

Reversed-phase high-performance Liquid Chromatography-ultraviolet Photodiode Array Detector Validated Simultaneous Quantification of six Bioactive Phenolic Acids in *Roscoea purpurea* Tubers and their *In vitro* Cytotoxic Potential against Various Cell Lines

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ABSTRACT

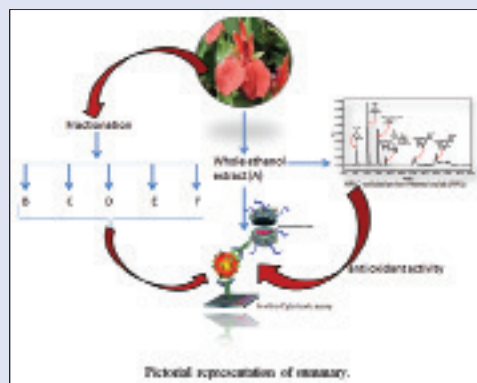
Background: *Roscoea purpurea* or *Roscoea procera* Wall. (*Zingiberaceae*) is traditionally used for nutrition and in the treatment of various ailments. **Objective:** Simultaneous reversed-phase high-performance liquid chromatography-ultraviolet (RP-HPLC) photodiode array detector identification of phenolic acids (PAs) was carried out in whole extract of tuber and their cytotoxic potential was estimated along with radical scavenging action. Bioactivity guided fractionation was also done to check the response potential against the same assay. **Materials and Methods:** Identification and method validation was performed on RP-HPLC column and *in vitro* assays were used for bioactivity. **Results:** Protocatechuic acid, syringic acid, ferulic acid, rutin, apigenin, and kaempferol were quantified as 0.774%, 0.064%, 0.265%, 1.125%, 0.128%, and 0.528%, respectively. Validated method for simultaneous determination of PAs was found to be accurate, reproducible, and linearity was observed between peak area response and concentration. Recovery of identified PAs was within the acceptable limit of 97.40–104.05%. Significant pharmacological response was observed in whole extract against *in vitro* cytotoxic assay, that is, Sulforhodamine B assay, however, fractionation results in decreased action potential. Similar pattern of results were observed in the antioxidant assay, as total phenolic content and total flavonoid content were highest in whole extract and decreases with fractionation. Radical scavenging activity was prominent in chloroform fraction, exhibiting IC₅₀ at 0.25 mg/mL. **Conclusion:** Study, thus, reveals that *R. purpurea* exhibit significant efficacy in cytotoxic activity with the potentiality of scavenging free radicals due the presence of PAs as reported through RP-HPLC.

Key words: Cytotoxic activity, phenolic acids, radical scavenging activity, reversed-phase high-performance liquid chromatography, *Roscoea purpurea*

SUMMARY

- Proto-catechuic acid, syringic acid, ferulic acid, rutin, apigenin and kaempferol were quantified as 0.774, 0.064, 0.265, 1.125, 0.128 and 0.528 %

- Preliminary cytotoxic activity revealed that whole extract of *R. purpurea* exhibit promising effect and after fractionation the potentiation of action reduces
- The radical scavenging potential of whole extract and fractions are well reflected by TPC, TFC and DPPH assay.



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INTRODUCTION

Roscoea purpurea or *Roscoea procera* Wall. is a perennial herb of family *Zingiberaceae*. The species is locally known as Kakoli, Red Gurkha, Dhawanksholika, Karnika, Ksheera, Madhura, Shukla, Svadumansi, Vayasoli and Vaysasha also. *R. purpurea* is widely available in The Himalayas and is native of Nepal and grown on steep, grassy hillsides, damp gullies, and stony slopes. *R. purpurea* is cultivated as an ornamental plant and traditionally in Northern India; fleshy roots are used for the treatment of malaria and urinary infection. In ethnobotanical practice, leaves, roots, and flowers are used for the treatment of diabetic, hypertension, diarrhea, fever, and inflammation. In Nepal, tubers are boiled for edible purpose and also used in traditional veterinary medicine.^[1,2] Tubers of *Roscoea* are major constituent of polyherbal Ayurvedic formulation, “Ashtavarga,” which according to *Nighandu Samhita* and *Indian Metria Medica* is like chawanprash having, antioxidant, anti-aging effect, and elevates overall

health status of a well-being.^[3] Pharmacological reports support the immunomodulatory^[4] and antidiabetic activity^[5] of *R. purpurea* tubers.

Natural phenolic acids (PAs) are the class of biologically active compounds possessing one or more ring structure with a variable

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number of the hydroxyl group. They are broadly diversified in plants and are one of the major secondary metabolites. PAs include a class of compounds (flavonoids, flavones, flavanones, tannins, and lignans, etc.), among which flavonoids are the most beneficial and richly available polyphenolic acids/phenolic acids in our diet. Classically, polyphenolic acids have been used as biologically active antioxidants; however, these compounds play several other remarkable activities also, for example antiallergic, anti-inflammatory, antimutagenic, and modulation of enzyme activities. Several workers have proven the efficacy of PAs as potent chemotherapeutic and chemoprotective agents.^[6-8] Isolated PAs, that is, quercetin, kaempferol, caffeic acid, apigenin, (\pm) catechin, and naringenin, etc., have well established and prominent anti-cancerous activities.^[9-11] Hence, chemoprevention or chemotherapy via a natural source with fortified radical scavenging activity is always welcomed.

