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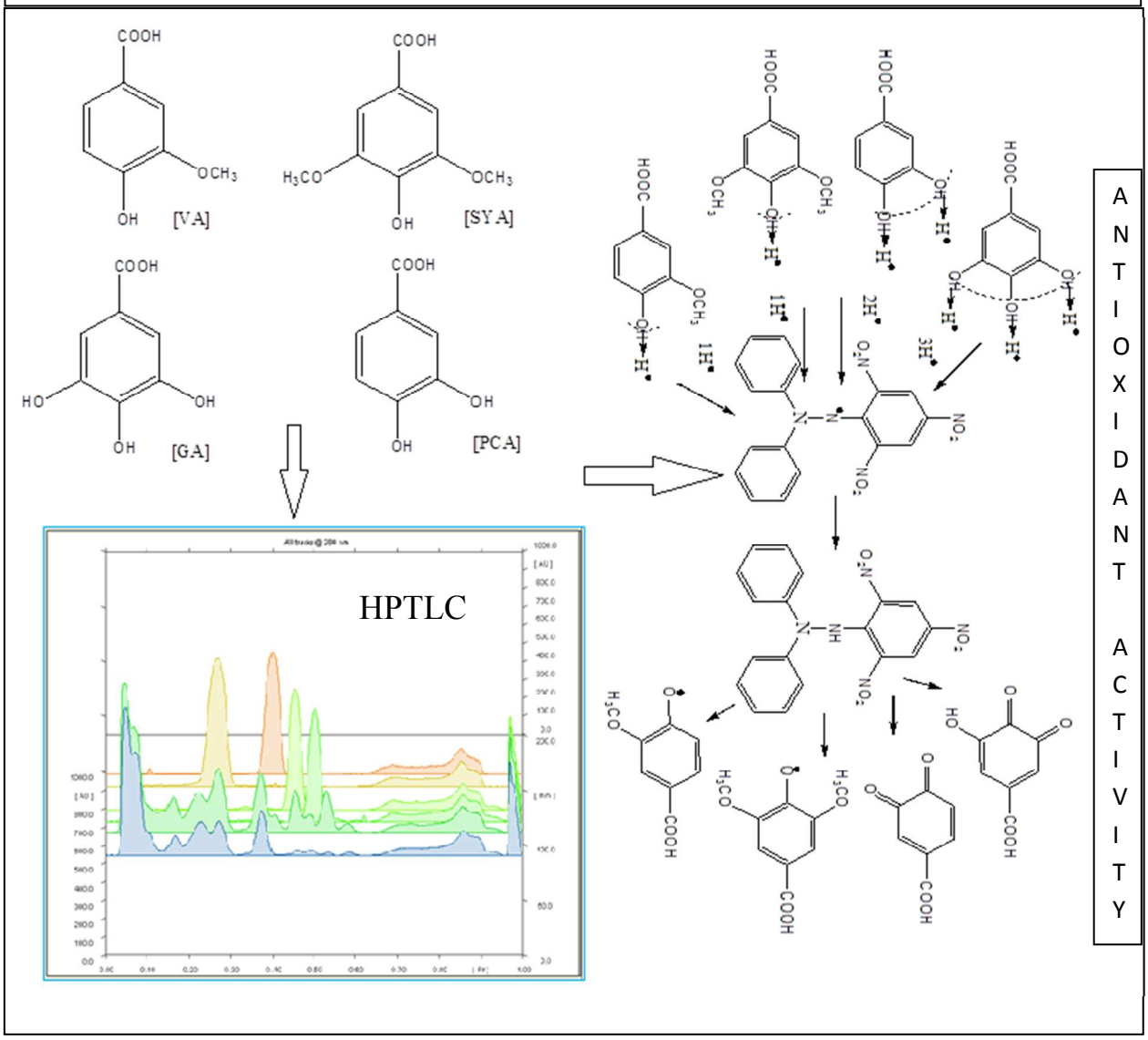
Graphical Abstract

We developed HPTLC method for quantification of Vanillic acid, Syringic acid, Gallic acid and Protocatechuic acid i and Kinetic studies on antioxidant potential in *Bergenia ciliata* and *Bergenia strachevi*



HPTLC-densitometric determination and Kinetic studies on antioxidant potential of Monomeric phenolic acids (MPAs) from *Bergenia species*

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Abstract

The aim of the present communication is the development of validated HPTLC method for simultaneous separation, detection, comparative quantification of monomeric phenolic acids (MPAs) such as Vanillic acid(VA), Syringic acid(SYA), Gallic acid(GA), Protocatechuic acid(PCA) in *Bergenia* species viz. *Bergenia ciliata* (*BC*) and *Bergenia stracheyi (BS)* (Paashanbheda; family Saxifragraceae) and Kinetics studies on antioxidant activity of focused metabolites. The analyses were performed on HPTLC pre-coated silica gel $60F_{254}$ plates with optimized solvent system toluene:ethyl acetate:formic acid (5:4:1 v/v/v) as mobile phase. Densitometric detection of MPAs was performed at wavelength (λ max) 280nm respectively. The contents of MPAs in both species were found (% in 10mg/ml) 0.007±0.1-0.003±0.4 (VA) (y=3.326x-1103, regression coefficient r=0.998), 0.017±0.4-0.002±0.5 (SYA) (y=3.410x-1009, r=0.998), 0.024±0.2-0.012±0.2 (GA) (y=5.349x-240.2, r=0.999) and 0.027±0.6-0.018±0.2 (y=3.6x-461.5, r=0.995). Quantitative variation is assumed as a result from samples collected from different altitudinal range. Two antioxidant assays DPPH and β-carotene were used kinetically in antioxidant potential assessment. Among both species *BC* had higher DPPH antioxidant activity and antiradical kinetics than *BS*, MPAs and positive controls (TOCO), (BHT). Whilst in β-carotene assay highest antiradical activity is reported in PCA kinetically despite BHT than others. However the deviation in C_{AA} values of *BC* and *BS* extracts are very close to the PCA value. EC_{50} values, rate constant (k), rate of reaction (dx/dt), half-life and average life were also measured in both assays. On the basis of finding it can be concluded that investigated MPAs actively involved in antioxidant properties. Kinetic studies of MPAs revealed that H atom transfer from phenolic moieties to the ROS predicts the reactivity of antioxidants.

Introduction

The genus *Bergenia* (family Saxifragraceae) and its species viz. *Bergenia ciliata (BC)* and *Bergenia stracheyi (BS,)* is an evergreen perennial herb, generally distributed in Central and East Asia. It is also found in temperate Himalayas from Kashmir to Bhutan at high altitude 7000-10000 feet and in khasia hill at 400 feet[1]. Previous studies on phytochemical analysis of *B. ciliata* have been shown the isolation of bergenin (C-glycoside of 4-*O*-methyl gallic acid), gallic acid (3,4,5 trihydroxybenzoic acid), (+)catechin, leucocyanidin, (+)-catechin-3-gallate, (+) catechin-7-*O*-beta-D-glucopyranoside, paashaanolactone, β-sitosterol, β-sitosterol-D-glucoside, and (+)afzelechin[2]. These phytochemicals have a range of biological activities such as antioxidant[3,4] antidiaarheol, anti-inflammatory, menorrhagia, excessive hemorrhage[5,6] antibacterial, antitussive[7,8] and in the treatment of pulmonary infections[9].

The pharmacological activity of extracts is dependent on the contents of active secondary metabolites in the plants. On varying the contents of secondary metabolites the activities also varies. Therefore, it is important to quantify the active secondary metabolites to find out the accurate pharmacological action of respective samples.

There is only limited paper available on analytical HPLC and HPTLC method development for quantification of Bergenin and Gallic acid in different *Bergenia* species[10,11,12]. No previous report available on simultaneous quantification of Vanillic acid (VA), Syringic acid (SYA), Gallic Acid (GA) and Protocatechuic acid (PCA) in *Bergenia* species using high performance thin layer chromatography (HPTLC) (Figure 1). Due to several advantage over other analytical methods such as the rapidity, less amount of test sample and extremely limited solvents waste, HPTLC has gained widespread interest as most acceptable technique for the

determination of pharmacologically interesting compounds in the biological matrices like plants and its different parts and even in formulations[13,14] .

