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EVALUATION OF *IN VITRO* ANTIOXIDANT ACTIVITY AND TOTAL PHENOLIC CONTENT OF ETHANOL AND AQUEOUS EXTRACTS OF POLYHERBAL FORMULATION

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ABSTRACT

To evaluate the *in-vitro* antioxidant potential of polyherbal preparation, a combination of six selected medicinal plants. The antioxidant potential of the aqueous extract and ethanolic extract of *Ocimum sanctum*, *Withania somnifera*, *Gymnema sylvestre*, *Tinospora cordifolia*, *Phyllanthus emblica* and *Coleus forskohlii*. Antioxidant potential was investigated by using *in vitro* assay like ABTS radical scavenging assay, DPPH radical scavenging activity and Total phenolic content. The effects were related with standard (Gallic acid), a known antioxidant. Various Phytoconstituents identified in the above selected plants extracts were poly phenols, flavonoids, terpenoids, tannins, alkaloids. The terpenoids were stated to possess the scavenging properties against the free radicals. The presence of these Phytoconstituents in selected plants might be responsible for antioxidant activity; gallic acid is used as a standard. *Conclusion:* Polyherbal formulation is better than individual plant extracts.

KEYWORDS: Polyherbal formulation (PHF), phytochemicals, Anti-oxidant activity and Gallic acid.

INTRODUCTION

Reactive oxygen species [ROS], sometimes called active oxygen species, are various forms of activated oxygen, which include free radicals such as superoxide ions (O_2^-) and hydroxyl radicals (OH.), as well as non-free .radical species such as hydrogen peroxide (H_2O_2).[1] These ROS play an important role in degenerative or pathological processes, such as aging,

cancer, coronary heart disease, Alzheimer's disease, neurodegenerative disorders, atherosclerosis, cataracts and inflammation.[2]

In living organism various ROSs are formed in different ways, including normal aerobic respiration, stimulated polymorphonuclear leukocytes, macrophages and perioxisomes. These appear to be the main endogenous sources of most of the oxidants produced by cells. Exogenous sources of free radicals include tobacco smoke, ionizing radiation, certain pollutants, organic solvents and pesticides.[1] The term antioxidant refers to the activity of numerous vitamins, minerals and other phytochemicals to protect the damage caused by ROS.[3] Antioxidant defence system scavenges and minimizes free radicals formation. The actions of free radicals are counteracted by antioxidants, either endogenous or exogenous.[4] The therapeutic effects of several medicinal plants are usually attributed to their antioxidant phytochemicals. It has been suggested that there is an inverse relationship between dietary intake of antioxidant rich food and incidence of human disease.[1] Synthetic antioxidant compounds, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are used in processed food but they have side effects.

Plant based antioxidants are now preferred to synthetic ones because of safety concerns.[5] Therefore, research regarding antioxidant potential from plant source is important. In the present study a polyherbal formulation of hot water extract and ethanolic extract of 6 selected plants is evaluated for the *in vitro* antioxidant properties using various experimental models.