As state of art suggested that tubers are effective as anti-aging, anti-inflammatory, and have immunomodulatory action, and if focused over the underlying cause of these disorders, free radicals were found as common agents. In addition to this, its traditional use as potential antioxidant further suspect for the presence of bioactive PAs. Hence, an attempt was made to identify, characterize, and quantify the PAs present in *R. purpurea* through reverse phase-high performance liquid chromatography (RP-HPLC). As stated above, polyphenolics are emerging source of chemotherapeutic agents, thus, the cytotoxic potential of *R. purpurea* tubers were evaluated for which scientific data is still lacking. In addition to this activity, guided fractionation was also carried out to determine the potentiation of action on targeted activity. Biologically active nature of identified PAs was further supported by radical scavenging assay.

In summary, the study was conducted with two major objectives, firstly for identification and quantification of PAs through validated RP-HPLC-ultraviolet (UV) photodiode array detector (PDA) developed method in whole extract (A) of species (tuber) and evaluated for preliminary cytotoxic activity. On the basis of results obtained, work was further extended with second objective of activity guided fractionation from tubers with solvents of differential polarity, that is, petroleum ether (B), chloroform (C), acetone (D), alcohol (E), and water (F) fractions as shown in Figure 1 and subjected to *in vitro* cytotoxic activity. The bioactive nature of identified PAs was further validated by *in vitro* antioxidant assay's, that is, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and determination of total PA and flavonoid content.

MATERIALS AND METHODS

Chemicals

Reference standards of protocatechuic acid (97%), syringic acid ($\geq 97\%$), ferulic acid ($\geq 99\%$), rutin ($\geq 95\%$), apigenin ($\geq 95\%$), kaempferol ($\geq 97\%$),

ascorbic acid ($\geq 97\%$), quercetin ($\geq 97\%$), 1-1-diphenyl-2-picrylhydrazyl ($\geq 99\%$, DPPH) and Vinblastine were purchased from Sigma-Aldrich. HPLC grade solvents viz., acetonitrile, methanol, water, and all other solvents/chemicals (AR grade) were purchased from Merck, Mumbai, India. Ham's Nutrient Mixtures F-12 medium, Roswell Park Memorial Institute-1640 (RPMI-1640), Dulbecco's modified Eagle medium (DMEM), heat-inactivated fetal bovine serum, and antibiotic antimycotic (Ref. No. 15240-062) were purchased from Invitrogen Bio Services India Pvt. Ltd.

Plant material

Fresh tubers of *R. purpurea* were collected in the month of October to November from the nearby area of Dhanaulti (Phytogeographical zone: Western Himalayas, Altitude: 1705 meter, latitude: 30° 21' 34.4", Longitude: 78° 23' 15.3"), Uttarakhand, India. It was authenticated, and a voucher specimen was deposited in institute's herbarium (LWG No. 254028).

Preparation of whole extract and bioactivity guided fractions

Collected sample was washed, shade dried, and coarsely powdered (40 mesh). About 5 g of defatted material (with petroleum ether) was macerated with ethyl alcohol at $30 \pm 2^\circ\text{C}$ for 24 h, filtered (Whatman No. 1 filter paper) and again macerated with fresh solvent. Extraction was repeated thrice, pooled, and concentrated in a vacuum with rotator evaporator (Buchi Labortechnik, Switzerland). The concentrated extracts were finally lyophilized to obtain ethanol soluble extractive yield (whole extract, A) of 41.85% of dry weight. For activity guided fractionation, about 5 g of powder was successively extracted through maceration (same conditions as above) with solvents in increasing order of polarity, that is, petroleum ether (B), chloroform (C), acetone (D), ethanol (E) and aqueous (F) having fraction yield (%) of 3.38%, 1.47%, 1.23%, 1.90% and 63.15%, respectively.

High-performance liquid chromatography method development and validation for phenolic acids in whole ethanol extract of *Roscoea purpurea* tuber

Preparation of standards and high-performance liquid chromatography conditions

The standard stock solutions (1 mg/mL) of protocatechuic acid, syringic acid, ferulic acid, rutin, apigenin and kaempferol were prepared in HPLC grade methanol and stored at 4°C , until used. Working solutions of lower concentration (0.1 mg/mL) were prepared by appropriate dilution of the stock solutions in methanol.

Waters RP-HPLC-PDA-2996 (Waters Corporation, Milford, MA, USA) was used for the qualitative and quantitative analysis of analytes. Chromatographic separation was performed on Supelco C_{18} column (4.6 mm \times 50 mm, 5.0 μm .) with C_{18} guard column in gradient mode with binary mobile phase, duly filtered through 0.22 μm Millipore filter and degassed ultrasonically for 15 min before delivered to column for separation. Data acquisitions were performed using Empower software version 2 (Waters, Milford, MA 01757, USA). The injection volume was 20 μL for standard and sample. Detection was observed at a maximum wavelength of λ_{285} nm.

