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EVALUATION OF *IN-VITRO* ANTIOXIDANT ACTIVITY OF PANCHAGAVYA: A TRADITIONAL AYURVEDIC PREPARATION

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ABSTRACT

Panchagavya, a classical Ayurvedic preparation, was evaluated for its antioxidant potential by HPTLC-DPPH bioautography method as well as assays for Ferric reducing antioxidant power (FRAP), DPPH – free radical scavenging activity (DPPH) and Superoxide radical scavenging activity. In addition total phenolic content was also estimated which was in fairly good amount. HPTLC-DPPH bioautography study revealed the presence of several antioxidant compounds in Panchagavya. In all the assays performed, it showed considerable antioxidant activity. On comparison of the data of three different batches of the samples studied, it showed 98.3 - 99.8% correlation between total phenolic content, FRAP and DPPH assays.

INTRODUCTION: Ayurveda, the ancient Indian system of medicine, with its holistic approach takes into account the aetiological factors, disease condition, patient's psycho-somatic condition, food and even activities altogether while deciding the line of treatment. It mentions use of various plant, animal and mineral products as such and in unique combinations for treatment and positive health maintenance. Many materials, which can hardly be termed as drug by prevalent norms, are also utilized in the treatment. Panchagavya- a peculiar combination of five cow products namely dung, urine, milk, curd and ghee [1], is one such drug which has been advocated in Ayurvedic classics as such in Vishamajvara [2] (malaria/typhoid), for detoxification of body/toxicity management [1] while in processed form (viz. Panchagavya ghrita) for psychogenic and neurogenic disorders [3].

In recent days, its use in management of cancerous conditions is also a practice in vogue.

Free radicals play an important role in degenerative diseases like cancer, cataract, immune system weakness and brain disorders, collectively called oxidative stress [4]. Amount/availability of free radicals are controlled by systems called antioxidants which can reduce oxidation rate considerably and are synthesized in the body as well as supplied by dietary sources and nutraceuticals. Applicability of antioxidants in management of oxidative stress related disorders has been suggested [5].

Since oxidative stress plays major role in aetio-pathogenesis of cancer, present study of *in vitro* antioxidant activity of Panchagavya was undertaken to evaluate its role in cancer therapy. This is probably the first report of antioxidant activity of Panchagavya.

MATERIALS AND METHODS:

Chemicals and Instrument: 2, 2 Diphenyl-1-picryl hydrazil (DPPH; Sigma-Aldrich, Germany), 2, 4, 6-Tri-(2-pyridyl)-s-triazine (TPTZ; SRL chemicals, Mumbai),

Nitroblue tetrazolium chloride (NBT), Riboflavin, Methionine, Gallic acid (Lobachemie, Mumbai), all other chemicals of Merck India pure or GR grade.

Absorbance was noted using a Perkin-Elmer Lambda 25 UV-Visible spectrometer.

The experiments were carried out at RMD Research and Development Center during October 2011 to January 2012.

Preparation of Panchagavya: 25 ml of freshly collected cow urine was added to 12.5 g freshly collected cow dung and uniformly mixed by stirring. The mixture was passed through a cotton cloth by squeezing and then strained repeatedly (7 times) through a cloth. To the filtrate 5 ml cow milk (boiled and cooled) and 5 ml curd (prepared by using cow milk) were added, mixed thoroughly, to it 4 ml molten cow ghee was added and again mixed well to prepare a homogeneous mixture. This freshly prepared Panchagavya was used for the study.

For the present study three batches of Panchagavya were prepared in three different days.

Preparation of sample: To 1 g Panchagavya 10 ml methanol was added, stirred well and sonicated for 60 minutes. It was filtered; the volume of the filtrate was made to 10 ml with methanol (PG) and was used for further study.

A. **Determination of Total Phenolic Content:** The total phenolic content (TPC) of the samples was determined with Folin-Ciocalteu reagent according to method described by Andrew[6] with slight modifications. It is based on the reduction of the reagent (a mixture of tungsten and molybdenum oxides) and measuring the absorbance of the product (blue colour) at 765 nm.