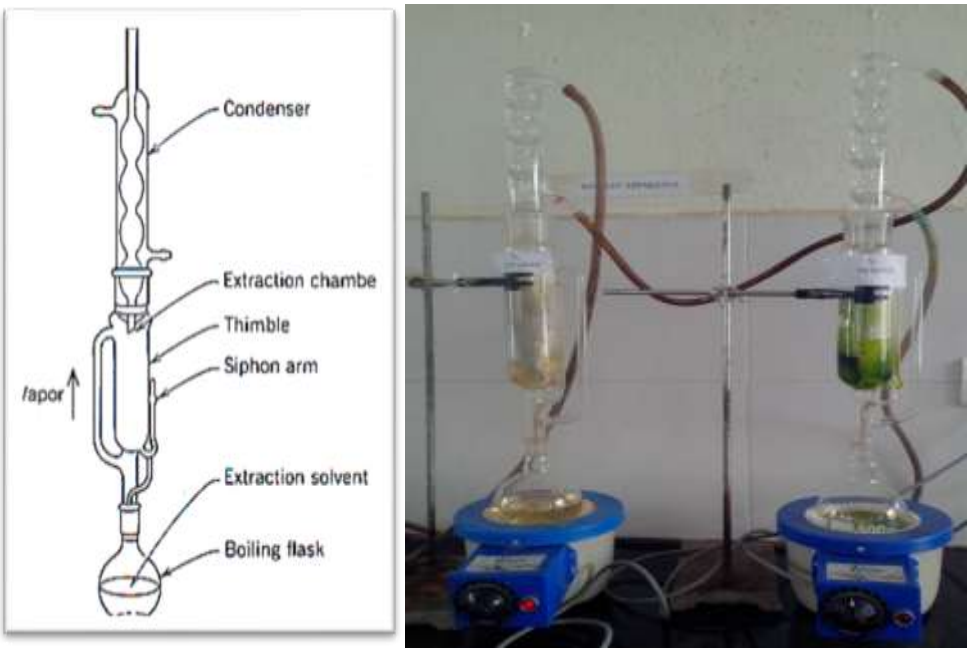
MATERIAL AND METHODS

Collection of plant material: The present study is aim to screen the synergetic Antidiabetic potential of six selected antidiabetic plants of western ghat. The plant materials were collected from western ghat and Bangalore rular zone. In the polyherbal preparation, leaves of *Tinnospora cordifolia, ocimum sanctum, Gymnema sylvestre* were selected, where as in the plants like *Withania somnifera* and *Coleus forskohlii*, the root part of the plant were selected, where as in the plant *Phyllanthus emblica*, the fruit portion of the plant was selected to prepare the polyherbal formulation to screen the antidiabetic and antioxidant therapeutic value through in vitro and in vivo studies.

PREPARATION OF PLANT'S EXTRACTS

Air dried plant material was crushed, powdered and extracted using soxhlet apparatus with polar and non-polar solvent. The solvent used were hot water, cold water, ethanol, methanol,

petroleum ether and n-hexane. Cold water extracts was obtained by adding distilled water to the crushed material in ratio 1:4 and kept in rotary shaker for 24h at 30 degree Celsius and at 130 rpm respectively. After 24 h it was filtered and the resulting extract was stored in refrigerator. Hot water extracts was obtained by soxhlet extraction were 400ml of distilled water to 100 grams of plant powders. Similarly medicinal powders were extracted using organic solvent in ratio 1:3 by soxhlet extraction.[6, 7]



METHODS

Test of extracted material from different part of plant (phytochemical analysis).

Test for the presence of different Bio-components such as alkaloids, flavonoids, glycosides, saponins, phenols, steroids and terpenoids were performed using standard procedures.

Table: 1 PHYTOCHEMICAL TEST

Sl.no	Test performed	Observation	Inference
1.	**Test for carbohydrates** **Molisch test:** To 1mlof extract add 2 drops of Molisch reagent mix well. Then add 3ml of concentrated sulphuric acid along the sides of the test tubes keeping the tube in an inclined position.	Formation of purple to violet ring appears at the junction of two liquids.	Carbohydrates present.
2.	**Test for glycosides** **Keller killani test:** To 1ml of extract add 0.4ml of glacial acetic acid cool then add 2 drops of ferric chloride solution and 2ml of concentrated sulphuric acid by sides of the tube	Formation of reddish brown colour at junction of two liquid layer	Indicates presence of cardiac glycosides.

3.	**Test for alkaloids** **Mayer's test:** Extract +4-5 ml of dilute HCL (0.1N) shake well and add Mayer's reagent.	White or yellow cream precipitate	Presence of alkaloids.
4.	**Steroids and triterpenoid test** **Salkowski test:** To 2ml of extract add 2ml of chloroform and few drops of concentrated sulphuric acid by sides of test tube	Red colour at lower layer. Yellow colour at lower layer.	Steroids present. Triterpenoid present.
5.	**Flavonoid test** **Shinoda test:** Extract with fragments of magnesium ribbon and concentrated Hydrochloric acid	Shows pink scarlet red or green to blue color after few minutes	Flavonoid present.
6.	**Phenol test** **Ellagic acid test:** Extract plus 3drops of 5% Sodium nitrate solution	Muddy or niger brown precipitate	Phenol present
7.	**Tannins test** **Ferric chloride test:** Extract plus 1% ferric chloride solution.	Blue color Green color	Hydrolysable tannins Condensed tannins
8.	**Test for saponin** **Froth test:** To 2ml of extract add 1ml of distilled water shake well	Formation of foam.	Indicates presence of saponins.

DPPH radical scavenging activity: The DPPH assay method is based on the reduction of DPPH, a stable free radical. The free radical DPPH with an odd electron gives a maximum absorption at 517 nm (purple color). When Antioxidants react with DPPH, which is a stable free radical becomes paired off in the presence of a hydrogen donor (e.g., a free radical-scavenging antioxidant) and is reduced to the DPPHH and as consequence the absorbance's decreased from the DPPH. Radical to the DPPH-H form, results in decolorization (yellow color) with respect to the number of electrons captured. More the decolorization more is the reducing ability. This test has been the most accepted model for evaluating the free radical scavenging activity of any new drug. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form (Diphenylpicrylhydrazine; non radical) with the loss of violet color.[8, 9, 10]