Optimization of method

A RP-HPLC-PDA-2996 (Waters, USA) was used for separation of reference compound on Chromatopak C_{18} column AQ (4.6 mm \times 50 mm, 5.0 μm .) using a gradient mode with 0.1% formic acid in water (A) and pure acetonitrile (B) as mobile phases. Separation of analytes was

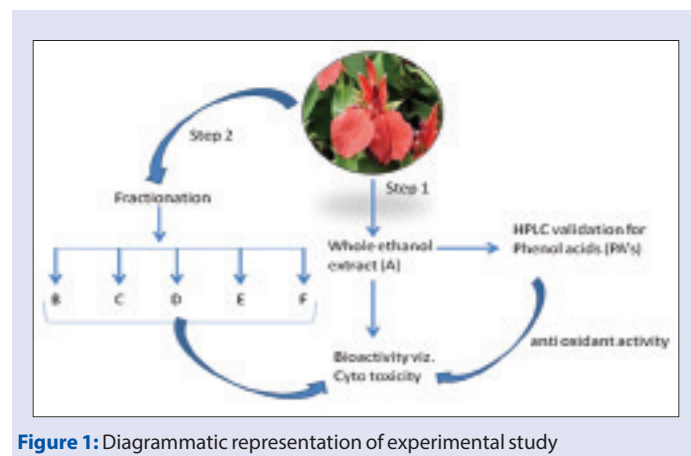


Figure 1: Diagrammatic representation of experimental study

carried out at 27°C using 20 µL of sample/standard injection volume. Total HPLC running time was 55 min and standards were eluted at their corresponding retention time viz.: 6.19, 10.56, 15.17, 18.51, 32.25, and 39.92 min for protocatechuic acid, syringic acid, ferulic acid, rutin, apigenin, and kaempferol, respectively. Peak areas versus reference standard concentration were subjected to regression analysis, and the slope, intercept, and correlation coefficient for the calibration curve was determined at 5 concentrations and quantified.

Validation of method

The HPLC method for quantification of PAs viz., protocatechuic acid, syringic acid, ferulic acid, rutin, apigenin, and kaempferol were determined by selectivity, linearity, precision, sensitivity, and accuracy.

Selectivity

A standard solution of six PAs was dissolved in 1 mL methanol, and 10 µL of the standard mixture was injected into the column, analyzed as per the method described above and peak purity was also accessed.

Linearity

Reference standards at the concentration range of 0.25–2.0 µg were injected into the HPLC system and calibration curves were plotted by linear regression of the peak area ratio (y) of each PA versus concentration (x) in µg/mL. Stability of the standard calibration mixture and extract was determined by injecting them repeatedly over a 24 h period. The percent relative standard deviation (% RSD) and percentage of change over the period were also determined for each compound.

Sensitivity

Sensitivity of method was determined with respect to limit of detection (LOD) and limit of quantification (LOQ), was calculated from the standard deviation (SD) of the response and slope of the calibration curve. The formulae used for LOD ($3.3 \sigma/S$) and LOQ ($10 \sigma/S$) were calculated with SD of the response (σ) and slope of the calibration curve (S).

Precision

Precision is a test for the distribution of concentrations measured. Two different concentrations of the stock solutions were exposed to five determinations on the same day. Repeatability of analysis was performed at five different concentrations ranging from 0.25 to 2.0 µg/mL for standards. All RSD values were below 3%, which is considered to be acceptable. To determine the precision of developed method, each standard and extract was analyzed six times on the same day to determine the intra-day precision. The same procedure was repeated over 3 days and compared by calculating the % RSD for the average values for each day to determine the inter-day precision.

Recovery

Accuracy of the method was analyzed by recovery studies, five different concentrations diluted from the stock solution were added to an extract with a known content of standards and the recovery of respective constituent was calculated.

Robustness

Precision is a test for the distribution of concentrations measured. Two different concentrations of the stock solutions were exposed to five determinations on the same day. Repeatability of analysis was performed in three concentrations of all standards. All RSD values were below 1%, which is considered to be acceptable to perform this test. The deviations were calculated on the basis of peak areas with parameters 0.8–1.2 mL/min and 27°C as reference values, which is the middle value of flow rate and temperature, respectively. Mean deviation is <3% which is acceptable for analysis.

Cytotoxic activity

Cell culture lumen lung carcinoma (A549), human cervical cancer (SiHa), Chinese hamster ovary cells (CHOK1), and rat glioma (C-6) cells were obtained from National Centre for Cell Science, Pune. A549 and CHOK1 cells were grown in Ham's F-12 medium, SiHa cells were cultured in RPMI-1640, and C-6 cells were grown in DMEM, supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotic antimycotic. The cells were maintained at 37°C in a 5% CO₂ humidified atmosphere.^[12,13]

Sulforhodamine B cytotoxic activity

The cell cultures were trypsinized and washed twice with phosphate buffer saline by centrifugation and incubated at a density of 20,000 cells/well in 96-well plates in 100 µL of complete medium. Several dilutions (10, 50, 100, and 150 µg/mL) of the whole extract and fractions were added in 100 µL of complete medium. Cell culture alone supplemented with the complete medium was used as negative control whereas; Vinblastine (1 µM) used as positive control for the assay. The plates were incubated at 37°C for 48 h in the CO₂ incubator. After 48 h, 50 µL of 50% trichloroacetic acid was added, and the plates were kept at 4°C for 1 h. The plates were flicked and washed 5–6 times with tap water and then air-dried. Subsequently, 100 µL of the sulforhodamine B solution (0.4% in 0.1% glacial acetic acid w/v) was added and incubated for 30 min at room temperature. After incubation, plates were washed rapidly 5–6 times with 1% acetic acid and air dried. 100 µL of 10 mM tris base was added to the wells. The absorbance was measured using microplate reader (BioTeK Synergy H1 Hybrid Reader) at a wavelength of 540 nm.^[14]

Antioxidant activity

Total flavonoids^[15] and phenolics^[16] were expressed in terms of mg/g of quercetin equivalent (QE) and g/g gallic acid equivalent (GAE) based on calibration curve of quercetin and gallic acid as standard. The radical scavenging potential^[17] of whole extracts (A) and fractions viz., B, C, D, E, and F of *Roscoea* tubers were analyzed using DPPH.

