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ORIGINAL ARTICLE

In vitro antioxidant activity evaluation and HPTLC profile of Cow dung

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Aim: The present study was undertaken to evolve suitable High Performance Thin Layer Chromatography (HPTLC) profile and to evaluate *in vitro* antioxidant activity of cow dung. **Materials and Methods:** Dung samples, collected from cows of Geer variety of *Bos indicus* on different days at predefined times, were subjected to development of HPTLC profile, estimation of total phenolic content and *in vitro* antioxidant activity by HPTLC-2, 2 Diphenyl-1-picrylhydrazil (DPPH) bioautography, Ferric Reducing Antioxidant Power (FRAP) and DPPH free radical scavenging methods. **Results:** Developed HPTLC profile revealed notable variations among the samples. HPTLC-DPPH Bioautography showed presence of number of antioxidant compounds, which was further confirmed by quantitative evaluation of *in vitro* antioxidant activity. The results of the study indicate presence of phenolic as well as non-phenolic natured bio-active compounds in cow dung. **Conclusion:** The HPTLC profile as well as antioxidant potential of cow dung is reported for the first time with notable intra-day and inter-day variations. The evolved chromatographic profile will be very useful for evaluating the quality of cow dung. It has potential as a natural antioxidant.

Key words: Antioxidant, cow dung, high performance thin layer chromatography, HPTLC-2, 2 Diphenyl-1-picrylhydrazil bioautography

INTRODUCTION

Cow dung is widely used since long time for its various day-to-day utilities. It is a very common choice as manure and has high religious value as per Indian traditions. Ayurveda attributes a number of therapeutic properties to it and prescribes it for both local and systemic uses in various ailments as well as, as an ingredient of various formulations.[1] Aqueous extract of cow dung is indicated for oral administration in the treatment of various clinical conditions viz. toxicity, Raktapitta (bleeding disorders), hiccough, dyspnoea, cough, conjunctivitis etc., while its nasal administration is indicated in cough and topical application is prescribed in epilepsy and discolouration of skin.[2] Inhibitive activity of cow dung against fungal pathogen[3] and antibacterial activity of aqueous extract of cow dung ash[4] have also been reported.

Amount/availability of free radicals, which play an important role in oxidative stress, are controlled by antioxidants, which can reduce oxidation rate considerably and are synthesised in the body as well as supplied by dietary sources and nutraceuticals.[5] Applicability of antioxidants in the management of oxidative stress-related disorders has been suggested.[6] Antioxidants are also widely used as preservative in packaged food items from oxidation prevention point of view.[7] Many synthetic compounds find their place as food supplements/preservatives such as ascorbic acid, butylated hydroxy-toluene (BHT), butylated hydroxy-anisole (BHA), etc., however, there are some reports about the possible carcinogenic effects of synthetic antioxidants in high doses and long-term use.[8-10] Hence, there is a need for search of natural antioxidants. The constant search for new, affordable, safe and effective bioactive agents from natural sources is going on throughout the world.

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Cow dung is one of the ingredients of Panchagavya and Panchagavya ghrita, which have been indicated for ailments like intoxication,[11] pyrexia[2] and epilepsy, psychosis, pyrexia, jaundice,[1] respectively. Analytical profile and *in vitro* antioxidant activity of Panchagavya[12,13] and Panchagavya ghrita[14] have been reported. However, reports directly focusing on analytical profile or bioactivity of cow dung are hardly available. The present study was undertaken to evolve suitable HPTLC profile and to evaluate *in vitro* antioxidant activity of cow dung.

MATERIALS AND METHODS

Sample Collection

Seventeen dung samples were collected from identified healthy, non-pregnant cow of Geer variety of *Bos*

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indicus (reared in house), on six consecutive days at three predefined times (around 3.30 am i.e., first excretion of the day, 8.30 am and 2.30 pm) and were used for the study as different samples.

Chemicals and Reagents

2, 2 Diphenyl-1-picrylhydrazil (DPPH; Sigma-Aldrich, Germany); 2, 4, 6-Tri-(2-pyridyl)-5-triazine (TPTZ; SRL Chemicals, Mumbai); Gallic acid and Folin-Ciocalteu's reagent (Lobachemie, Mumbai); all other chemicals of Merck India, pure or GR grade.

Preparation of Sample

To 2 g of sample, 20 ml of methanol was added, stirred well and sonicated for 60 minutes. Further, the mixture was filtered, volume of the filtrate was made to 20 ml with methanol and the extracts were kept in refrigerator till used for the study.