Reagents/Solutions: Working Folin-Ciocalteu reagent - 50% v/v aqueous solution of Folin-Ciocalteu reagent, Sodium carbonate solution - 20% w/v, Standard Gallic acid solution - 0.1 mg/ml (freshly prepared).

To 0.1 ml of the sample (PG), 3.9 ml distilled water and 0.25 ml of working Folin-Ciocalteu reagent were added. After 5 minutes (but before 8 minutes), 0.75 ml of sodium carbonate solution was added, mixed and incubated at room temperature for 60 minutes. The absorbance was measured at 765 nm. Different volumes of Gallic acid solution were used in same manner for calibration of standard curve and quantification was done in terms of mg equivalent of Gallic acid. The blank was prepared by using distilled water in place of sample/standard.

B. **Evaluation for Antioxidant Activity:**

i. **Rapid screening for *in vitro* Antioxidant activity by HPTLC-Bioautography method:** For preliminary screening of the sample, the antioxidant activity was evaluated by using HPTLC-DPPH – bioautography method [7, 8]. HPTLC was performed on 10 cm x 10 cm aluminum backed plates coated with 0.2 mm layers of silica gel $60F_{254}$ (Merck, Germany). Defatted methanol extract of Panchagavya was applied in band with a Linomat V applicator (CAMAG, Switzerland), equipped with a 100-µl syringe. Plates were developed vertically, in a CAMAG twin trough chamber previously saturated with mobile phase vapor for 20 min at room temperature. Toluene - Ethyl acetate - Glacial acetic acid (6:4:0.2) was used as mobile phase. After development the plates were dried at room temperature, sprayed with 0.2% methanolic DPPH solution and observed after keeping the plate in dark for 30 minutes. Presence of antioxidant compounds were detected by yellowish spots against a purple background.

ii. ***In vitro* Antioxidant Assay:**

a. **Assay for *in vitro* Ferric Reducing Antioxidant Power (FRAP assay):** The FRAP assay was carried out by the method described by Benzie and Strain[9] with slight modifications. It is based on the principle of reduction of Fe^{3+}-TPTZ to Fe^{2+}-TPTZ complex at low pH which gives blue color and can be measured at 593 nm.

Preparation of FRAP working reagent: Acetate buffer of 300 mM concentration and pH 3.6 was prepared by using appropriate volumes of sodium acetate anhydrous, glacial acetic acid and distilled water. TPTZ solution of 10 mM concentration was prepared in 40 mM hydrochloric acid. Aqueous ferric chloride

solution of 24.998 mM concentration was prepared using ferric chloride anhydrous. Acetate buffer, TPTZ solution and freshly prepared ferric chloride solution were mixed in 10: 1: 1 proportions to prepare the FRAP working reagent.

0.1 ml of PG was added to 3.0 ml of FRAP working reagent, mixed well and absorbance was measured after 10 minutes. Freshly prepared aqueous ascorbic acid solution (0.1 mg/ml) was used as standard. Different volumes of ascorbic acid solution (equivalent to 10 – 80 µg) were used in same manner for calibration of standard curve and quantification was done in terms of mg equivalents of ascorbic acid. The blank was prepared by using distilled water in place of sample/standard.

b. **Assay for *in vitro* DPPH- Free Radical Scavenging activity (DPPH Assay):** DPPH radical gives strong absorbance at 517 nm (deep violet color) due to its unpaired electron. When this radical pairs off in presence of a free radical scavenger, the absorption vanishes and the resulting discoloration is stoichiometric with respect to the number of electrons taken up.

DPPH – free radical scavenging activity assay of Panchagavya was carried out using reported method [10-12] with suitable modifications.

Reagent/solutions: DPPH solution - 0.3 mM in methanol (freshly prepared), Standard Ascorbic acid solution – 1 mg/ml in methanol.

Sample preparation: 1 ml of PG was dried on mild heat in a water bath; the residue was taken with methanol to make 1mg/ml (PGE1) and used for the test.

