	$900.47\pm13.19^{\alpha\beta\gamma}$
5.	Aqueous stem	$503.440\pm23.69^{\alpha\beta\gamma\delta}$



GRAPH 8: SCAVENGING ACTIVITIES OF STANDARD AND DIFFERENT EXTRACTS



GRAPH 9: IC_{50} VALUE OF STANDARD AND DIFFERENT EXTRACTS

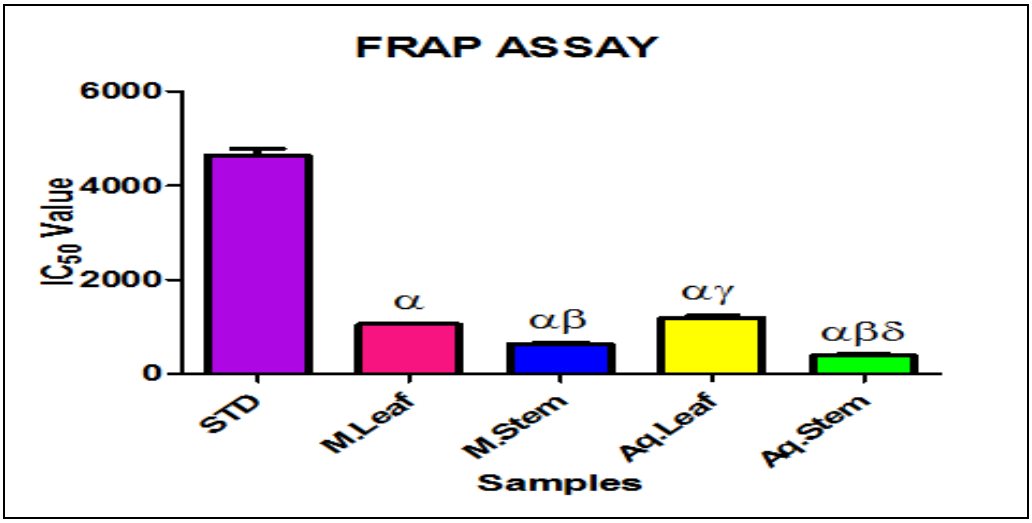
All values are Mean ± SD, $^{\alpha}p$ <0.05 with STD, $^{\beta}p$<0.05 with M. leaf, $^{\gamma}p$ <0.05 with M. stem, $^{\delta}p$<0.05 with Aq. leaf.

The plant extracts exhibited varying degree of antioxidant activity (**Table 13**). Methanolic stem extract showed the minimum IC_{50} value of 407.33µg/ml while aqueous leaves extract showed the maximum IC_{50} value of 900.47µg/ml with minimum percentage radical effect

FRAP Assay:

TABLE 14: FRAP VALUE OF DIFFERENT EXTRACTS AND STANDARD

S. No.	Standard/Extracts	FRAP Value
1.	Ferrous sulphate (standard)	4635.667±263.81
2.	Methanolic leaf	$1055.003\pm42.12^{\alpha}$
3.	Methanolic stem	$636.5\pm50.12^{\alpha\beta}$
4.	Aqueous leaf	$1199.72\pm61.55^{\alpha\gamma}$
5.	Aqueous stem	$391.07\pm17.25^{\alpha\beta\delta}$



GRAPH 10: IC $_{50}$ VALUE OF STANDARD AND DIFFERENT EXTRACTS

All values are Mean ± SD, $^{\alpha}p$<0.05 with STD, $^{\beta}p$<0.05 with M. leaf, $^{\gamma}p$<0.05 with M. stem, $^{\delta}p$<0.05 with Aq. leaf.

FRAP values was obtained by comparing the absorption change in the test mixture with those obtained from increasing concentration of Fe^{3+} and expressed as Mm (millimole) of Fe^{2+} equivalent per liter of sample. The aqueous leaf extract had highest FRAP value of 1199.72±61.55µmole/l, thus aqueous leaf extract showed good antioxidant activity by FRAP method.