Morever, VA, SYA, GA and PCA were reported to possess various pharmacological effects which may be closely correlated with its antioxidant activities[15,1]. It has been well recognized that, several biochemical reactions involve the generation of ROS (reactive oxygen species) in human body. However, the balance between the generations of diminution of ROS under normal conditions is controlled by antioxidant defense system. In case of certain pathological conditions, when ROS are not effectively eliminated by the antioxidant defense system, the dynamic balance between the generation and diminution of ROS is broken. Excessive ROS and free radicals attack on carbohydrates, proteins, lipids, DNA and result into oxidative stress, which leads to various disorders and diseases[17]. Antioxidants are the compounds capable to either delay or inhibit the oxidation processes which generate free radicals and reactive oxygen species. For the protection of bio-molecules against the attack of ROS, a no. of synthetic antioxidants such as 2- and 3-tert-butyl-4-methoxyphenol (i.e. butylated hydroxytoluene BHT, and tert-butylhydroquinone TBHQ) have been added to foodstuffs and also used for industrial processing in recent periods, but because of their toxicity issues, their use is being questioned[1.]

Yet, these synthetic antioxidants have been suspected of being harmful[19] and cause severe side effects. Thus, considerable focus has been given to searching for natural antioxidants from plants in the recent years. The plant derived antioxidants can be phenolic acid (flavonoids and tannins) nitrogen –containing compounds such as alkaloids, chlorophyll derivatives, amino acids, peptids), DL-α-Tocopherol acetate or ascorbic acid and its derivatives[20]. Natural phenolic compounds are now proven as potent antioxidants which inhibit the generation of free ROS

quickly, compare to synthetic compounds. Therefore, plant extracts rich in polyphenolics are increasingly of interest to the food industry because being capable to retard the oxidative degradation of lipids and thereby improve the quality and nutritional value of the edible materials.

Consolidated comparative quantitative studies of MPAs using high performance thin layer chromatographic (HPTLC) and their antioxidant activity evaluation allows the analysts to determine the potency of each component within total extract[21]. Furthermore, it also allows the recovery of most active compounds and decides upon the best technology development of extraction which enhances the quantity of potent compounds and to formulate the products with these properties[22]. In order to evaluate antioxidant activity it is important to understand the mechanism of reaction involved in scavenging of free radicals. According to DPPH assay the order of antioxidant activity (AA) is *BC*~GA>PCA>SYA>*BS*>BHT~TOCO~VA of tested MPAs and extracts and in β-carotene the order of (AA) is BHT>PCA>TOCO>*BC*>GA>SYN>VA>*BS*. The results obtained from these two assays differ despite of similar conditions used in experiments. It seems important to notice that the compound which is more active in DPPH assays, may not show the same potency in case of β-carotene assay. This contradiction can be only obvious, since the (AA) is not an inherent property of a particular compound, but depends on the nature of free radical that is reacting with it. Free radical originated from hydrophilic reactions prefers polar compounds and those generated from lipophilic reactions like to neutralize by non-polar antioxidants. Kinetic study is preferred to understand the order and mechanism of reaction and it's also help in estimation of different parameters required for stability study of compounds. Therefore, it was considered important to

assess the comparative scavenging activity of each benzoic acid derivatives and extracts. For assessing the antioxidant activity DPPH and β-carotene assay are used.

Test

Chemicals

Vanillic acid (purity: 98% w/w), Syringic acid (purity: 99% w/w), Gallic acid (purity: 98% w/w) Protocatechuic acid (purity: 99% w/w), 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) were procured from Sigma-Aldrich USA, DL-α-Tocopherol acetate (TOCO), Butylated hydroxy (BHT) from Laboratory Rasayan, linoleic acid, β-carotene from MP Biomedicals LLC and Tween 40 were procured from Merck. All the solvents used were of analytical grade from Rankem India.

Preparation of crude extracts

B. ciliata and *B.stracheyi* were collected from Lansdowne and Juda ka talab, Uttrakhand, India in the month of August and December 2012, deposited (voucher specimen no. 254021 and 262557) in repository of CSIR-National Botanical Research Institute, Lucknow (Uttar Pradesh) India(Table-1). After washing with tap water, rhizomes were chopped and dried under shade conditions. The dried rhizomes (100 g) were crushed into powdered and soaked in absolute methanol (4 x 250 mL) at room temperature (25 ± 2^0C) for 5 days. The suspension was filtered and evaporated to dryness by using rotary evaporator (Buchi, USA). Methanolic extract of *B. ciliata* and *B.stracheyi* were further hydrolyzed in acidic medium as per reported by Srivastava et al., 2014.

Acid-hydrolysis

Two grams extract was added into 10 ml of methanol containing 2N HCl respectively. These mixtures were refluxed in a thermostatically controlled water bath linked magnetic stirrer with continuous stirring at 80 ^{0}C for 30 min. The samples were cooled at room temperature and dried

over vacuum to yield solid residue. Then the extract was washed and dissolved in distill water and eventually extracted thrice with ethyl acetate.

HPTLC method

Apparatus

Camag Linomat V automated TLC applicator, Camag twin trough glass chamber, ascending. Camag TLC scanner model 3 equipped with Camag Wincats IV software were used during the study at temperature 27± 2^0C, relative humidity.

Chromatographic experiments

Sample solution and standards were applied on precoated silica gel $60F_{254}$ HPTLC plates with 6 mm band width using Camag 100 microlitre sample syringe (Hamilton, Switzerland) with a Linomat 5 applicator (Camag, Switzerland) under a flow of N_2 gas. The Linear ascending development was carried out with Toluene/ethyl acetate/formic acid (5:4:1 *v/v/v*) as a mobile phase in a Camag glass twin trough chamber (20 x 10 cm).The saturation time of the TLC chamber in the mobile phase was optimized to 20 min for a good resolution of the tested markers and total run time was about 25 minutes at room temperature (27 ± 2°C), 50 % $\pm$ 2 % relative humidity. After run, plates were dried over hair drier and TLC image was taken on wavelength λ_{max} 254 and 365 (Figure-8). Scanning of TLC plate were performed by using Camag TLC Scanner 3 at λ_{max} 280 nm in UV absorbance mode for all tracks, TLC plate were developed at distance of approximately 80 mm from the point of application and slit dimensions were 4 mm × 0.45 mm. Quantification evaluation of the plate was performed using peak area with linear regression of amount 1-6µg/band (Table-2). Peak profiling was done in ultra violet region at 280 nm (Variable wavelength was used to get best absorbance range) check the identity of the bands,

UV absorption spectrum of each standard was overlaid with the corresponding band in the samples track (Figure-3). Standards solid chromatogram is illustrated in (Figure-4).

Assessment of antioxidant activity

Assays of total phenolic contents (TPC)

Total phenolic content (TPC) was quantified as described by the method of Singh et al., 2010[23] and expressed as mg gallic acid equivalents (GAE)/mg extract. The extract (1 mg/ml), Folin–Ciocalteau's reagent (1N) and 20% sodium carbonate were added subsequently. The test mixture was mixed properly on cyclomixer and left at room temperature for 30 min. Then the volume of mixture was maintained up to 25 ml with deionized water. The absorbance of test mixture was recorded at A_{720} nm using ''Thermo Scientific" Vis–UV spectrophotometer. TPC was determined using a standard curve with Gallic acid (0–50 μg/ml) as the standard.

Free radical scavenging activity (FRSA) assay

FRSA of the extracts was measured by using $DPPH^{\bullet}$ stable radical (Yen and Duh, 1994)[24]. Briefly, each 0.1 ml extract was added to freshly prepared 2.9 ml $DPPH^{\bullet}$ solution (6 x 10^{-5}M) and mixed vigorously. The reduction of the $DPPH^{\bullet}$ radical was measured by monitoring absorbance continuously at A_{517} nm until stable values was obtained. The percentage of remaining $DPPH^{\bullet}$ ($DPPH^{\bullet}_{rem}$) was calculated as $\%DPPH^{\bullet}_{rems} = DPPH^{\bullet}_{t} = 60/DPPH^{\bullet}_{t} = 0$ and plotted against the sample concentration (Figure-5). Results were expressed in the terms of percent inhibition and efficiency concentration (EC_{50}).

β-carotene bleaching assay

Antioxidant activity of the extract was performed by autoxidation of β-carotene and linoleic acid coupled reaction method as reported by Singh et al., 2009[25]. Briefly, 2 mg of β-carotene was dissolved in 20 ml chloroform. Three milliliters of β-carotene solution were added to 40 mg of

linoleic acid and 400 mg of tween 40 emulsion followed by the addition of 100 ml distilled water. The solution was thoroughly mixed and 3 ml aliquot of this emulsion were mixed with the extract (1 mg/ml) and incubated in a water bath at 50 ^{0}C for 60 min. Oxidation of this emulsified reaction mixtures were monitored by measuring the absorbance at A_{470} nm. The control contained solvent only in place of the extract. AOA was expressed as per cent inhibition relative to the control.