Different volumes (equivalent to 5 - 300 µg) of PGE1/standard were taken in a set of test tubes and methanol was added to make the volume to 3 ml. To this, 1 ml of DPPH reagent was added mixed thoroughly and absorbance was recorded at 517 nm after 30 minutes incubation in dark at room temperature. 1 ml of DPPH reagent diluted to 4 ml with methanol was taken as reagent blank. Percent scavenging activity was calculated as

$$\% \text{ Scavenging} = \frac{A_0 - A_S}{A_0} \times 100$$

Where, A_0 = Absorbance of reagent blank, A_S = Absorbance of sample/standard

c. **Assay for Superoxide radical scavenging:** The method is based on scavenging of superoxide anion generated in light induced reaction mixture having absorbance at 560 nm due to blue complex formed by NBT; antioxidants inhibit blue complex formation. The decrease of absorbance at 560 nm in presence of sample thus indicates the consumption of superoxide anion in the reaction mixture [13, 14]. The method was adapted with minor modifications.

Reagent/solutions: Stock solutions of 130 µM riboflavin, 1 mg/ml NBT and 40 µM ethylene di-amine tetra acetic acid (EDTA) di-sodium salt (all in distilled water) were prepared. Phosphate buffer 50 µM, pH 7.8 was prepared by using appropriate quantities of di-sodium hydrogen phosphate (Na_2HPO_4) and potassium di-hydrogen phosphate (KH_2PO_4).

1.539 ml of Riboflavin, 6.132 ml of NBT, 2 ml of EDTA stock solutions were mixed in this sequence. To it 65-70 ml of phosphate buffer was added and mixed thoroughly. To this mixture, 193.96 mg of methionine was dissolved completely and the resultant solution was diluted to 120 ml with phosphate buffer and was used as reaction mixture.

Freshly prepared 1 mg/ml aqueous solution of ascorbic acid was used as standard.

Sample preparation: 1 ml of PG was dried on mild heat in a water bath, the residue was taken with water to make 1mg/ml, sonicated for 15 minutes to prepare a homogeneous suspension (PGE2) and used for the test in different concentration range. Different volumes of PGE2/standard were taken in test tubes, 3 ml of the reaction mixture were added, mixed and phosphate buffer was added to make the volume to 4.5 ml.

After mixing the tubes were exposed to fluorescent light (30 W) for 40 minutes (taking care of equal illumination exposure) and the absorbance was measured at 560 nm. 3 ml of reaction mixture diluted to 4.5 ml with phosphate buffer was taken as reagent blank. Percent scavenging activity was calculated as

$$\% \text{ Scavenging} = \frac{A_0 - A_S}{A_0} \times 100$$

Where, A_0 = Absorbance of reagent blank, A_S = Absorbance of sample/standard

RESULTS AND DISCUSSION:

1. **Total Phenolic Content of Panchagavya:** The total phenolic content was determined by Folin-Ciocalteu reagent. The method used is the one basically used for determination of total phenol content in wine industry and capable of differentiating inter-batch differences [6]. The standard curve of gallic acid has been presented in **Figure 1**.

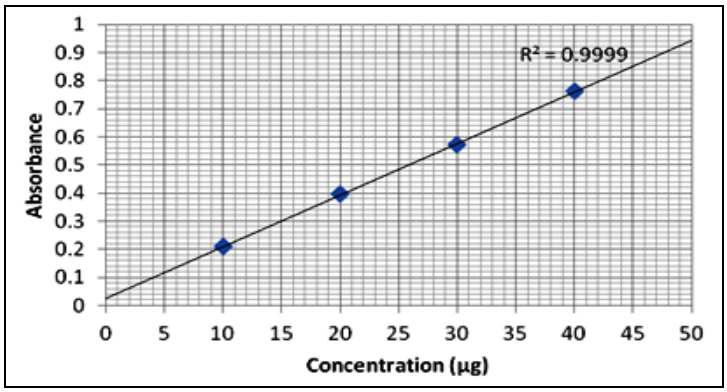

FIGURE 1: STANDARD CURVE OF GALLIC ACID

The total phenolic content of the samples, expressed in terms of mg gallic acid / g of Panchagavya has been presented in **Table 1**.