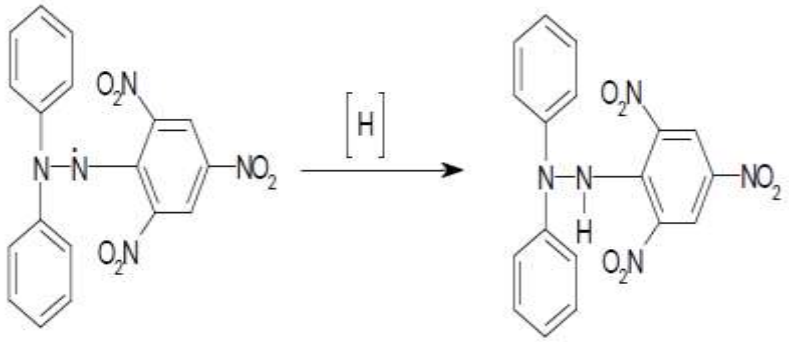


Fig. 1: Reduction of 1,1- Diphenyl-2-picryl hydrazyl (DPPH) Free Radical

Principle

The antioxidant reacts with stable free radical, DPPH and converts it to 1,1-diphenyl–2-picryl hydrazine. The ability to scavenge the stable free radical DPPH is measured by decrease in the absorbance at 517 nm. The degree of discoloration indicates the scavenging potential of the antioxidant compounds in terms of hydrogen donating ability.[11]

Materials required

❖ DPPH (1, 1-diphenyl-2-picryl hydrazyl, D9132, Sigma, USA, (Store at -20°C).

❖ Positive control: Gallic acid [3, 4, 5-Trihydroxy benzoic acid] (G7384, Sigma, USA, store at RT)

❖ Microwell plate - 96well flat, clear plate (Cat. no. 980040, Tarsons)

❖ Methanol (HPLC grade, M0275, Ranbaxy, India, store at RT)

Preparation of working solutions

DPPH (0.659mM): 2.6mg is dissolved and made up to 10ml in HPLC grade methanol.

(a)Positive control (1000µg/ml): 1mg of Gallic acid is dissolved and made up to 1 ml HPLC grade methanol.

(b)Sample preparation: 5mg of the sample was dissolved in 5mL of water. Subsequent dilutions were made in water and the final concentrations at which the sample was tested were 200, 400, 600, 800 and 1000µg/mL.

Procedure

24mg of DPPH (1, 1-Diphenyl –2-picrylhydrazyl) was dissolved in 100 ml of methanol; it was protected from light by covering the test tubes with aluminium foil.2ml of DPPH solution was added to 1ml of methanol and absorbance was taken immediately at 517nm for control reading. Various concentrations of hot water and ethanol extracts of PHF (200-1000µg/ml) as well as standard compound Gallic acid were taken and diluted with 1ml of methanol and to each 2ml of DPPH was added. Absorbance was taken after 48 hrs at 517nm using methanol as blank on UV-visible spectrophotometer.[12] The IC50values for each drug compounds as well as standard preparation were calculated. The DPPH free radical scavenging activity was calculated using the following formula:

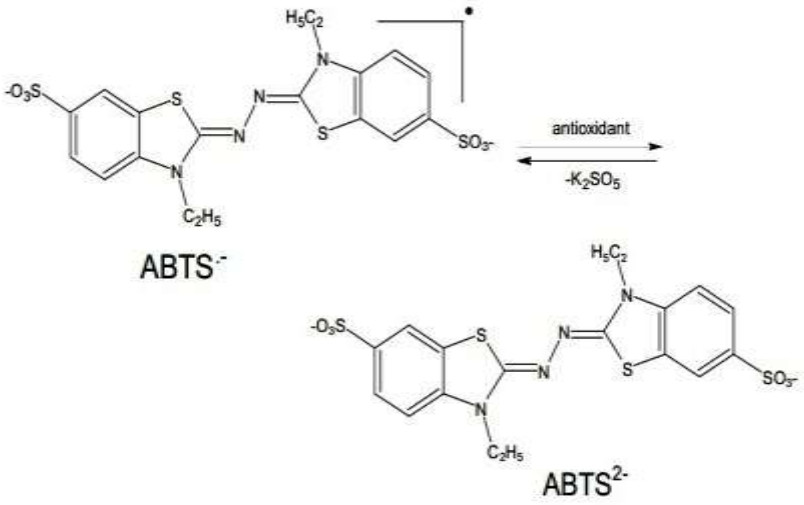
% scavenging activity = [1-Absorbance of sample/Absorbance of control] × 100

The effective concentration of sample required to scavenge DPPH radical by 50% (IC50value) was obtained by plotting graph of % scavenging activity Vs concentrations