Statistical analysis

For HPLC interpretation, results were reported as means \pm SD of at least three replicates of the whole extract. Data were subjected to one-way analysis of variance (ANOVA) and the least significant difference between the extracts at $P < 0.01$ was calculated by *post-hoc* comparison test (SPSS 11.5 SPSS Inc. Chicago, USA).

In the antioxidant activity, results were expressed as mean \pm SD. Linear regressions analysis was carried out for standards to calculate TPC and TFC, and GraphPad Prism 5 (San Diego, CA, USA) software was used to calculate the IC₅₀ values. One-way ANOVA followed by Student's *t*-test ($P < 0.01$) was used to find the significance of standard and sample.

RESULTS

High-performance liquid chromatography method validation

Method development began with the optimization of chromatographic conditions, including mobile phase composition and column type. Versatility, suitability, and robustness of method were checked with several C₁₈ columns of various manufacturers (data not shown) and it was found that chromatographic resolution, selectivity, and sensitivity were good with Supelco C₁₈ column (4.6 mm \times 50 mm, 5.0 µm). Feasibility of various mixtures (s) of solvents such as ammonium acetate, acetic acid, and formic acid with variable pH range of 0.4–6.6, along with altered flow-rates (in the range of 0.4–1.5 mL/min) was tested for complete chromatographic resolution of PAs. Finally, mobile phase A

comprising 0.1% formic acid in water and B was pure acetonitrile were selected, and conditions were as follows: Eluent A; eluent B; gradient, 0–30 min (5–25% B) at 0.80 mL/min flow rate, 30–40 min (25–45% B) at 0.90 mL/min flow rate, 45–50 min (50–40% B) at 0.90 mL/min, 45–

50 min (40–20% B) at 1.0 mL/min 50–55 min (20–5% B) at 1.2 mL/min flow rate, and then equilibrated with 8% B for 10 min at 1.0 mL/min flow rate.

Table 1: RP-HPLC quantification of phenol acids in whole extract of *Roscoea purpurea* rhizomes

Standard	Retention time (min)	Concentration* (%)
Proto catechuic acid	6.196	0.077±0.01
Syringic acid	10.563	0.064±0.01
Ferulic acid	15.173	0.265±0.05
Rutin	18.510	1.125±0.01
Apigenin	32.252	0.128±0.01
Kaempferol	39.926	0.528±0.05

*Values are Mean±S.D. RP-HPLC: Reverse phase- High performance liquid chromatography

A typical HPLC chromatogram of the standard and extract is shown in Figures 2 and 3. The identification of PA's was based on a comparison of retention times and UV spectra of reference standards with the corresponding peaks in the extract [Figure 4]. The retention time (R_t) for standards were obtained according to the optimized method as described in experimental section. Peak purity was accessed by PDA data at λ_{285} nm and purity factor obtained for extract reveals the presence of pure peak without contamination of other co-eluting compounds. Quantification of PA compounds ranges from 0.064% to 0.528% [Table 1] in triplicate.

The HPLC quantification method for PA's was validated, and all the included parameters are within the specified limit [Table 2]. The selectivity validation of method is well demonstrated by the excellent separation of analytes in reference mixture and as explained above, the peak purity was also good; it indicates that there is no merging of any unidentified peak with known identified PA (analyte), and thus confirms the selectivity of method. Standard solutions of PA's in methanol showed a linear correlation between the peak area and concentration, correlation coefficient was found to be satisfactory while plotting of calibration for standards. To estimate the stability, % RSD of standard mixture and extract was calculated and results (not more than 3%) shows that the analytical solutions are stable for duration of 24 h at a temperature of 4°C in autosampler unit. Value of correlation coefficient for protocatechuic acid, vanillic acid, ferulic acid, apigenin, and kaempferol indicates good linearity [Figure 5]. LOD and LOQ values depict that method is sensitive [Table 2]. A fixed concentration of extract was used to access the precision and accuracy of method, inter and intra-day RSD (%) indicate the values within the limit range (not more than 5%) as shown in Table 3. Recovery studies of PA's, as shown in Table 4, vary from 97.40% to 104.05%, respectively. The observed values are within the specified limit (95–105%) and hence the method was found to be accurate, precise, and reproducible. To the best of our knowledge and available literature, this is first reporting on the development of a simple, rapid, and sensitive method on quantification of protocatechuic acid, vanillic acid, ferulic acid, rutin, apigenin, and kaempferol in whole extract of *R. purpurea* tubers. The outcome supports the evidence that developed method is accurate and reproducible in specified conditions for quantification of targeted PA's.