TABLE 1: TOTAL PHENOLIC CONTENT OF PANCHAGAVYA

Batch	TPC /g*	Mean ± SD
Batch I	0.576	0.551 ± 0.031
	0.561	
	0.516	
Batch II	1.525	1.655 ± 0.115
	1.742	
	1.698	
Batch III	0.694	0.712 ± 0.023
	0.703	
	0.739	

*in terms of Gallic acid equivalents (mg)

The total phenolic content in three batches of Panchagavya is in between 0.55 to 1.65 mg/gm in terms of gallic acid equivalents, which is significant as Panchagavya daily dose is about 150 ml i.e. about 160 g and is comparable with plant materials of known antioxidant activity [13, 15-20].

The response of Folin-Ciocalteu reagent depends on chemical structures of phenolics which are of various types and of different polarities. Panchagavya being a natural product combination, variation is expected.

2. **Antioxidant activity**:

A. **HPTLC-DPPH Bioautography method**: The preliminary screening of Panchagavya for antioxidant activity was carried out by HPTLC-DPPH bioautography method. Spraying the plate with DPPH solution revealed several yellowish colored spots (**Figure 2**) indicating the presence of number of antioxidant compounds in Panchagavya.

FIGURE 2: CHROMATOGRAM AFTER DPPH SPRAY

i. ***In vitro* antioxidant assay**:

a. **Ferric Reducing Antioxidant Power (FRAP):** The FRAP activity was determined in terms of ascorbic acid equivalents. The ascorbic acid standard curve for FRAP is shown in **Figure 3**.

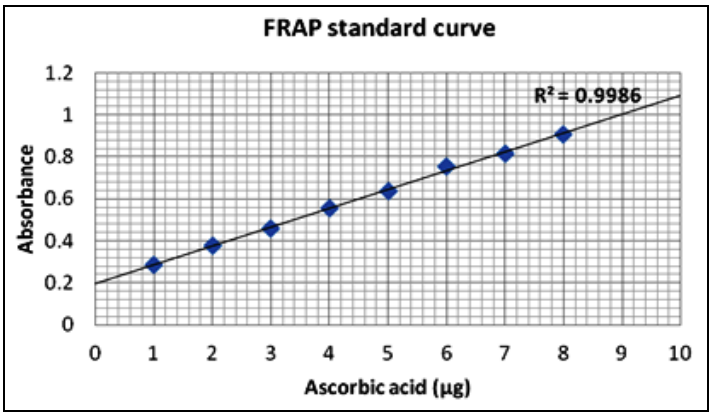

FIGURE 3: STANDARD CURVE OF ASCORBIC ACID

The FRAP value varied between 0.22 - 0.89 mg/g of Panchagavya (**Table 2**), keeping a correlation with their phenolic content.

TABLE 2: FRAP VALUE OF PANCHAGAVYA SAMPLES

Batch	FRAP value/g*	Mean ± SD
Batch I	0.256	0.222 ± 0.029
	0.203	
	0.208	
Batch II	0.784	0.891 ± 0.093
	0.940	
	0.951	
Batch III	0.401	0.434 ± 0.044
	0.419	
	0.484	

*in terms of Ascorbic acid equivalents (mg)

ii. **DPPH Radical Scavenging Activity:** For DPPH scavenging activity, ascorbic acid was used as standard and the standard curve is presented in **Figure 4**.