Statistical analysis

The sample extracts were named into *BC* (hydrolyzed *B.ciliata*) and *BS* (hydrolyzed *B.starcheyi*) extract. Quantitative variation of MPAs in two species *B. ciliata* and *B.stracheyi* was done. Data were analyzed by employing ANOVA at $p<0.05$ significance level for analyzing the results statistically.

Result and Discussion

Method validation

Specificity

The specificity of the methods was determined by analyzing the standards and samples bands. The bands for the MPAs (VA, SYA, GA, and PCA) in sample solution were confirmed by comparing the R_f and UV-spectra with the reference standards. A densitometer is used for providing whether the spot contains one compound or more by measuring its UV-spectrum at the up slope (peak start), apex (peak apex) and down slope (peak end). The value of correlation coefficient of up slope to apex ($r_{sm)}$ and apex to down slope (r_{me}) are found (≈0.99), so it can be conclude that the peak is pure (Table-3).

Calibration and quantification

The calibration curves for each standard MPAs (VA, SYA, GA, and PCA) were linear in the concentrations range of 1-6 µg/spot with correlation coefficient (r^2) 0.998, 0.997, 0.999 and 0.991 respectively. The regression data obtained showed a good linear relationship (Table-2). Plate development and spot scanning as well as quantification were performed as mentioned in section 2.3.2. and calibration curve was constructed (Table-4).

Accuracy

The accuracy of the methods was determined by analyzing the percentage recovery of the MPA in the samples. To obtain it, three sets were prepared from each species, i.e. *B.ciliata* and *B.stracheyi.* The samples were spiked with similar concentrations: 400ng for each standard MPA (VA, SYA, GA, and PCA). The spiked samples were recovered in triplicate and then analyzed by proposed HPTLC method. The average recoveries for each MPAs (VA, SYA, GA, and PCA) in *B.ciliata* were found to be 102.57, 104.26, 99.52, 101.14%, whereas in *B.stracheyi* the average recoveries for each MPAs were found to be 101.92, 100.82, 97.49, 99.16% respectively, within the acceptable RSD% (Table-5).

Precision

Instrumental precision was checked by repeated scanning of the spot of standards MPAs (VA, SYA, GA, and PCA) five times each. The repeatability of the sample application and measurements of peak area was expressed in terms of percent relative standard deviation (% RSD). Intra-day precision study was achieved at different concentrations levels of 1-6 µg/spot of each standard MPAs (VA, SYA, GA, and PCA) were spotted in three times within 24 h and expressed in terms of percent relative standard deviation %RSD (Table-6). For inter-day precision study, same concentrations levels of 1-6 µg/spot of each MPA were used over a period

of 5 days and expressed as %RSD. The results showed no significant inter and intraday variation was observed in the analysis of the MPAs (VA, SYA, GA, and PCA).

Limit of detection (LOD) and quantification (LOQ)

In order to estimate the limit of detection (LOD) and limit of quantification (LOQ), the signal to noise ratio was determined. LOD was considered as 3:1 and LOQ as 10:1. In the present study LOD for MPAs (VA, SYA, GA, and PCA) estimation in samples was found to be 510.70, 778.06, 275.23, and 602.83ng/band, respectively whereas LOQ for MPAs (VA, SYA, GA, and PCA) estimation in samples was found to be 1547.58, 2357.76, 834.03 and 4457.30ng/band (Table-2).

Robustness

Robustness is a measure of the method to remain unaltered by small but deliberate variations in the method conditions, and is indicate of the reliability of the method. For robustness study different mobile phase composition, developing TLC distance and different TLC plate lots were assessed (Table-7).

Kinetic studies

Previously many reports had shown strong antioxidant activity of plant polyphenols in various model systems. We were quite interested to probe if polyphenols rich hydrolyzed extracts of *Bergenia* species would be showed antioxidant potential in *in vitro* system, and result showed the higher quantity of TPC in extracts of *B*C, greater is the antioxidant activity compares to *BS* extract. In our previous report, it is documented that acid hydrolysis is important practical approach to recover optimum quantity of phenolic compounds[22]. On the basis of previous finding we prepared the acid hydrolyzed samples and analyzed total phenolic contents.

The TPC varies from 24.2 to 179.1 µg GAE/mg extract (Figure 6). The order of TPC was descended in following order: *BC*>*BS*. Incidentally, *BC* with highest poly-phenolic contents also had higher amounts of targeted compounds (i.e. VA, SYA, GA and PCA) as evident from HPTLC analysis.

The DPPH$^{\bullet}$ radical has been widely used to estimate the free radical scavenging capacity of various antioxidants. The free radicals are scavenged by antioxidants that provide stability to the free radicals by electron or hydrogen donation. The un-reacted or remaining level of DPPH$^{\bullet}$ in the reaction medium was calculated by using the following relation.

% of remaining DPPH$^{\bullet}$ = 100×[*A*s517nm (t=30)/*A*c517nm] (Equation-1), where *A*s represents absorbance of sample at 517nm (λ max) measured at (t=30 min) and *Ac* represents absorbance of control at 517nm (λ max) measured at (t=0). It was observed that the % of remaining DPPH$^{\bullet}$ level linearly decreased with increased *B.ciliata* and *B.stracheyi* concentrations to a certain level then leveled off. Along with extracts each identified MPAs (VA, SYA, GA and PCA) concentration effect on % of remaining DPPH$^{\bullet}$ was also assessed. The effectiveness of the extracts of both species of *Bergenia* in scavenging the free radicals was estimated as the half maximal effective concentration (EC_{50}) (which refer to the concentration of drug which induce a response half way between the baseline and maximum after a specified exposure time) of both extracts separately in the reaction mixture that caused the decrease in the initial concentrations of DPPH$^{\bullet}$ by 50%, denoted as EC_{50}. EC_{50} values for the extracts of both species of *Bergenia* and MPAs (VA, SYA and PCA) along with TOCO and BHT (used as positive control) are presented in (Table-7). The results of investigated extracts, MPAs, TOCO and BHT, showed stronger free radical scavenging activity of *BC* extract over *BS* extracts, MPAs, TOCO and BHT. The effect of extracts, MPAs (VA, SYA and PCA), positive control TOCO and BHT on the kinetics of free

radical scavenging capacity for the investigated antioxidants is compared in (Figure-7). In Figure-8 the values of *A*s517nm (t=x)-*A*c517nm as the function of time are presented as concentrations of antioxidants, MPAs and positive control TOCO and BHT in the reaction mixture of amounting 0.1mg/mL. In (Figure-7) Y-axis value *A*s517nm (t=x)-*A*c517nm refers to the concentrations of DPPH$^{\bullet}$ scavenged at variable time interval (t=x). From the (Figure 5), it is clear that in the presence of extracts of *BC* extract rapid initial decrease of DPPH$^{\cdot}$ concentrations are followed by slow gradual disappearance of DPPH$^{\bullet}$. Antioxidants quench the free radicals by two major mechanisms: by hydrogen atom transfer or via electron transfer that may also occur in parallel[26]. However, end result is the same regardless of the mechanism, but kinetics differ[27]. The contribution of particular mechanism is depending upon the compound involved (Figure-9). DPPH$^{\cdot}$ quenching is considered to be mainly based on electron transfer mechanism whilst hydrogen atom transfer mechanism is marginal reaction pathway[28]. Reaction initiate with transfer of either electron or hydrogen atom from antioxidants to the free radicals. As it is clear from (Figure-7) that there are significant variations between the slopes after the completion of initial fast step that do not rank in the way as the EC_{50} values do. These variations are related to the role of slow secondary reactions which may dimerization or disproportionation of initially formed phenol-derived radicals. To analyze the first rapid step of DPPH$^{\cdot}$ quenching, different kinetic models has been proposed[29,30]. To study the dynamic behavior of the system being analyzed, some mathematical models proposed by Saguy and Karel, 1980[31]. It has already been established that antioxidant acidity follows the first order kinetics[32].