ABTS radical scavenging assay

Introduction: Free radicals are generally very reactive molecules possessing an unpaired electron which are produced continuously in cells either as by-products of metabolism or by leakage from mitochondrial respiration.[13] The free radicals produced *in-vivo* include the active oxygen species such as super-oxide radical O_2^-, hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). Oxygen free radicals have been shown to be responsible for many pathological conditions.[14] Free radicals and Reactive Oxygen Species (ROS) cause DNA damage, lipid peroxidation, protein damage. They are known to be involved in the pathogenesis of a wide variety of clinical disorders such as cancer, cardiovascular diseases, inflammatory diseases, asthma and aging.[15, 16] Free radicals like the hydroxyl radical, hydrogen peroxide, super-oxide anion etc. mediate components of the inflammatory response, with production of migratory factors, cyclic nucleotides and eicosanoids. Super-oxide radicals amplify the inflammation process, increasing vascular permeability, adhesion of polymorph nuclear leucocytes to the endothelium and stimulation of platelet aggregation.[17]

Principle: ABTS assay is based on the scavenging of light by ABTS radicals. An antioxidant with an ability to donate a hydrogen atom will quench the stable free radical, a process which is associated with a change in absorption which can be followed spectroscopically. The relatively stable ABTS radical has a green colour and is measured at 734nm.[18, 19]



Materials required

- ABTS [2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] store at RT.
- Positive control: Gallic acid [3,4,5-trihydroxy benzoic acid] store at RT.

Other reagents

- Phosphate Buffer Saline
- Ammonium persulphate (store at RT).
- Microwell plate: 96well flat, clear plate.

Preparation of working solutions

Phosphate buffer saline 10mM [PBS] (pH-7.4 at 25°C): One sachet of PBS is dissolved in 1000ml of de-ionized water.

Ammonium persulfate [APS] (2.45mM): 5.59mg of APS is dissolved & made up to 10ml of 10mM PBS pH 7.4.

(a) Positive control (1000µg/ml)

Stock 1: 1mg of Gallic acid is dissolved & made up to 1 ml 10mM PBS pH 7.4. (1000µg/ml). Further dilutions are made as required.

(b) Sample preparation: 12.5 mg of sample was dissolved in 5 ml PBS. Further dilutions were prepared with vehicle buffer. The final concentration of sample tested was 10, 25, 50,100 and 200µg/ml.

ABTS (7mM):38.41mg of ABTS is dissolved in 10ml of 10mM PBS pH 7.4.

ABTS radical solution

❖ **Stock 1(3.5mM ABTS):**10ml of 7mM ABTS + 10ml of 2.45mM APS is mixed & incubated in dark (store in an amber colored bottle) for 16hrs at 25°C in order to produce the ABTS radicals.

❖ **Working solution (0.238mM ABTS):** 680µl of Stock 1 is made up to 10ml with 10mM PBS pH 7.4. The working solution should have an absorbance of ≥1.0 at 734nm.

Procedure: The assay was performed as per Auddy (2003).[20] In brief, to a 250µl total reaction volume containing 20µl of 10mM PBS pH 7.4/ vehicle buffer/ positive control / test

solutions of various concentrations, add 230µl of ABTS radical solution (0.238mM). Mix and immediately read at 734nm using microplate reader (Molecular devices Versamax microplate reader).

Calculation of results

$$\% \text{ inhibition} = \frac{\text{Absorbance (control)} - \text{Absorbance (test)}}{\text{Absorbance (control)}} X \quad 100$$

Note

i. IC_{50} is calculated using log-probit analysis.
ii. Gallic acid should be prepared fresh prior to analysis and stored in dark
iii. Methanol can be used up to 8% of the total reaction volume.
iv. DMSO can be used up to 8% of the total reaction volume.
v. Appropriate solvent and colour corrections should be done (in case of colour samples & non-aqueous solutions).
vi. ABTS reagent should be stored in an amber colour bottle and prepared fresh.

ESTIMATION OF TOTAL PHENOLIC CONTENT

Introduction: Phenolics include simple phenols, phenolic acids (benzoic and cinnamic acid derivatives), coumarins, flavonoids, stilbenes, hydrolysable and condensed tannins, lignans, and lignins. These compounds are among the most widely occurring secondary metabolites in the plant kingdom, acting mainly as phytoalexins, attractants for pollinators, and contributors to plant pigmentation, antioxidants, and protective agents against UV light, among others.[21] Colorimetric reactions are widely used in the UV/VIS spectrophotometric method, which is easy to perform, rapid and applicable in routine laboratory use, and low-cost.[22] However, it is important that colorimetric assay need to use a reference substance, and then this method measures the total concentration of phenolic hydroxyl groups in the plant extract.