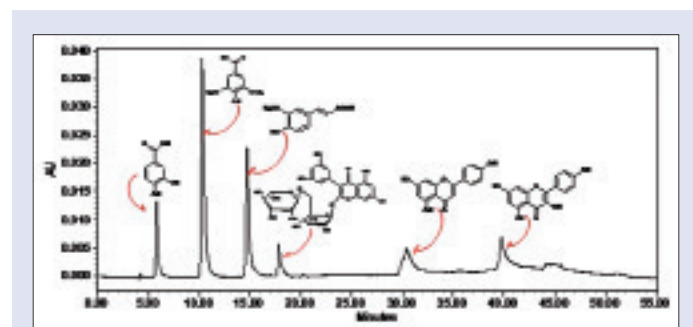


Figure 2: High-performance liquid chromatography-ultraviolet photodiode array detector chromatograms of standards

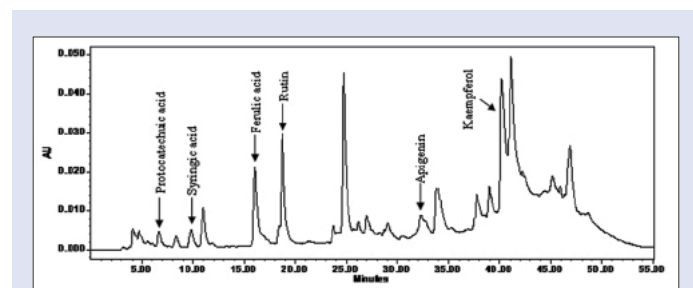


Figure 3: High-performance liquid chromatography-ultraviolet photodiode array detector chromatograms of whole extract of *Roscoea purpurea* rhizome

Cytotoxic activity of whole extract and fraction (s)

In vitro cytotoxicity was carried out against four different cell lines viz., A549, SiHa, CHOK1, and C-6 cells at 10, 50, 100, and 150 $\mu\text{g/mL}$,

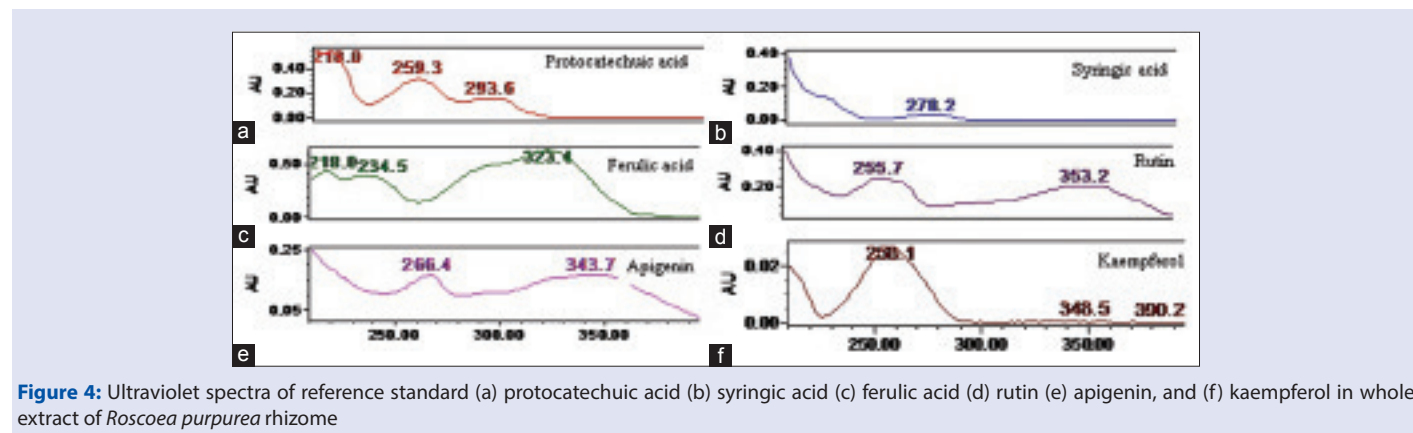


Figure 4: Ultraviolet spectra of reference standard (a) protocatechuic acid (b) syringic acid (c) ferulic acid (d) rutin (e) apigenin, and (f) kaempferol in whole extract of *Roscoea purpurea* rhizome

respectively. The percentage of cellular growth inhibition was calculated by measuring the absorbance of respective incubated cells [Table 5]. Potential activity was observed in the whole extracts against the tested cell lines exhibiting IC_{50} value of <10, 49.5, 83.8, and 141.6 $\mu\text{g}/\text{mL}$ against A549, SiHa, C-6, and CHOK1, respectively. These interesting results, lead to the idea of fractionating the phytomolecules present in the whole extract on the basis of solvent polarity for identification of the nature of molecules responsible for targeted bioactivity. Data reveals that all the tested fractions exhibit dose-dependent cytotoxicity against the cell lines. Among them fraction B exhibit the highest activity (69.1 ± 0.6 and

60.5 ± 1.5) on SiHa and CHOK-1 cells at concentration of 100 and 150 $\mu\text{g}/\text{mL}$, respectively, and C showed the highest activity (62.4 ± 1.0) on A549 cells at a concentration of 100 $\mu\text{g}/\text{mL}$. However, the activity against C-6 cells is similar in B and C fractions [Table 5].