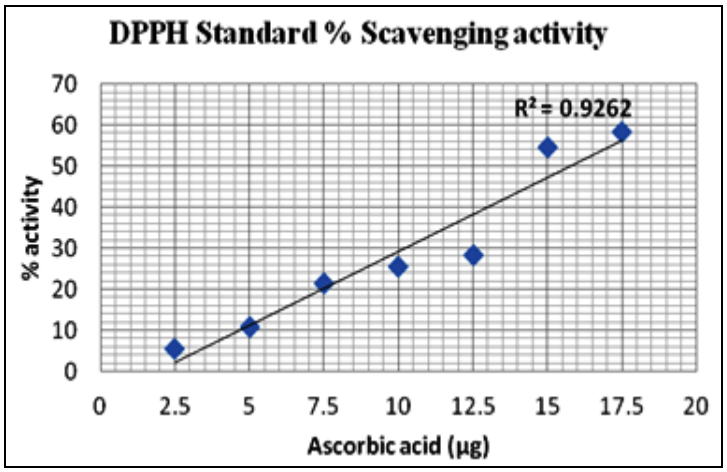

FIGURE 4: STANDARD CURVE OF ASCORBIC ACID (DPPH)

DPPH scavenging activity of Panchagavya samples has been presented in **Table 3**.

TABLE 3: DPPH SCAVENGING ACTIVITY OF PANCHAGAVYA SAMPLES

Batch	Concentration (µg)	% scavenging
Batch I	100	9.25
	150	11.63
	200	14.42
Batch II	100	9.32
	150	18.08
	200	22.91
Batch III	100	9.05
	150	17.07
	200	21.08

The DPPH scavenging activity of PGE1 revealed a peculiar pattern. The activity tends to decrease beyond the concentration 200 µg (**Figure 5**).

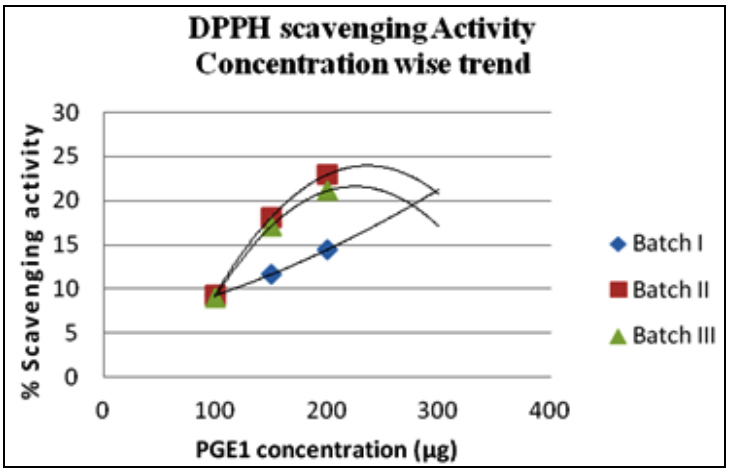

FIGURE 5: DPPH ACTIVITY TREND

The usual trend to show scavenging activity is by indicating IC_{50} (Half-maximal Inhibitory concentration) which Panchagavya is unable to show. The reasons may be of interference by other chemical molecules competing for reduction by DPPH or having higher internal activity scavenging chain reaction, not permitting DPPH be donated with an electron. IC_{50} for ascorbic acid was 15.78 µg.

iii. **Superoxide Radical Scavenging Activity:** The Superoxide radical scavenging activity of the samples has been presented in **Table 4**.

TABLE 4: SUPEROXIDE SCAVENGING ACTIVITY OF PANCHAGAVYA SAMPLES

Sample ID	Concentration (µg)	% scavenging
Batch I	30	4.55
	60	19.89
	90	23.65
Batch II	30	5.57
	60	23.04
	90	20.36
Batch III	30	23.57
	45	24.07
	60	17.69

By this method Panchagavya showed concentration dependent antioxidant activity in the range of 20-100 µg and then activity decreases with increase of concentration like in the case of DPPH scavenging activity. Superoxide scavenging activity of Panchagavya in the range of 20-100 µg varied from 4 to 24 % (**Figure 6**). Required concentration of ascorbic acid standard for 24 % scavenging activity was about 45 µg.