Polyphenols in plant extracts react with specific redox reagents (Folin-Ciocalteau reagent) to form a blue complex that can be quantified by visible-light spectrophotometry.[23] The reaction forms a blue chromophores constituted by a phosphotungsticphosphomolybdenum complex[24], where the maximum absorption of the chromophores depends on the alkaline solution and the concentration of phenolic compounds.[23]

Materials required

- **Folin-Ciocalteau reagent**
- **7.5% Sodium bicarbonate**
- **Gallic acid**
- **Phosphate buffer saline**
- **UV-Spectrophotometer.**

Preparation of working solutions

- **Folin-Ciocalteau reagent**

Dissolve 10 g sodium tungstate and 2.5 g sodium molybdate in 70 ml water. Add 5 ml 85% phosphoric acid and 10 ml concentrated hydrochloric acid. Reflux for 10 hr. Add 15 g lithium sulfate, 5 ml water and 1 drop bromine. Reflux for 15 min. Cool to room temperature and bring to 100 ml with water.

- **Phosphate buffer saline 10mM [PBS] (pH-7.4 at 25°C)**

One sachet of PBS is dissolved in 1000ml of de-ionized water.

- **Positive control (1000µg/ml)**

Stock 1: 1mg of Gallic acid is dissolved & made up to 1 ml 10mM PBS pH 7.4. (1000µg/ml). Further dilutions are made as required.

Sample preparation: 12.5 mg of sample was dissolved in 5 ml PBS, further dilutions were prepared with vehicle buffer. The final concentration of sample tested was 1.0 ml.

Procedure: The total phenolic content in Hot Water and Ethanol extracts of PHF was estimated by Folin-Ciocalteau method[25] with some modifications, 1.5ml of Folin-Ciocalteau reagent was added to 1 ml of each extracts and placed for 5 minutes. Then 4ml of 7.5% Sodium bicarbonate was added and the total volume was made up to 10ml using distilled water. The above solution was kept for incubation at room temperature for 30 minutes. Absorbance was measured at 765 nm using 1 cm cuvette in a UV-VIS lambda 25 spectrophotometer. Gallic acid solution (1.0-100µg/ml) was used to produce standard curve. The total phenolic content was expressed in mg of Gallic acid Equivalents (GAE) mg / g of extract.

RESULTS

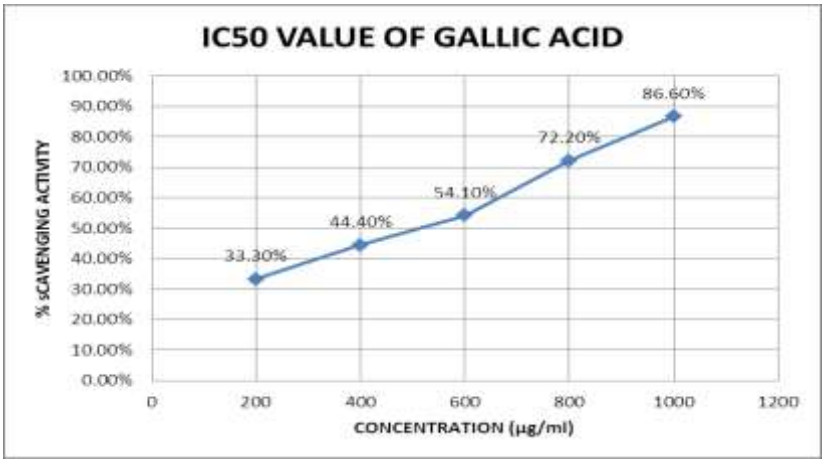
PHYTOCHEMICAL SCREENING OF THE POLYHERBAL FORMULATION

As a first part of study from the phytochemical analysis, it is evident that both hot water and ethanolic solvent showed presence of carbohydrates, glycoside, alkaloids, sterioids, triterpenoids, flavonoids, phenol, saponins and tannins. Out of the phytochemical, tannins showed maximum, which is further screened to know the type of tannin which indicated the presence of hydrolysable tannins.

DPPH scavenging activity: The DPPH radical scavenging activity was recorded in terms of % Inhibition as shown in Table-3. It is evident from the table that Ethanolic extract of PHF showed minimum DPPH scavenging activity (54.5%) and Hot water PHF showed has maximum DPPH scavenging activity (83.60%). The Results obtained were compared with standard used (Gallic acid) which exhibited 86.60% scavenging activity. Higher % Inhibition indicates better scavenging activity or antioxidant potential. The amount of extract required for 50% inhibition of DPPH activity (IC50) is also shown in table-3. It is evident from the results hot water extract showed percentage of inhibition, which is similar to selected standard Gallic acid. Hence the polyherbal formulation using hot water extract can be used as safe alternative for the synthetic standard gallic acid.

Table-2: DPPH radical scavenging activity of Gallic acid at 1mg/ml concentration.