HPTLC Profile

HPTLC was performed on 20 × 10 cm aluminum backed plates coated with 0.2 mm layers of silica gel $60F_{254}$ (Merck, Germany). Seven microlitres of sample solution was applied in band with a Linomat 5 applicator (CAMAG, Switzerland), equipped with a 100-µl syringe. Plates were developed vertically, in a CAMAG twin trough chamber, previously saturated with mobile phase vapour for 20 minutes at room temperature. Various mobile phases were tried to obtain better resolution. Chloroform: Methanol (85:15) system, giving optimum resolution, was used as mobile phase. Densitometric scanning was performed with CAMAG TLC Scanner 4 at 366 nm operated by WinCats software version 1.4.6 and details were noted. The source of radiation utilised was deuterium and tungsten lamp emitting a continuous spectrum between 200 and 700 nm.

Determination of Total Phenolic Content

The total phenolic content of the samples was determined by Folin-Ciocalteu's method.[15] It is based on the reduction of the reagent (a mixture of tungsten and molybdenum oxides) in strong alkaline medium and measuring the absorbance of the product (blue colour) at 765 nm.

Reagents/Solutions: Folin-Ciocalteu's reagent working solution - 50% v/v aqueous solution of Folin-Ciocalteu's reagent; Sodium carbonate solution - 20% w/v; Standard gallic acid solution - 0.1 mg/ml (freshly prepared).

To 0.1 ml of the sample, 3.9 ml distilled water and 0.25 ml of working Folin-Ciocalteu's 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 the 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 methanol in place of sample/standard.

Evaluation of *In Vitro* Antioxidant Activity

Screening by HPTLC-DPPH Bioautography Method

The preliminary screening for antioxidant activity of the samples was carried out by using HPTLC-DPPH Bioautography method.[16,17] After development, as mentioned above for HPTLC profile, the plate was dried at room temperature, sprayed with 0.2% methanolic DPPH solution and observed after keeping in dark for 30 minutes.

Ferric Reducing Antioxidant Power Assay

The FRAP assay was carried out by the method described by Benzie and Strain[18] 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 colour 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 20 mM concentration was prepared by 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.

To 0.1 ml of the sample solution, 3.0 ml of FRAP working reagent was added, 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 methanol in place of sample/standard.

Assay for In Vitro DPPH – free Radical Scavenging Activity (DPPH Assay)

DPPH radical gives strong absorbance at 517 nm (deep violet colour) due to its unpaired electron; when this radical pairs off in presence of a free radical scavenger, the absorption vanishes and the resulting discolouration is stoichiometric with respect to the number of electrons taken up. DPPH – free radical scavenging activity assay was carried out using reported method[19,20] with suitable modifications.

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

Different aliquots of sample/standard solutions were taken in series in a set of test tubes and methanol was added to make the volume up to 3 ml in each case. 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. One millilitre of DPPH reagent diluted to 4 ml with methanol was taken as reagent blank. Percent scavenging activity was calculated as

$$\% \text{ Scavenging} = \frac{\left(A_0 - A_s\right)}{A_0} \times 100$$

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

The graphs of percent scavenging activity against concentration of sample in mg were plotted for each series and the half minimal inhibitory concentrations (IC_{50}) were calculated.

RESULTS AND DISCUSSION

HPTLC Profile

Natural products contain multiple chemical substances with varied chemical nature and it is very difficult to pinpoint a particular compound to which, the complete biological activity of the product can be attributed. In such cases, chromatographic profile is very useful and widely used for its quality evaluation. Our literature survey did not reveal any report on HPTLC profile of cow dung and hence the HPTLC profile has been evolved. The mobile phase Chloroform: Methanol (85:15) gave optimum resolution and was used for developing the plate. The chromatograms (at 366 nm) as well as comparative 3D-graph of the samples are presented in Figures 1 and 2, respectively. The chromatographic profile is more or less similar in almost all samples irrespective of time of collection though in one of the samples (Track 6), noticeable qualitative difference is observed. The bright blue-coloured fluorescent spot (R_f 0.91) is the major spot in this sample and is also present in one more sample (Track 4) among 17 samples in total. Another notable difference is the absence of the spot at R_f 0.98 only in sample 6. Few quantitative differences were also noted; however, it has hardly any correlation with time/day of collection.