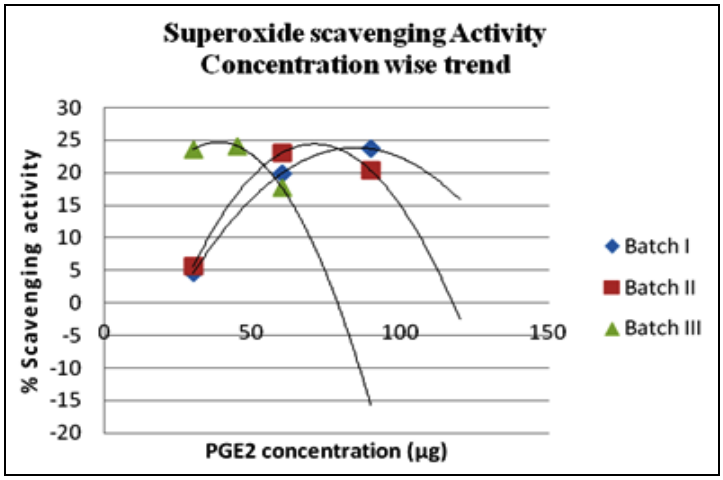

FIGURE 6: SUPEROXIDE SCAVENGING ACTIVITY CONCENTRATION WISE TREND

Superoxide anion is a reduced form of molecular oxygen and has a role in oxidation reaction associated with ageing [21]. Superoxide can directly initiate lipid peroxidation [22].

The batch wise trend of TPC as well as FRAP and DPPH activity was compared and is shown in **Figure 7**.

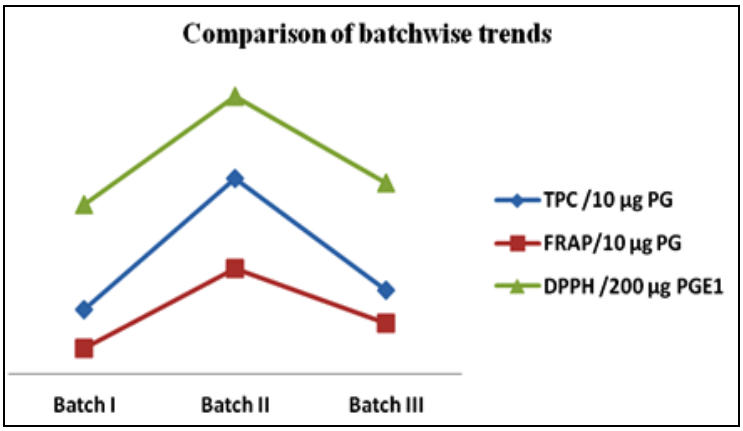

FIGURE 7: COMPARISON OF BATCHWISE TRENDS

Their correlation level was between 98.3 to 99.8 % (**Table 5**).

TABLE 5: CORRELATION BETWEEN BATCHWISE TRENDS

Correlation between	Percentage
TPC and FRAP	98.38%
TPC and DPPH	99.86%
FRAP and DPPH	99.19%

Among naturally occurring antioxidants, both water soluble and lipid soluble antioxidants are present. The major water soluble antioxidants are glutathione, α-lipoic acid, ascorbic acid, polyphenols and bio-flavonoids while lipid soluble antioxidants are vitamin E, α-lipoic acid, co-enzymeQ10, polyphenols and bio-flavonoids.

Free radicals are generated in both aqueous and lipid portions of intracellular and extracellular environments; so it is crucial for the body to have a combination of water soluble and lipid soluble antioxidants to acquire the full range of protection. Some types of antioxidants are synthesized by the body while others are obtained from external sources like food, nutraceuticals [5].

Panchagavya is a unique preparation having both water based (colloidal milk without fat portion, urine, curd and dung) and fat based (ghee, milk with fat particles) products. It is likely to provide both polar and non-polar natural antioxidants.

The data of the antioxidant study revealed uncommon activity limiting behavior. The probability of its higher activity in low concentration at cellular level cannot be ruled out but requires detail study.

Since both water and lipid soluble antioxidants are needed by the body for intra and extracellular clearance of the oxidative stress and Panchagavya contains both water based and lipid based products, it has advantage as potential antioxidant.

The study indicates some basis for the practice of using Panchagavya in cancer.

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