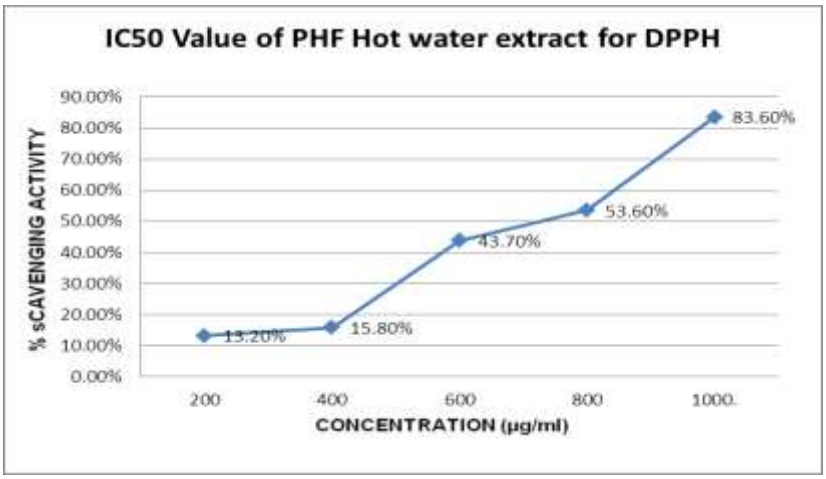
	Concentration(µg/ml)	% Inhibition	IC50 Value
Gallic acid	200	33%	481µg/ml
	400	44.4%	
	600	54.1%	
	800	72.2%	
	1000	86.6%	



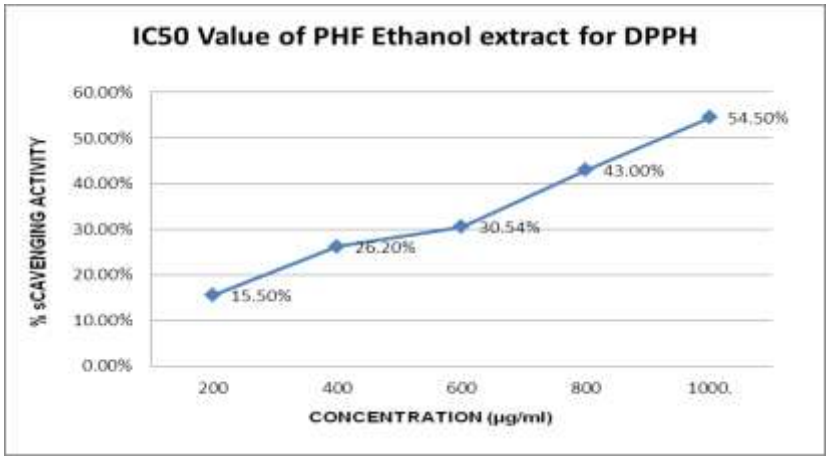
Graph-1: IC50 Value of Gallic acid at 1mg/ml

Table-3: DPPH radical scavenging activity of PHF

	Extract	Concentration(µg/ml)	% Inhibition	IC50 Value
11	Hot water	200	13.2%	760µg/ml
		400	15.8%	
		600	43.7%	
		800	53.60%	
		1000	83.60%	
	Ethanol	200	15.5%	939µg/ml
		400	26.2%	
		600	30.54%	
		800	43.00%	
		1000	54.5%	



Graph-2: IC50 Value of PHF Hot water extract for DPPH



Graph-3: IC50 Value of PHF Ethanol extract for DPPH

ABTS radical scavenging assay

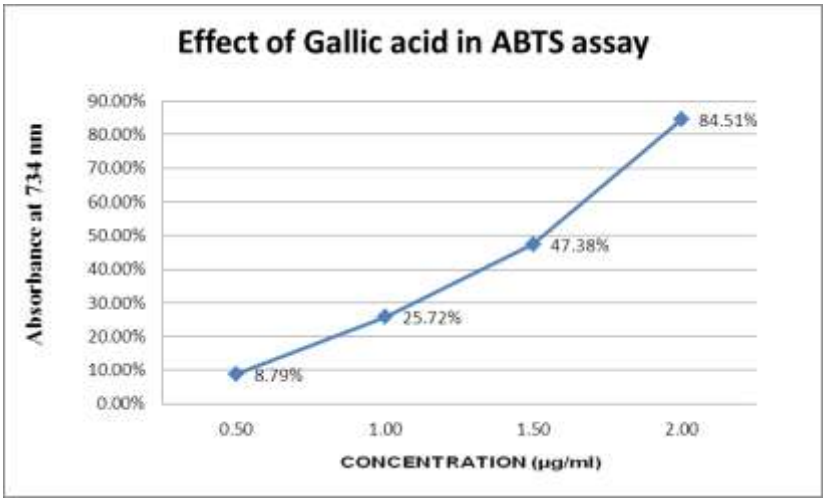
The ABTS radical scavenging activity was recorded in terms of % Inhibition as shown in table-5 and graph-5, graph-6. It was observed from the table that Ethanolic extract of PHF showed maximum ABTS scavenging activity (86.5%) and Hot water PHF has minimum

DPPH scavenging activity (82.30%). The Results obtained were compared with standard used (Gallic acid) which exhibited 84.51% scavenging activity. Higher % Inhibition indicates better scavenging activity or antioxidant potential. The amount of extract required for 50% inhibition of ABTS activity (IC50) is also shown in table-5 and graph-5, graph-6.