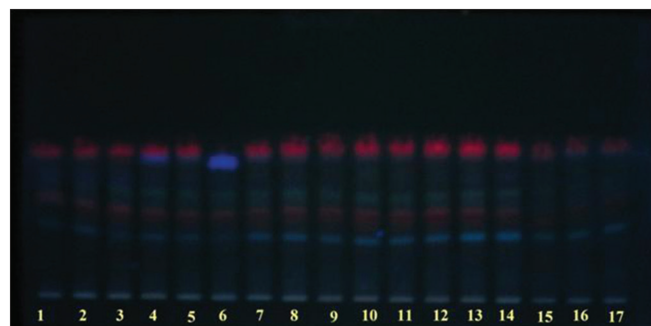

Figure 1: HPTLC Profile (at 366 nm) of cow dung samples

Determination of Total Phenolic Content

The estimated total phenolic content of all the samples, expressed in gallic acid milligram equivalents with respect to per gram of original sample, is presented in Table 1. The total phenolic content varies between 0.479 and 1.699 mg/g, with sample 6 exhibiting the highest content, closely followed by sample 10; being the average value as 1.238 mg/g. A large portion of the samples, i.e., 8 out of 17, has the total phenolic content between 1.3 and 1.6 mg/g.

Evaluation of *In Vitro* Antioxidant Activity

Initial screening was carried out by using HPTLC-DPPH Bioautography method. Further, quantitative estimation of *in vitro* antioxidant activity was carried out by Ferric-reducing Antioxidant Power (FRAP) method and DPPH – free radical scavenging activity (DPPH) method.

Screening by HPTLC-DPPH Bioautography Method

The HPTLC-DPPH Bioautography method is very useful for rapid screening of natural products for antioxidant potential. In this method, the presence of antioxidant compounds are detected by yellowish spots on the chromatograms against a purple background. In the present study, all the samples revealed presence of yellowish spots after spraying with DPPH reagent, indicating existence of antioxidant compounds in them.

Ferric-reducing Antioxidant Power Assay

The estimated FRAP value for all the samples, expressed in ascorbic acid milligram equivalents with respect to per gram of original sample, is presented in Table 2. It varies between 0.565 and 1.625 mg/g (average: 1.015 mg/g), with sample 6 having the highest FRAP value, followed by sample 10. The value for majority of the samples, i.e., 9 out of 17, lies between 0.98 and 1.28 mg/g.

Assay for *In Vitro* DPPH – Free Radical Scavenging Activity (DPPH assay)

The calculated IC_{50} values expressed in milligrams of original sample are presented in Table 3. The IC_{50} varies between 12.810 and 41.554 mg, with sample 10 at the lowest i.e., with highest activity, followed by sample 6; being average value as 24.836 mg. Majority of the samples i.e., 13 out of 17, have the IC_{50} value below 30 mg. IC_{50} of ascorbic acid, under similar conditions, was found to be 20.13 µg.

All the samples revealed antioxidant activity in the methods used in the study, though at varying degree. Samples of

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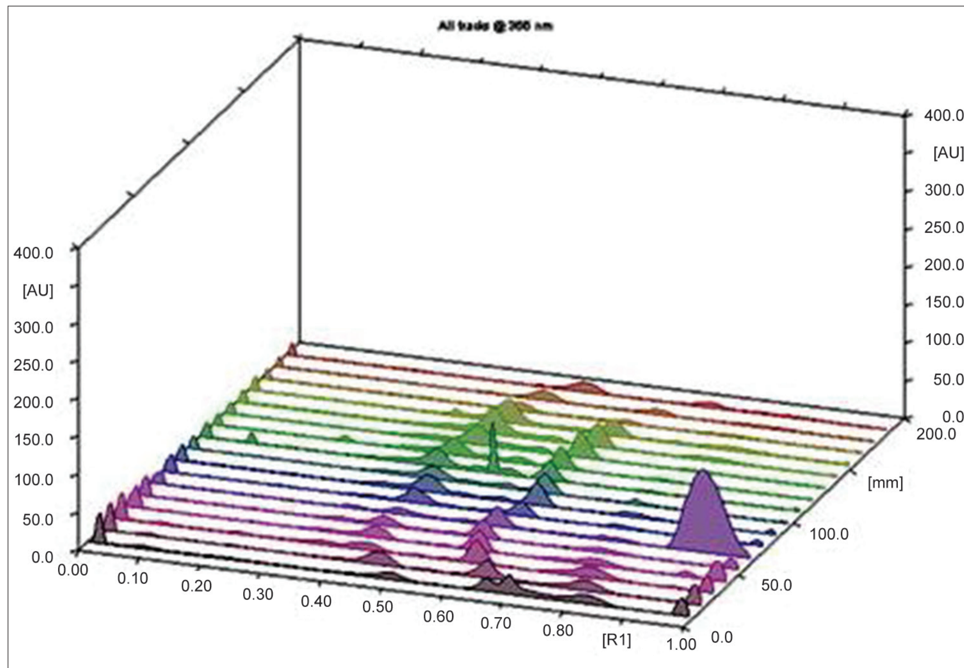