To evaluate the mechanism and time dose-response of antioxidants in this investigation, a general reaction rate equation for first order kinetics can be written as follow $-dx/dt=kf(x)^m$ (Equation-2), where x representing the concentration of reactant at time t, k represents rate of

reaction of order m. In the above equation m=1 and rate constant k are calculated at different time intervals depicted in (Table-8) Half-life of each were also calculated by using first order half-life equation $t_{1/2}$ =0.693/k. Half-life of any compound represents the concentration remains half at that time. Similarly the average life of all were also calculated using the equation Ƭ=1/k.

The rate of reaction (*Rs*) was calculated at different time intervals t=0-0.25min (initial rate), t=0.25-5min (reaction propagation), t=5-10min (after the completion of initial step) and at t=15, 20, 25min and t=30min (at the end of the observation when the reactions are presumably completed) depicted in (Table-7). To find the accurate EC_{50}, the graph (Figure-5) was plotted in between % inhibition and concentrations at different intervals and positive correlation coefficient of linear equation showed the value (r^2 =>0.9). EC_{50} values were calculated by taking mean of minimum base to maximum range on Y-axis to the X-axis.

To understand the Kinetics of antioxidant activity we plotted a graph between % inhibition and rate of reaction showed positive correlation in case of DPPH$^{\bullet}$ antioxidant activity. Higher the value of rate of reaction, more will be the activity. Yet this relationship has not been reported in previous studies. In first order kinetics the rate of reaction is directly proportional to the concentration of reactants at time t. Similarly, proportional relations were observed in the % inhibition and concentration. This relation lead into the correlation of % inhibition is proportional to the rate of reaction (Figure 10). In DPPH$^{\bullet}$ scavenging activity, the availability of proton is responsible for the attending the stability of free radicals. The decreasing absorbance value indicates the stability of free radical achieved by proton donated by targeted samples. In other words, de-colorization of sample solution shows the positive antioxidant activity. Higher the ability of samples to decolorize the DPPH$^{\bullet}$ solution more will be the potency of the samples. The overall results of kinetic study were summarized in the (Table-7). The order of DPPH$^{\bullet}$

antioxidant activity is *BC*~GA>PCA>SYA>*BS*>BHT~TOCO~VA. Results of (Figure-5) concentrations Verses time also support the above statements.

Similarly kinetic approach also used for assessing antioxidant activity of extracts, MPAs (VA, SYA, GA, PCA), positive control TOCO and BHT in β-carotene antioxidant assay. In plant and living system multiple phases in which lipids and water coexist with some emulsifier, therefore it become important to study the antioxidant assay using a heterogeneous system or emulsion is also required. The antioxidant activity using emulsions are defined as β-carotene antioxidant assay. Emulsion system of linoleic acid was used to estimate the antioxidant activity of the extracts, MPAs and positive controls. Temperature of reaction was mentioned under 50^0C to avoid or minimize the formation of side products. In the reaction mixture free radicals (peroxy radicals-ROO^-) formed from oxidation of linoleic acid that attack on β-carotene (target molecule) and result into rapid de-colorization of reaction medium. The mechanism of de-colorization of β-carotene can be slowed down by subsequent addition of antioxidants which donates hydrogen atom to quench the free peroxy radicals by converting it into lipid derivatives RCOOH via following mechanism.

$ROO^{\bullet}$ + β-carotene-----bleaching....Scheme (1),

$ROO^{\bullet}$+ AH----------RCOOH+ $A^{\cdot}$…Scheme (2)

The Kinetic profile of auto-oxidation of polyunsaturated fatty acid was evaluated using the observed data from β-carotene-assay. β-carotene was exposed to free peroxy radicals (Scheme 1 and 2) formed from emulsion of linoleic acid in the presence of antioxidants i.e. extracts, MPAs and positive control TOCO and BHT. The Kinetic of β-carotene assay was assessed as same as in case of $DPPH^{\bullet}$ quenching using the same expression [$-dx/dt=kf(x)^m$] m=1 (Equation-2). The value *Ac*470nm-*A*s470nm (t=x) refer to the change in the concentrations of β-carotene was

obtained by measurement of the absorbance of the sample, *as* 470nm (t=x) at t=20, 40, 60, 80, 100 and 120 min. The curve was plotted between value *Ac*470nm-*A*s470nm (t=x) as function of time in (Figure-8). The extracts concentrations used in emulsion were of 1mg/ml. Like, DPPH˙ free radical scavenging kinetics, the mathematical model that most satisfactorily describes the time dependence of *Ac*470nm-*A*s470nm (t=x) for extracts, MPAs, positive controls TOCO and BHT is the as function of time.

The antioxidant activity coefficient (C_{AA}) was calculated according to following (Equation-3)

$C_{AA} = 1-[A_{S470nm\ (t=0)}-As_{470nm\ (t=120)}/Ac_{470nm(t=0)}-Ac_{470nm\ (t=120)}]$.

In (Equation-3) $A_{S470nm\ (t=0)}$ denotes the initial absorbance of the sample along with antioxidants at time = 0 and $As_{470nm\ (t=120)}$ denotes the absorbance of sample at t = 120min. Similarly $Ac_{470nm(t=0)}$ shows the absorbance of control at t = 0 and $Ac_{470nm\ (t=120)}$ shows the absorbance of control at t = 120min. Results obtained from both assays are almost similar and extracts *BC* was found more active over *BS*, MPAs, TOCO despite BHT. The following order of activity based on C_{AA} was achieved BHT>PCA>TOCO>*BC*>GA>SYN>VA>*BS*. Higher the value of $C_{AA,}$ higher will be the β-carotene bleaching activity. In contrast to DPPH• antioxidant activity, the relation between inhibition and rate was not found positive as in β-carotene. Correlation coefficient was obtained from graph of %inhibition and rate showed the R^2=0.87. It has already been noted in the DPPH• antioxidant activity that greater the capacity of de-colorization of sample solution, higher will be the activity whilst in the case of β-carotene inverse of DPPH• observation was found, lower the capacity of de-colorization or higher the color retention of sample solution, higher will be the antioxidant activity (Figure 11).

The need of the kinetic study is to resolve the problem of present time, use of single-time dose response of one commercial antioxidants as calibration curve to compute the equivalently

antioxidant activity of sample is considered as common and incorrect practice due to availability of computational applications that provide the adequate tools to work with different variables in non-linear models also. Reduction to study the dose-response at one single time and expect to find linear relation often lead to unreliable values hiding the real aspects of the actual responses. Multiple times dependent dose response can be used to find linear regression curve and that can be used to describe the whole kinetic profile.

Conclusion

Simple, precise and reproducible HPTLC method for simultaneous separation and quantification of biologically active phenolics acids (VA, SYA, GA and PCA), was developed and validated for first time in *Bergenia species*. High contents of targeted MPAs were observed in *B.ciliata* compare to *B.stracheyi* and were also validated through measurement of TPC contents. On the basis of findings the higher antioxidant activity was reported in the same having high contents of TPC. In conclusion, PCA was proved to be more effective than other tested compounds in both lipid and aqueous mediums. Its contents varied from *BC* to *BS* and proved that PCA play major role in antioxidant activity of *BC*. Antioxidant activities were evaluated using kinetic approach to establish the relationship between quenching ability is directly associated with concentrations of active metabolites. Correlation between rate of reaction and % inhibition is established at first time in the present communication.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Legend to Tables

Table-1 Details of collection of *Bergenia* species.

Table-2 Statistical analysis of calibration curves in the HPTLC determination of MPAs (VA, SYA, GA & PCA)

Table-3 Peak purity test for MPAs (VA, SYA, GA & PCA)

Table-4 Quantification of MPAs (VA, SYA, GA and PCA) in *Bergenia* species.

Table-5 Recovery study to evaluate accuracy of method

Table-6 Inter- and Intra-day precision of MPAs (VA, SYA, GA & PCA)

Table-7 Robustness testing of the HPTLC method

Table-8 Antioxidant activity evaluation using first order kinetic for DPPH and β-carotene assay, rate constant, rate of reaction, ED_{50}, C_{AA}, half-life, average life

Legend to Figures

Figure-1 Chemical structure of MPAs (VA=Vanillic acid, SYA=Syringic acid, GA=Gallic acid and PCA=Protocatechuic acid.

Figure-2 Photograph of TLC plate at wavelength λ_{max}=254 and λ_{max}=365

Figure-3 Overlay spectra comparison of MPAs (VA, SYA, GA & PCA) with sample track (*BC* & *BS*).