The IC_{50} of tested fractions ranges between < 10 and > 150 $\mu\text{g}/\text{mL}$ [Table 6]. In a nutshell, from above preliminary cytotoxic activity, it was observed that whole extract of *Roscoea* exhibits promising effect and after fractionation, the potentiation of action reduces and variable responsible was observed. The underlying mechanism of action was not clearly known and may be suspected due to the synergistic effect of PA's with other bioactive metabolites in whole extract. Although fractionation results in segregation of metabolites, and thus the differential response was achieved. Previously, workers had reported the cytotoxic/anti-cancerous activity of pure PA's which are identified in the whole extract of *Roscoea* also. The preventive effect of apigenin in chemotherapy is also established.^[18] Further studies in near future will reveal the precise cellular and molecular mechanisms induced by the extract. The inhibitory effect of natural PA's in carcinogenesis and tumor growth may be through two main mechanisms: (1) Modifying the redox status and (2) interfering with basic cellular functions (cell cycle, apoptosis, inflammation, angiogenesis, invasion, and metastasis) and both the action are mediated through their ability to scavenge the reactive oxygen

Table 2: Validation parameters for HPLC analysis of Protocatechuic acid, Syringic acid, Ferulic acid, Rutin, Apigenin and Kaempferol

Standard*	Calibration curve	correlation coefficient (r^2)	LOD (μg)	LOQ (μg)
Protocatechuic acid	$y=977338x+35447$	0.9999	0.029	0.097
Syringic acid	$y=4E+06x-96038$	0.9993	0.067	0.225
Ferulic acid	$y=2E+06x+241311$	0.9995	0.076	0.254
Rutin	$y=571390x+97.63$	0.9988	0.086	0.289
Apigenin	$y=2E+06x+136560$	0.9999	0.028	0.094
Kaempferol	$y=1E+06x+84378$	0.9987	0.090	0.303

*Mean \pm S.D ($n=5$). HPLC: High performance liquid chromatography; LOD: Limit of detection; LOQ: Limit of quantification

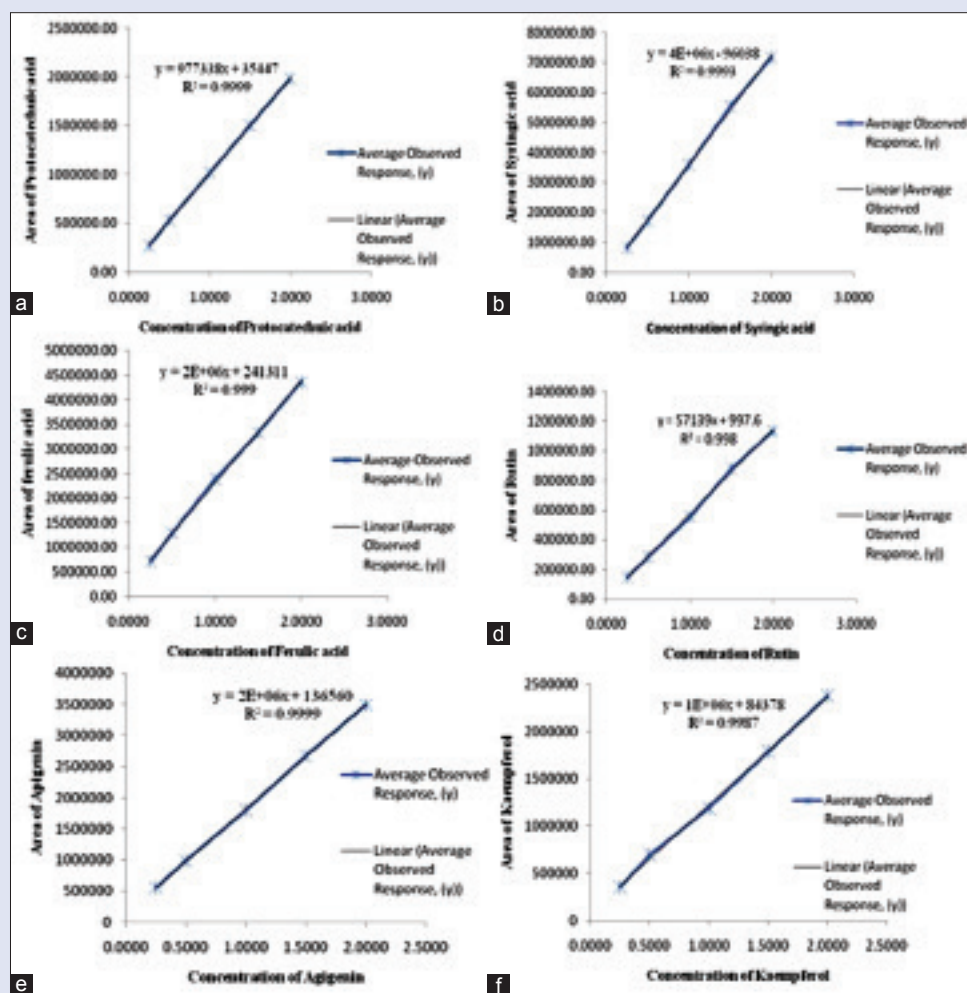


Figure 5: Calibration curve of phenolic acids (a) protocatechuic acid (b) syringic acid (c) ferulic acid (d) rutin (e) apigenin, and (f) kaempferol in whole extract of *Roscoea purpurea* rhizome

species/free radicals at one or other steps of cancer pathology. The study, however, needs to be extended further on SAR and QSAR lineage to depict the mode of action of the compound responsible for same.^[19]

Antioxidant potential

Presence of natural phenolic and flavonoid moiety in the plants serves as an indicator of their free radical scavenging activity and quantification of

the same can be useful in accessing the antioxidant capacity of species. Total flavonoid content (TFC) and total phenolic content (TPC) were significantly ($P < 0.01$) rich in whole extract of *Roscoea*, that is, 26.78 mg/g QE and 3.03 g/g GAE, respectively. After fractionation, flavonoid content decreases in order of F fraction and then E, D, B, and C fractions having 9.15, 1.71, 1.39, 0.27, and 0.216 mg/g QE, respectively. Phenolic content was also highest in F (0.565 g/g GAE) fraction, followed by D (0.135 g/g GAE), E (0.106 g/g GAE), C (0.100 g/g GAE), and B (0.040 g/g GAE).