TABLE 15: COMPARISON OF IC_{50} VALUE OBTAINED BY DIFFERENT ANTIOXIDANT METHOD OF STANDARD AND EXTRACTS

Standard and Extracts	Different methods used for anti-oxidant activity				
	Total Phenolic Content (mg of Gallic acid/g of extract)	Total Flavonoid Content (mg of Rutin/g of extract)	IC_{50} Value (µg/ml) DPPH	IC_{50} Value (µg/ml) ABTS	FRAP Value (µmol/l) FRAP
Standard			22.09333±6.4	131.61±.900	4635.667±263.81
M. Leaf	113.33±21.6	331.54±95.1	27.283±8.603	648.953±6.27	1055.003±42.12
M. Stem	236.22±43.1	212.80±35.8	79.603±5.278	407.33±18.27	636.5±50.12
Aq. Leaf	82.5±28.16	245.73±95.3	74.86±7.98	900.47±13.19	1199.72±61.55
Aq. Stem	49.83±10.53	89.02±20.37	263.74±30.28	503.440±23.6	391.07±17.25

DISCUSSION: WHO survey indicates that about 70–80% of the world's populations depend on non-conventional medicine, mainly of herbal origin, for their primary healthcare.[42] These medicinal plants are rich sources for naturally occurring antioxidants especially phenolic and flavonoid content.[43] These agents have ability to scavenge free radicals, super oxide and hydroxyl radicals etc, thus they enhance immunity and antioxidant defence of the body.[44]

For acceptance of medicinal plants into scientific medicine, it is necessary that their effectiveness and safety be evaluated and confirmed through active ingredient testing. The extractive capability of phenolic and flavonoid components of plant material is considerably depended on the type of solvent.[44] Highest content of phenolic and flavonoids in methanolic extract in comparison to aqueous solvent, make this organic solvent (methanol) an ideal and selective to extract a great number of bioactive phenolic compounds from the plant *Hamelia patens*.

In present study, the methanolic extracts of leaf and stem showed the high concentration of phenols and flavonoids. Therefore, methanolic leaf and stem extracts of *Hamelia patens* have greater potential to reduce or scavenge free radicals or produces more beneficial effects as compared to aqueous extracts of leaf and stem. A positive correlation was noted between the total phenolic content and antioxidant activity in both the DPPH and ABTS assay, while no significant correlation was observed between the DPPH, ABTS, and FRAP assay and total flavonoid, suggesting that the level of antioxidant activity in these plants varies greatly but the total phenolic in the plant extracts provided a substantial antioxidant activity.

CONCLUSION: The plant *Hamelia patens* justifies its role in traditional claims due to presence of polyphenols, flavonoids, alkaloids etc. It is noticed that the highest concentration of phenolic compounds in the extract were obtained using solvents of high polarity; the methanolic extract manifested greater power of extraction for phenolic compounds from the plant *Hamelia patens*. The high content of phenolic compounds and significant linear correlation between the values of the concentration of phenolic compounds and antioxidant activity of leaf and stem of *Hamelia patens* indicated that these compounds contribute to the strong antioxidant activity and thus can be a source of safer natural antioxidants. Further investigation and proper isolation of more active principles might help in the finding new lead compounds which will be effective against free radical mediated diseases. Also, further studies of this plant species should be directed to carry out *in-vivo* studies of its medicinal active components in order to prepare natural pharmaceutical products of high value.

ACKNOWLEDGEMENTS: The author wish to acknowledge the department of herbal drug technology (HDT), DIPSAR , New Delhi for providing the environment, materials, technologies to make this work a success.

DISCLOSURE: The authors have no conflicts of interest to declare.

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How to cite this article:
Singh S and Vyas M: Comparative *In-Vitro* Biological Study of Aerial Parts of Plant *Hamelia Patens.* Int J Pharm Sci Res 2016; 7(4): 1793-08.doi: 10.13040/IJPSR.0975-8232.7(4).1793-08.