Figure-4 All track chromatogram at wavelength λ_{max}=280nm. Abbreviation-VA=Vanillic acid, SYA= Syringic acid, GA= Gallic acid and PCA= Protocatechuic acid, *BC=Bergenia ciliata* and *BS=Bergenia stracheyi.*

Figure-5 Comparison of DPPH % Inhibition of each MPAs (VA, SYA, GA and PCA) at various concentrations

Figure-6 Comparative total phenolic contents in Hydrolyzed and Un-hydrolyzed BC and BS extract

Figure-7 The dependence of Ac 517 nm _ As 517 nm(t=x) on time of incubation at a *BC* and *BS* extract and MPAs concentrations in the reaction mixture of 0.1 mg/mL. Symbols represent experimental values; curves are plotted according to the parameters from Equation (1).

Figure-8 The dependence of Ac 470 nm - As 470 nm(t=x) on time of incubation at a *BC* and *BS* extract and MPAs concentrations in the reaction mixture of 1 mg/mL. Symbols represent experimental values, curves are plotted according to the parameters from Equation (1).

Figure-9 Availability of hydrogen free radicals from MPAs to DPPH free radicals.

Figure-10 Correlation of rate of reaction of DPPH free radical scavenging activity with % Inhibition.

Figure-11 Correlation of rate of reaction of *β-carotene* free radical scavenging activity with % Inhibition.

Abbreviations

BC=*Bergenia ciliate,* BS =*Bergenia stracheyi,* MPAs=Monomeric phenolic acids, VA= Vanillic acid, SYA=Syringic acid, GA=Gallic acid, PCA=Protocatechuic acid, ROS = Reactive Oxygen species, RNS=Reactive Nitrogen species, BHT=Butylated hydroxytoluene, TOCO =α-Tocopherol acetate

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Table-1 Details of collection of *Bergenia* species

Sample no.	Plant	State	Region explored	Collection stage	GPS information	Material
254021	*B. ciliata*	Uttrakhand	Lansdowne	Pre-flowering	9400 feet, N $31^0$03.116' E $78^0$11.096'	Whole Plant
262557	*B. stracheyi*	Uttrakhand	Juda ka talab	Pre-flowering	5400 feet, 29°50'N 78°41'E / 29.83°N 78.68°E	Whole Plant

Table-2 Statistical analysis of calibration curves in the HPTLC determination of MPA's (VA, SYA, GA & PCA)

Parameters	VA	SYA	GA	PCA
Accuracy	102.57	104.26	99.52	101.14
Rf value	0.47±0.02	0.43±0.01	0.23±0.01	0.38±0.01
Regression equation	y=3.326x-1103	y=3.410x-1009	y=5.349x-240.2	y=3.6x-461.5
Slope	3.326	3.41	5.349	3.6
Intercept	1103	1009	240.2	461.5
Linearity range	1-6µg	1-6µg	1-6µg	1-6µg
95% Confidance limits of intercept	-518.0196009	-94.16249112	267.4136872	1364.360849
Correlation coefficient (r)	0.998	0.998	0.999	0.995
LOD	510.7	778.06	275.23	602.83
LOQ	1547.58	2357.76	834.03	4457.3
SE of Intercept	210.9533511	329.5067881	182.8382463	657.6350141
SD of Intercept	514.7261768	803.9965629	446.1253209	1604.629434
P-value	0.006	0.03	0.0259173678	0.0521490957

Table- 3 Peak purity test for MPA's (VA, SYA, GA & PCA)

MPA	Standard track r (s, m)	Sample track r (s, m) *BC*	Sample track r (s, m) *BS*	Standard track r (e, m)	Sample track r (e, m) *BC*	Sample track r (e, m) *BS*
VA	0.999969	0.999263	0.998793	0.999942	0.997806	0.991686
SYA	0.998756	0.998426	0.999878	0.996367	0.996143	0.999623
GA	0.998256	0.998843	0.997241	0.996321	0.997944	0.987834
PCA	0.998779	0.999037	0.998944	0.9973	0.997562	0.99383

Table-4 Quantification of MPA's (VA, SYA, GA and PCA) in *Bergenia* species

S.No.	Sample	Extract (MeOH)	Applied Sample volume	% Content of MPAs in extract (10mg/ml)			
			10mg/ml; 10µl	VA	SYA	GA	PCA
1-	*B.ciliata*	Hydrolyzed extract	10µl	0.007±0.1	0.017±0.4	0.024±0.2	0.027±0.6
2-	*B.stracheyi*	Hydrolyzed extract	10µl	0.003±0.4	0.002±0.5	0.012±0.2	0.018±0.2

Table-5 Recovery study to evaluate accuracy of method

MPA	Amount present in BC in µg	Amount present in BS in µg	Amount added into sample	Theoretical value in BC	Theoretical value in BS	Average amount found in mixture of *BC*	Average amount found in mixture of *BS*	Average recovery in *BC*	Average recovery in *BS*
VA	740	290	400	1140	690	1169.3	710.5	102.5701754	102.9710145
SYA	1720	220	400	2120	620	2210.4	625.1	104.2641509	100.8225806
GA	2410	1170	400	2810	1570	2796.6	1530.6	99.52313167	97.49044586
PCA	2670	1820	400	3070	2220	3105	2201.4	101.1400651	99.16216216

Table- 6 Inter- and Intra-day precision of MPA's (VA, SYA, GA & PCA)

MPA	Concentration (ng/spot)	Intraday		Interday	
		RSD%	Mean RSD%	RSD%	Mean RSD%
VA	4000-6000	2.65	99.80±2.64	2.29	100.22±2.29
SYA	4000-6000	2.74	99.72±2.73	2.75	100.7±2.78
GA	4000-6000	2.01	99.27±1.99	1.37	100.28±1.37
PCA	4000-6000	4.71	99.82±4.67	2.13	102.19±1.37

Table-7 Robustness testing of the HPTLC method

Parameters	RSD% of peak area			
	(VA)	(SYA	(GA)	(PCA)
Time interval difference between spotting and plate development	0.32	0.36	0.43	0.56
Mobile phase composition	0.39	0.7	0.73	0.76
Time interval between drying and scanning	0.37	0.5	0.67	0.78

RSD= Relative standard deviation

Table- 8 Antioxidant activity evaluation using first order kinetic for DPPH and β-carotene assay, rate constant, rate of reaction, EC_{50}, C_{AA}, half-life, average life

	Rate constant^{D} K (Mean±SD) (1×10^{-2}(min^{-1}))	Rate of reaction^{D} (Average)	$EC_{50}{}^{D}$ (t=0.25,10,20,30min)	Half Life^{D} $t_{1/2}$ (Mean)	Average life^{D} (τ) (Mean)	Rate constant$^{\beta}$ K (Mean±SD) (1×10^{-3}(min^{-1}))	Rate of reaction$^{\beta}$ (Average)	$C_{AA}{}^{\beta}$	Half Life$^{\beta}$ $t_{1/2}$(Mean)	Average life$^{\beta}$ (τ) (Mean)
BC	88.27±0.05	0.144788	$54.08277^{t=0.2}$ $54.90861^{t=10}$ $55.06106^{t=20}$ $52.24576^{t=30}$	0.768055	1.108305	30.129218±0.07	0.01697	0.856718	24.82295	35.81955
BS	88.82±0.03	0.021078	$53.84259^{t=0.25}$ $66.86047^{t=10}$ $72.03846^{t=20}$ $5.586592^{t=30}$	0.76422	1.102771	22.473847±0.04	0.014521	0.764849	31.09774	44.87409
VA	76.30±0.05	0.027918	$223^{t=0.25}$ $87.75^{t=10}$ $41.18519^{t=20}$ $151.6875^{t=30}$	0.863595	1.246168	35.6869547±0.05	0.186336	0.822632	20.88828	30.14185
SYA	83.96±0.06	0.190908	$7.87879^{t=0.25}$ $8.83268^{t=10}$ $47.70115^{t=20}$ $64.01786^{t=30}$	0.799937	1.154311	34.978968±0.06	0.017452	0.829644	23.03764	33.24335
GA	102.11±0.05	0.057927	$57.5^{t=0.25}$ $194.7475^{t=10}$ $41.79063^{t=0}$ $43.31719^{t=30}$	0.681032	0.98273	34.964833±0.05	0.016605	0.849656	23.41162	33.78209
PCA	91.51±0.07	0.110352	$56.63452^{t=0.25}$ $54.69366^{t=10}$ $54.13303^{t=20}$ $55.33425^{t=30}$	0.745764	1.076139	36.06857±0.06	0.014859	0.913298	24.06178	34.72118
BHT	108.73±0.03	0.036225	$83.33333^{t=0.25}$ $53.97154^{t=10}$ $56.96815^{t=20}$ $58.56125^{t=30}$	0.645982	0.932153	25.405815±0.05	0.009022	0.981896	28.90811	41.71444
TOCO	103.51±0.06	0.019808	$89.57143^{t=0.25}$ $80.30435^{t=10}$ $109.2903^{t=20}$ $87.16667^{t=30}$	0.673302	0.971576	29.313845±0.04	0.017703	0.861469	25.81279	37.24722