The amounts of ABTS activity (IC50) were recorded as 38.6 µg/ml, 72.76 µg/ml in ethanolic extract and hot water extract. It is evident that IC50 value of the extract shows a safe administration when compared to the IC50 value of the standard drug (Gallic acid) is 1.34 µg/ml (table-4, graph-4).

Table-4: ABTS radical scavenging activity of Gallic acid

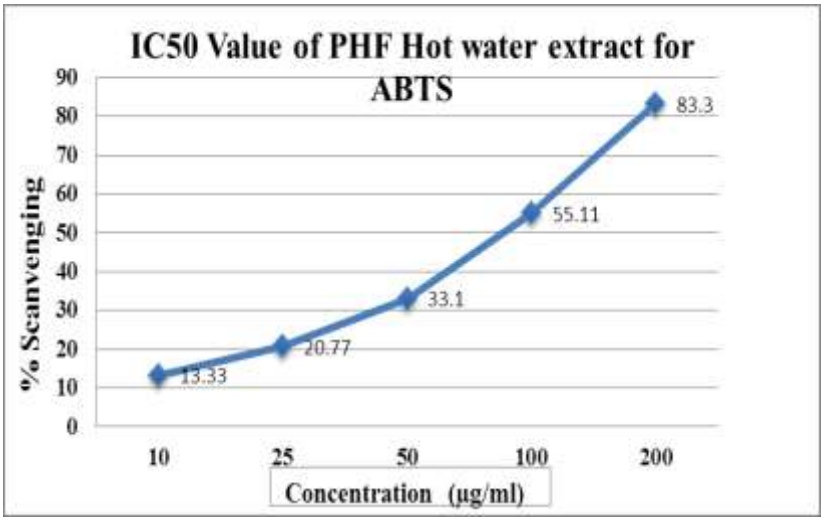
	Concentration(µg/ml)	% Inhibition	IC_{50} (µg/ml) (95% C.I.)
Standard (Gallic acid)	0.5	8.79%	1.34 (1.23-1.46)
	1.0	25.72%	
	1.5	47.38%	
	2.0	84.51%	



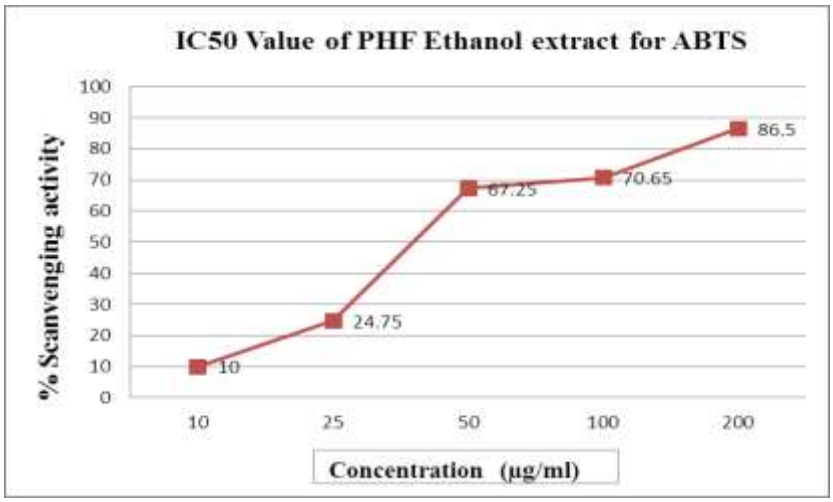
Graph-4: Standard Gallic Acid

Table-5: ABTS radical scavenging activity of PHF

	Extract	Concentration(µg/ml)	% Inhibition	IC50 Value
Polyherbal formulation	Hot water	10	13.33%	72.76µg/ml
		25	20.77%	
		50	33.10%	
		100	55.11%	
		200	82.30%	
	Ethanol	10	10.0%	38.6µg/ml
		25	24.75%	
		50	67.25%	
		100	70.65.%	
		200	86.5%%	