Figure 2: HPTLC Profile of cow dung samples - 3D graph at 366 nm

Table 1: Total phenolic content of cow dung samples

Collection schedule	Total phenol content (mg gallic acid/gm)	Mean±SEM
Day 1, 3.30 am	1.133	1.238±0.092
Day 1, 8.30 am	0.479	
Day 1, 2.30 pm	1.521	
Day 2, 3.30 am	0.777	
Day 2, 8.30 am	1.429	
Day 2, 2.30 pm	1.699	
Day 3, 3.30 am	1.372	
Day 3, 8.30 am	1.494	
Day 3, 2.30 pm	1.395	
Day 4, 3.30 am	1.685	
Day 4, 8.30 am	1.520	
Day 4, 2.30 pm	1.540	
Day 5, 3.30 am	1.483	
Day 5, 8.30 am	1.129	
Day 5, 2.30 pm	0.723	
Day 6, 8.30 am	0.929	
Day 6, 2.30 pm	0.729	

SEM – Standard error of means

Table 2: FRAP value of cow dung samples

Collection schedule	FRAP (mg eq. ascorbic acid/g of dung)	Mean±SEM
Day 1, 3.30 am	0.565	1.015±0.067
Day 1, 8.30 am	0.811	
Day 1, 2.30 pm	0.983	
Day 2, 3.30 am	1.275	
Day 2, 8.30 am	1.083	
Day 2, 2.30 pm	1.625	
Day 3, 3.30 am	0.991	
Day 3, 8.30 am	1.168	
Day 3, 2.30 pm	1.032	
Day 4, 3.30 am	1.349	
Day 4, 8.30 am	1.198	
Day 4, 2.30 pm	1.161	
Day 5, 3.30 am	1.063	
Day 5, 8.30 am	0.846	
Day 5, 2.30 pm	0.710	
Day 6, 8.30 am	0.647	
Day 6, 2.30 pm	0.746	

SEM – Standard error of means; FRAP – Ferric reducing antioxidant power

the present study were collected randomly from various cows, on different days at varied times. Considerable variations are seen in total phenolic content as well as *in vitro* antioxidant activity of the samples indicating both inter-day and intra-day variations. Considering the dependency of results upon various factors like physiological condition of the cow as well as diurnal and individual variations, such variation is not unexpected.

Samples 6 and 10 were found to exhibit more promising antioxidant potential as compared to other samples. As far as chromatographic profile is concerned, a distinguished component in significant proportion, was found in sample 6, which was absent in sample 10. As total phenolic content of these two samples is almost comparable, it can be inferred that more than one bioactive compounds probably of non-phenolic nature have also been contributing to the attributes discussed.

The HPTLC profile as well as antioxidant potential of cow dung is reported for the first time. The evolved chromatographic profile will be very useful for evaluating

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Table 3: IC_{50} in DPPH assay of cow dung samples

Collection schedule	DPPH (IC_{50} in mg)	Mean±SEM
Day 1, 3.30 am	28.534	24.836±2.052
Day 1, 8.30 am	35.236	
Day 1, 2.30 pm	29.888	
Day 2, 3.30 am	26.733	
Day 2, 8.30 am	21.536	
Day 2, 2.30 pm	15.368	
Day 3, 3.30 am	20.137	
Day 3, 8.30 am	16.667	
Day 3, 2.30 pm	16.924	
Day 4, 3.30 am	12.810	
Day 4, 8.30 am	16.967	
Day 4, 2.30 pm	19.675	
Day 5, 3.30 am	21.582	
Day 5, 8.30 am	28.781	
Day 5, 2.30 pm	35.377	
Day 6, 8.30 am	41.554	
Day 6, 2.30 pm	34.439	

SEM – Standard error of means; DPPH – Diphenyl-1-picrylhydrazil

the quality of cow dung. It has potential as a natural antioxidant.

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