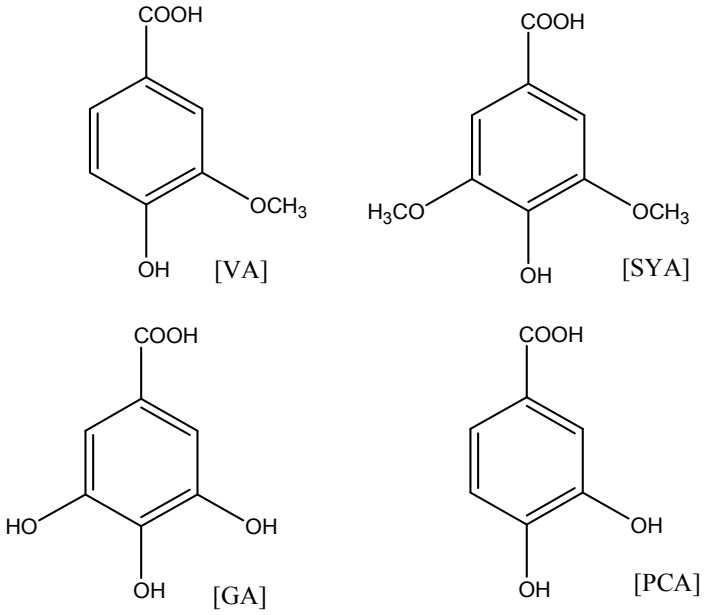


Figure-1 Chemical structure of MPA's (VA=Vanillic acid, SYA=Syringic acid, GA=Gallic acid and PCA=Protocatechuic acid

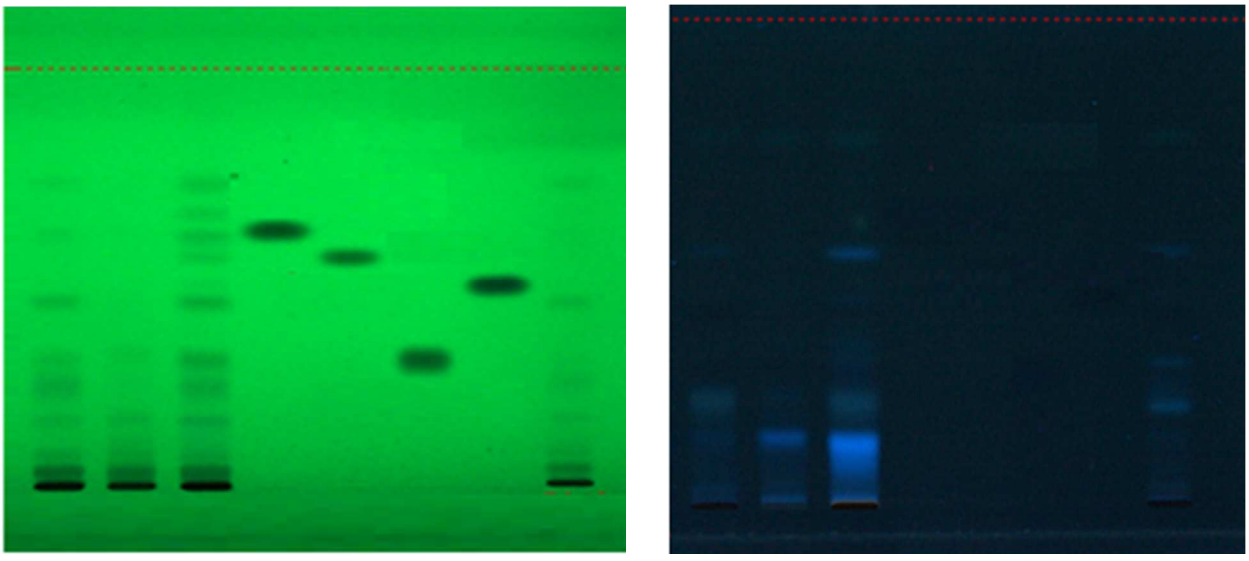
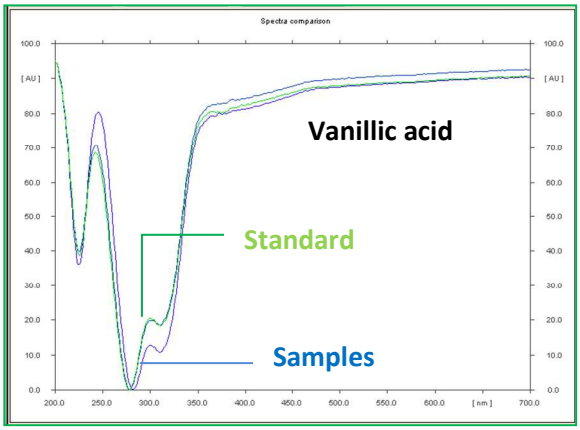
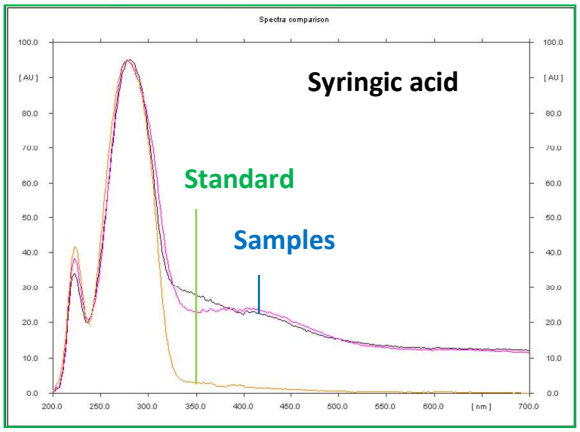
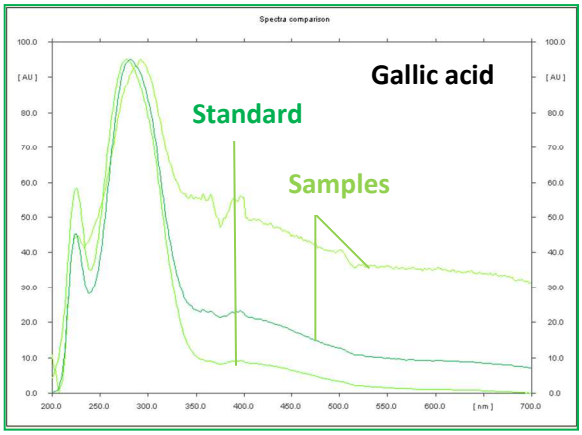


Figure-2 Photograph of TLC plate at wavelength λ_{max}=254 and λ_{max}=365







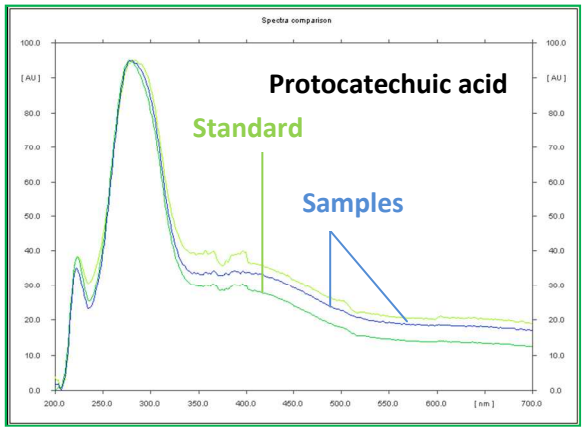
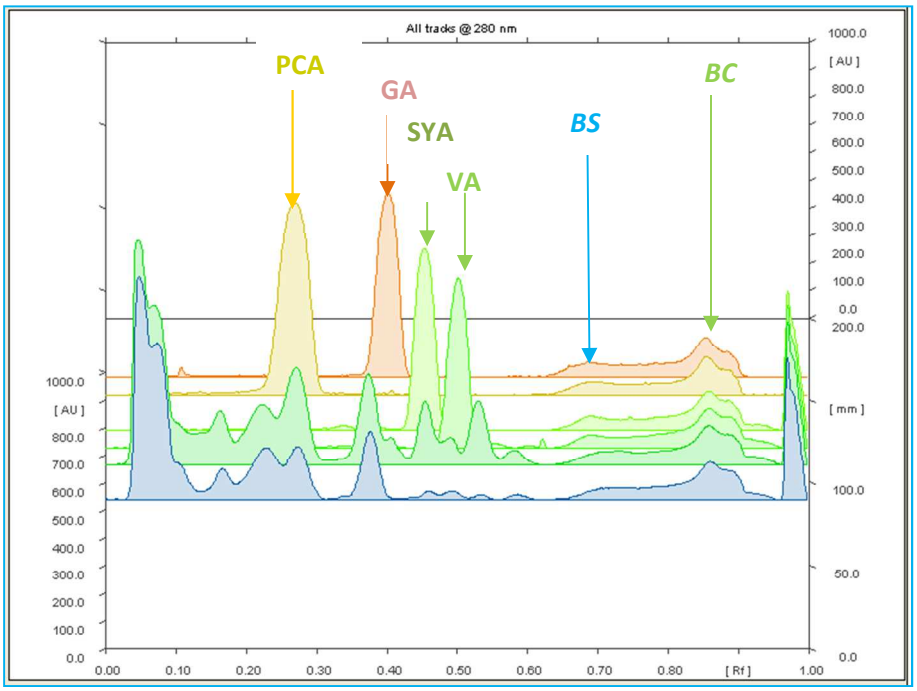