Bioactive PAs are the class of compounds that effectively inhibits free radicals because of their scavenging activity and therapeutically beneficial as they are common underlying cause of several disorders viz., cardiovascular, diabetes, aging, arthritis, cancer, and inflammatory disorders, etc.^[20] Thus, in order to estimate the radical scavenging effect of the whole extract and fractions, DPPH radical was used. Data show that activity varied considerably among the whole extract and fractions of tubers [Figure 6] when compared to standard reference viz., ascorbic acid, quercetin, and rutin. In standards, maximum inhibition of free radicals was observed in ascorbic acid (77.57%, IC_{50} : $3.86 \pm 0.057 \mu\text{g/mL}$), followed by quercetin (72.43%, IC_{50} : $5.93 \pm 0.115 \mu\text{g/mL}$), and rutin (71.48%, IC_{50} : $6.80 \pm 0.173 \mu\text{g/mL}$). Whole extract exhibits IC_{50} at $0.925 \pm 0.005 \text{ mg/mL}$. Among the *Roscoea* fractions, inhibition of radicals varies from 5.2% to 83.21% [Figure 6]. Fraction C possesses significant IC_{50} value at 25 mg/mL [Table 7].

Table 3: Inter day and Intraday precision table of standard compounds

Standards	Inter day precision (n=5), Mean % RSD		Intraday precision (n=5), Mean % RSD					
	T _R ^a	P _A ^b	Day one		Day two		Day three	
Protocatechuic acid	0.13	0.14	T _R ¹	P _A ¹	T _R ²	P _A ²	T _R ³	P _A ³
Syringic acid	0.12	0.12	0.22	0.38	0.29	0.46	0.19	0.55
Ferulic acid	0.19	0.18	0.25	0.37	0.28	0.41	0.18	0.69
Rutin	0.13	0.22	0.29	0.32	0.28	0.47	0.17	0.45
Apigenin	0.13	0.13	0.20	0.31	0.27	0.54	0.20	0.87
Kaempferol	0.13	0.13	0.20	0.22	0.27	0.67	0.20	0.78

T_R: Retention time of analyte; P_A: Peak area response; ¹Day one, ²Day two, ³Day three

Table 4: Recovery studies (%) of phenol acids in *Roscoea purpurea*

Amount of Protocatechuic acid in sample (ng)	Calculated amount of Protocatechuic acid (ng)	Analyzed Protocatechuic acid (ng)	Recovery (%)	RSD%
77	77	78	101.29	2.09
77	97	99	102.06	
77	117	119	101.17	
77	127	124	97.63	
77	157	162	103.31	
Amount of Syringic acid in sample (ng)	Calculated amount of Vanillic acid (ng)	Analyzed Vanillic acid (ng)		
64	64	65	101.56	2.77
64	74	77	98.48	
64	104	102	104.05	
64	154	150	97.40	
64	194	199	102.57	
Amount of Ferulic acid in sample (ng)	Calculated amount of Ferulic acid (ng)	Analyzed Ferulic acid (ng)		
265	265	269	101.50	1.13
265	295	299	101.35	
265	325	333	102.46	
265	365	364	99.72	
265	465	477	102.58	
Amount of Rutin in sample (ng)	Calculated amount of Apigenin (ng)	Analyzed amount of Apigenin (ng)		
1125	1125	1129	100.35	0.39
1125	1525	1520	99.67	
1125	2025	2020	99.75	
1125	2525	2538	100.51	
1125	3025	3018	99.76	
Amount of Apigenin in sample (ng)	Calculated amount of Kaempferol (ng)	Analyzed amount of Kaempferol (ng)		
128	178	174	99.75	0.72
128	228	230	100.87	
128	278	283	101.79	
128	328	330	100.60	
128	378	381	100.79	
Amount of Kaempferol in sample (ng)	Calculated amount of Kaempferol (ng)	Analyzed amount of Kaempferol (ng)		
528	528	531	100.56	0.33
528	628	629	100.15	
528	728	732	100.54	
528	928	926	99.78	
528	1028	1033	100.48	

Table 5: Cytotoxicity exhibited by whole extract and fractions of *R. purpurea* (rhizomes) against different cells by SRB assay