Graph-5: IC50 Value of PHF Hot water extract for ABTS

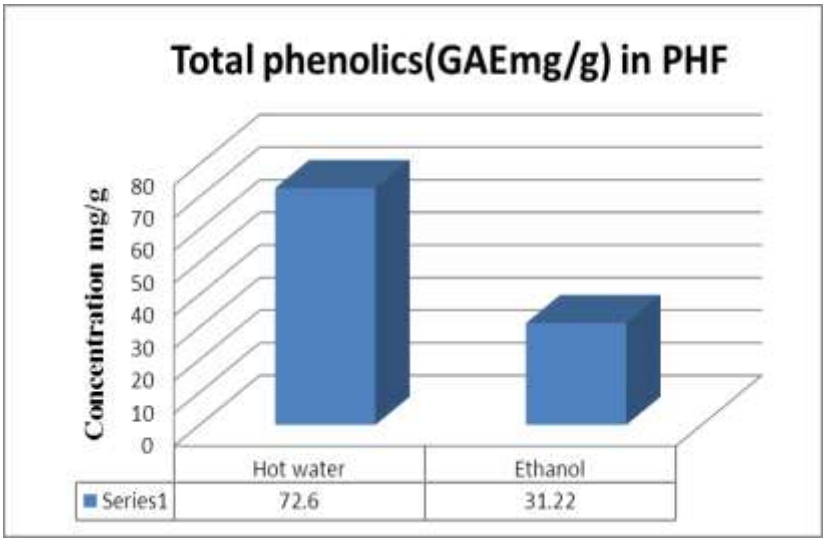


Graph-6: IC50 Value of PHF Ethanol extract for ABTS

Estimation of total phenolic content: Since there is a strong correlation between the antioxidant activity of plant extracts and their phenolic content, hence the total phenols were determined using Folin-Ciocalteau reagent. Gallic acid was used as standard compound. The total phenols in two extracts of two PHF was expressed as mg/g Gallic acid equivalent using the standard curve equation: **y=0.0116x+0.0478 (y = mx + c)**, where y is the absorbance at 765nm and x is the total phenolic content in different extracts of PHF expressed in mg/g. The phenol content in Ethanolic extract of Polyherbal formulation was found to be 31.22. The maximum phenolic content was found in PHF Hot water extract (72.60mg/g) shown in Table-6 and graph-7. Indicating its remarkable antioxidant activity.

Table-6: Total phenolic content of PHF in different extracts

Extracts	Concentration(mg/g)
Hot water	72.60
Ethanol	31.22



Graph: 7. Total phenolic content in PHF extracts

DISCUSSION

The usefulness of herbs in several disorders might be due to their antioxidant activity. To support the use of the selected plant extracts in herbal mixture and in Ayurveda and naturopathy, the antioxidant potential of the aqueous extract and ethanolic extract of *Ocimum sanctum, Withania somnifera, Gymnema sylvestre, Tinospora cordifolia, Phyllanthus emblica* and *Coleus forskohlii.* of Indian origin was invested in comparison with the known antioxidant gallic acid following I*in vitro* studies. The antioxidant activity of ascorbic acid was well established.[30] The DPPH radical scavenging activity was recorded in terms of % Inhibition as shown in Table-3. It was observed from the table that Ethanolic extract of PHF showed minimum DPPH scavenging activity (54.5%) and Hot water PHF has maximum DPPH scavenging activity (83.60%) . The Results obtained were compared with standard used (Gallic acid) which exhibited 86.60% scavenging activity. Higher % Inhibition indicates better scavenging activity or antioxidant potential. The amount of extract required for 50% inhibition of DPPH activity (IC50) is also shown in table-3.

The ABTS radical scavenging activity was recorded in terms of % Inhibition as shown in Table. It was observed from the table that Ethanolic extract of PHF showed minimum ABTS scavenging activity (67.25%) and Hot water PHF has maximum DPPH scavenging activity (86.65%) . The Results obtained were compared with standard used (Gallic acid) which exhibited 84.51% scavenging activity. Higher % Inhibition indicates better scavenging activity or antioxidant potential. The amount of extract required for 50% inhibition of ABTS activity (IC50) is also shown in table-5. The amount of total phenols was determined using Folin-Ciocalteau reagent. Gallic acid was used as standard compound. The total phenols in

six extracts of two PHF was expressed as mg/g Gallic acid equivalent using the standard curve equation: **y=0.0116x+0.0478**, where y is the absorbance at 765nm and x is the total phenolic content in different extracts of PHF expressed in mg/g. The total phenol varied from 8.81mg/g to 72.60mg/g in the extracts. The maximum phenolic content was found in PHF Hot water extract (72.60mg/g) shown in Table 6 and graph-7.

The literature reports reveal that alkaloids, carbohydrates, polysaccharides and saponin glycosides present in the plant extract known to possess Flavonoids possess antioxidant activity.[26, 27, 28, 29] The present investigation also observed antioxidant potential of test extract may be due to the presence of similar Phytoconstituents which was evident by preliminary phytochemical screening.

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