Figure- 3 Overlay spectra comparison of MPA's (VA, SYA, GA & PCA) with sample track (*BC* & *BS*)



Figure–4 All track chromatogram at wavelength λ_{max}=280nm. Abbreviation- VA=Vanillic acid, SYA= Syringic acid, GA= Gallic acid and PCA= Protocatechuic acid, *BC=Bergenia ciliata* and *BS=Bergenia stracheyi*

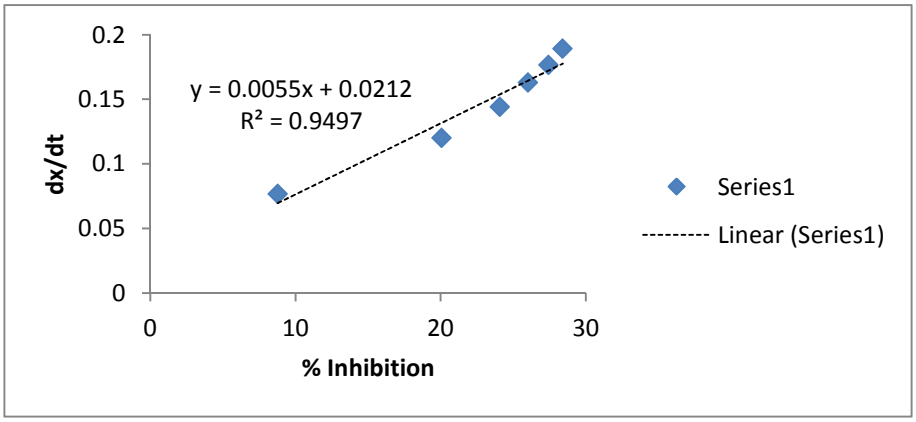


Figure-5 Comparison of DPPH % Inhibition of each MPA's (VA, SYA, GA and PCA) at various concentrations

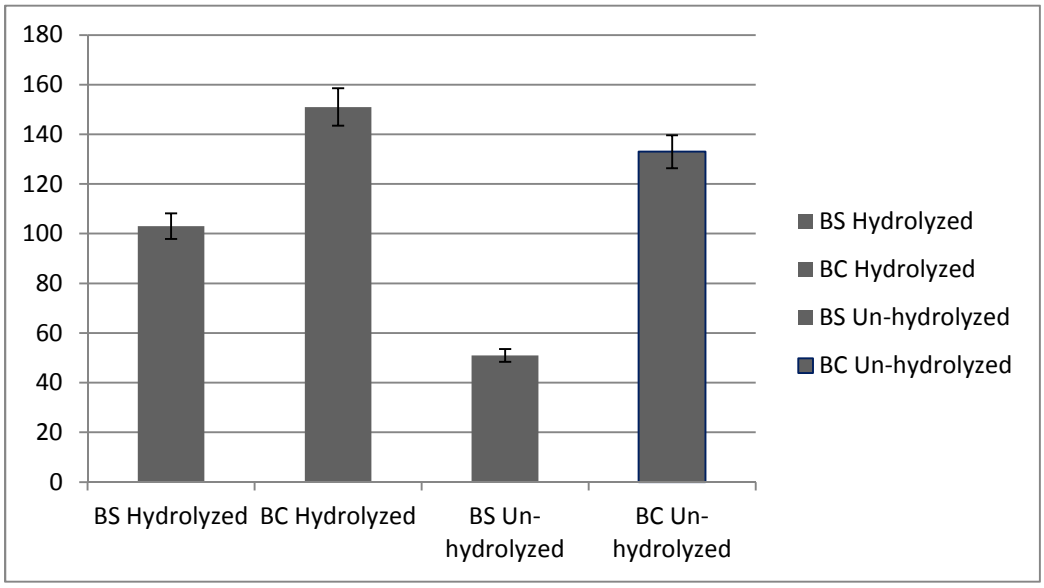


Figure 6 Comparative total phenolic contents in Hydrolyzed and Un-hydrolyzed BC and BS extract

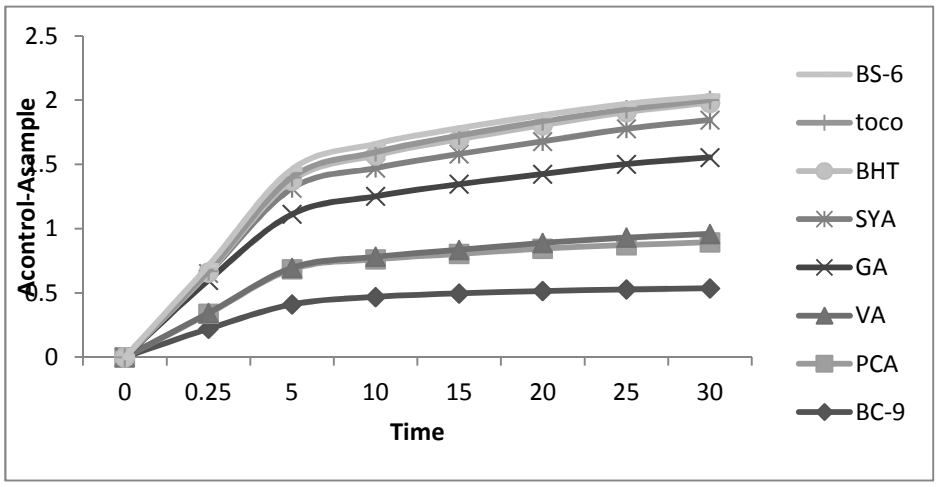


Figure-7 The dependence of Ac 517 nm-As 517 nm(t=x) on time of incubation at a *BC* and *BS* extract and MPA's concentrations in the reaction mixture of 0.1 mg/mL. Symbols represent experimental values, curves are plotted according to the parameters from Equation(1).

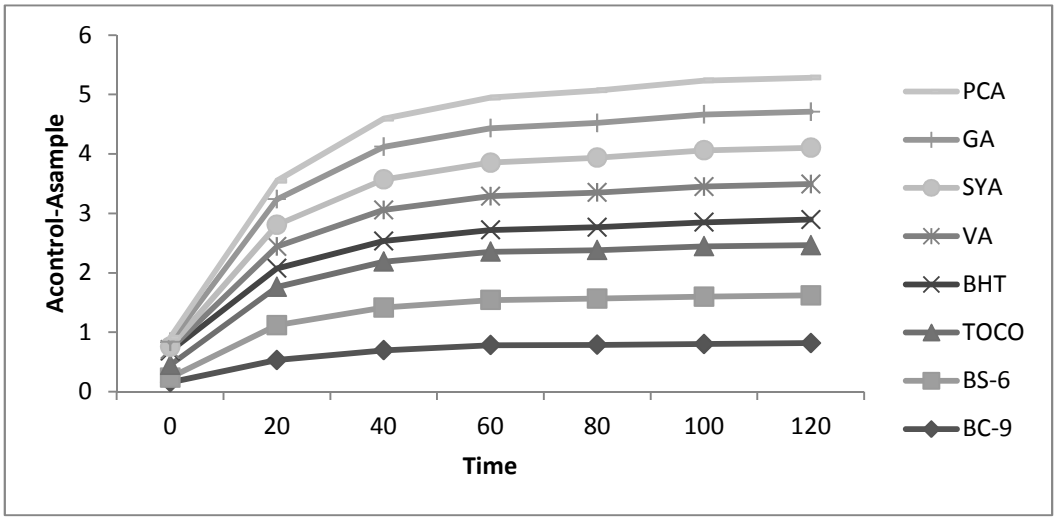


Figure- 8 The dependence of Ac 470 nm - As 470 nm(t=x) on time of incubation at a *BC* and *BS* extract and MPA's concentrations in the reaction mixture of 1 mg/mL. Symbols represent experimental values, curves are plotted according to the parameters from Equation.(1).