<i>R. purpurea</i>	Concentration	Cell lines for cyto toxic activity			
		A549	SiHa	C-6	CHOK1
A (µg/ml)	10	51.1±3.6	29.1±2.1	21.9±2.9	20.6±3.4
	50	59.0±2.2	50.1±2.3	26.4±4.1	34.3±3.7
	100	60.1±1.7	63.7±3.1	61.3±1.5	41.6±4.9
	150	55.5±4.0	68.7±3.1	64.4±4.0	51.7±1.9
B (µg/ml)	10	45.3±3.3	34.4±3.0	21.3±0.6	27.8±4.5
	50	51.8±2.9	53.8±0.4	51.2±2.9	41.6±3.6
	100	54.4±0.8	69.1±0.6	57.5±2.4	48.5±4.4
C (µg/ml)	10	50.6±3.3	65.9±2.2	60.8±1.0	60.5±1.5
	50	47.1±3.4	33.3±4.7	30.7±2.7	29.6±1.9
	150	55.3±0.5	56.9±1.6	62.0±4.6	36.3±2.6
D (µg/ml)	10	48.2±2.9	63.5±1.1	61.8±3.8	39.6±3.1
	50	39.4±2.3	52.6±1.8	60.4±4.8	43.6±4.9
	100	49.3±1.2	25.2±3.6	26.7±2.4	19.7±0.6
	150	59.9±0.8	31.5±2.2	24.9±1.0	26.4±2.0
E (µg/ml)	10	62.4±1.0	35.3±2.3	22.3±1.8	34.4±3.9
	50	57.3±4.4	40.6±1.3	25.3±4.1	35.8±4.2
	100	51.9±1.2	28.4±1.6	26.0±2.1	20.1±2.5
	150	61.9±3.1	33.6±3.7	27.2±1.4	26.8±4.0
F (µg/ml)	10	61.0±2.1	28.1±1.9	21.4±2.0	26.4±0.8
	50	56.2±3.7	30.7±3.8	22.1±4.8	32.3±2.8
	100	51.1±3.5	11.4±3.7	16.6±4.4	12.3±4.6
	150	59.0±2.2	35.8±1.2	20.1±2.3	18.8±1.6
Vinblastin (µM)	1	60.1±1.3	25.7±2.1	23.5±4.0	22.5±0.6
		55.5±3.8	21.8±3.9	13.8±3.7	26.9±0.5
		67.6±1.3	63.5±1.6	90.5±2.5	60.5±3.1

Mean±S.D (n=3). CHOK1: Chinese hamster ovary cells; SRB: Sulforhodamine B

Table 6: IC₅₀ value of whole extract (A) and fractions (B, C, D, E and F) of *Roscoea rhizomes* against A549, SiHa, C-6 and CHOK1 cells in µg/ml

Cell line*	A	B	C	D	E	F
A549	<10	38.9	24.1	12.6	<10	13.4
SiHa	49.5	42.2	38.3	>150	>150	>150
C-6	83.8	48.4	34.7	>150	>150	>150
CHOK1	141.6	106.2	>150	>150	>150	>150

*Values are mean (n=3)

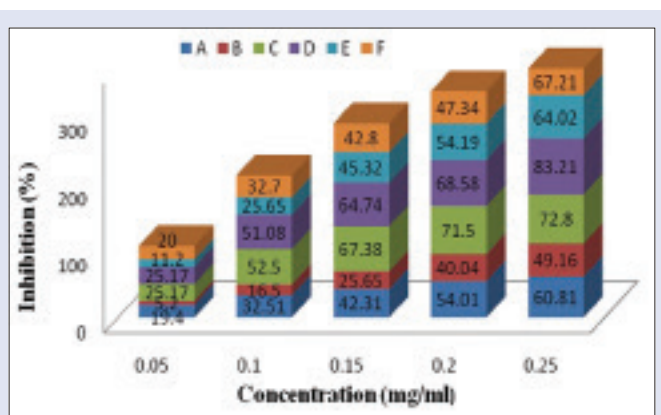
Table 7: IC₅₀ value of *R. purpurea* whole extracts and fractions in DPPH radical scavenging assay

<i>R. purpurea</i>	IC ₅₀ (mg/ml)*
Crude extract (A)	0.925±0.005
Fraction B	1.25±0.005
Fraction C	0.25±0.005
Fraction D	0.48±0.005
Fraction E	0.77±0.011
Fraction F	1.04±0.005

*Values are mean±S.D (n=3). DPPH: 1,1-diphenyl-2-picrylhydrazyl

CONCLUSION

HPLC method developed for simultaneous quantification of six PA's reveals the presence of protocatechuic acid, syringic acid, ferulic acid, rutin, apigenin, and kaempferol in a concentration range of 0.064–1.125%. It is evident that the developed protocol is accurate and reproducible under specified conditions and can be utilized as a quality control method for quantification of PA's marker in the species. On the basis of data from preliminary cytotoxic study of whole extract, significant results have been noticed against various cell lines viz., A549, SiHa, CHOK1, and C-6 cells, and thus may be explored as a source of cytotoxic agent. The observed above activity response of whole

**Figure 6:** 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of *Roscoea* whole extract and fractions

extracts may be suspected due to the synergistic effect of PA's with other phytochemicals present in the extracts. However, fractionation leads to segregation of phytochemicals, and thus the activity response reduced prominently. The radical scavenging potential of the whole extract and fractions are well reflected by TPC, TFC, and DPPH assays.

The work is novel as this is the first ever report on simultaneous identification and quantification of PA's in the species, in addition to this cytotoxic potential of identified PA's was not reported yet. We summarized that *R. purpurea* (whole extract and fractions) revealed significant efficacy in cytotoxic activity with potentiality of scavenging free radicals due to the presence of six bioactive PA's as reported via RP-HPLC-UV PDA validated method.

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Conflicts of interest

There are no conflicts of interest.

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