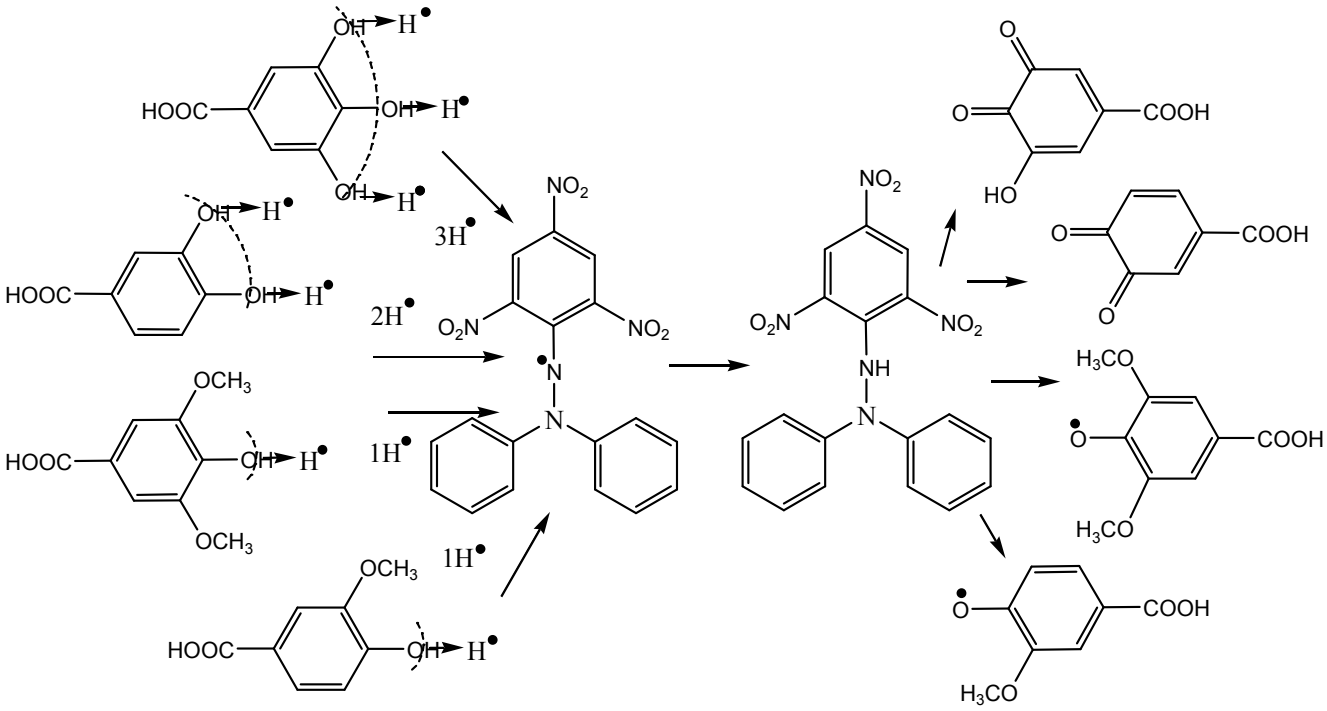


Figure- 9 Availability of hydrogen free radicals from MPA's to DPPH free radicals

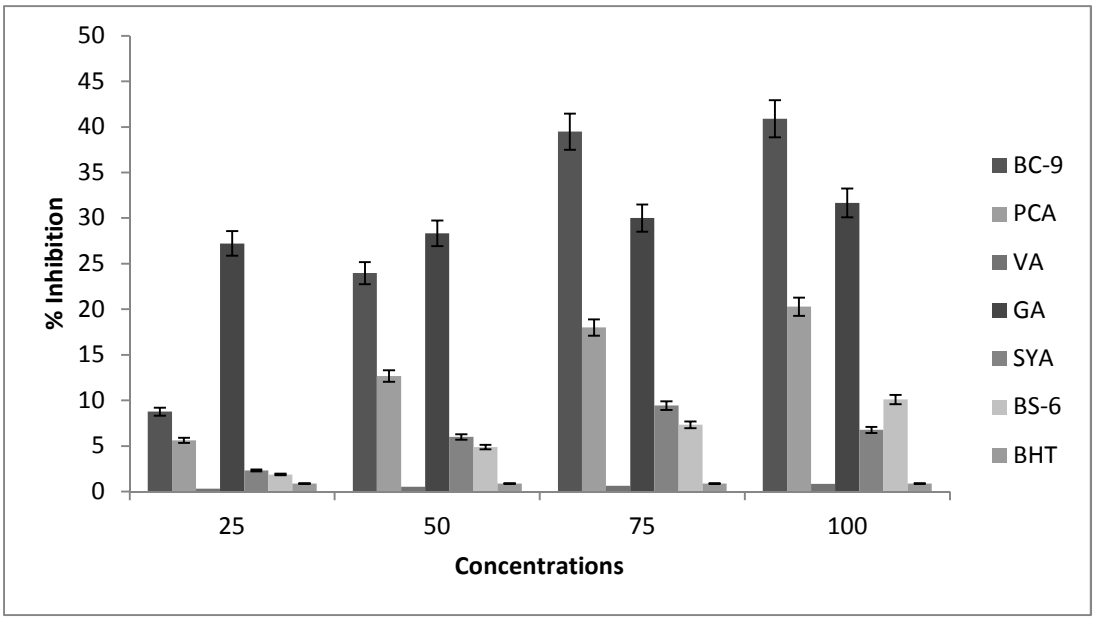


Figure-10 Correlation of rate of reaction of DPPH free radical scavenging activity with % Inhibition

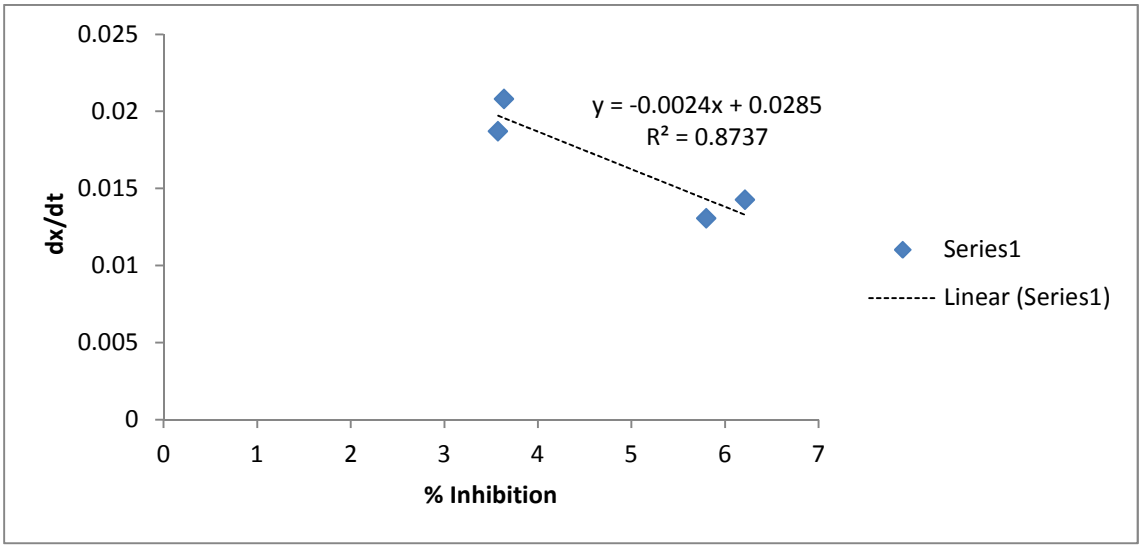


Figure-11 Correlation of rate of reaction of *β-carotene* free radical scavenging activity with % Inhibition