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# Simultaneous Detection and Validation of Analytical Markers of *Swertia chirata* by HPLC-DAD to Evaluate the Potency of Extracts and Fractions against Antioxidant Potential

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The present study is based on the selection of extract and fraction of *Swertia chirata* plant for the antioxidant potential with HPLC fingerprinting, which includes the simultaneous detection and quantification of four analytical markers protocatechuic acid (PCA), swertiamarin (SM), mangiferin (MF) and amarogentin (AG) by HPLC-DAD. The yield of water extract (SWA), hydroalcoholic extract (SHA) and fractions of hydroalcoholic extracts were evaluated for their antioxidant potential against 2,2-diphenyl-1-picrylhydrazyl-hydrate free radical assay (DPPH assay), 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid radical scavenging assay (ABTS assay), total reducing assay (TRA), ferric reducing antioxidant potential assay (FRAP assay), total antioxidant capacity assay (TAC assay). The hydroalcoholic extracts (SHA) can be a better choice as compared to water extract (SWA) due to higher yield of extract (13.680 ± 0.548%) and higher antioxidant activity against DPPH assay, ABTS assay, TRA assay, FRAP assay and TAC assay. In hydroalcoholic extract (SHA), ethyl acetate fraction (SEA) showed most potent activity against DPPH ($IC_{50}$ = 0.008 ± 0.002 mg/mL) and ABTS (0.025 ± 0.001 mg/mL). *n*-Hexane fraction of SHA showed higher FRAP (28.664 ± 3.153 µmol/mL) and TAC (3.263 ± 0.325 µmol/mL) value (equivalent to ascorbic acid in µmol/mL) but showed very low yield (0.468 ± 0.018%), SBU showed higher TRA value (0.413 ± 0.309 mg/mL). The ethyl acetate fraction (SEA) can be a choice for antioxidant as it showed second highest FRAP (19.547 ± 2.119 µmol/mL) and TAC (2.750 ± 0.466 µmol/mL) with better yield (2.473 ± 0.594%) as compared to *n*-hexane (SH) fractions (0.468 ± 0.018%).

**Keywords: *Swertia chirata*, Protocatechuic acid, Swertiamarin, Mangiferin, Amarogentin, HPLC-DAD, Antioxidant assay.**

## INTRODUCTION

Swertia genus of Gentianaceae family was first introduced by Roxburgh in 1814 [1]. The genus of Swertia has vast range of annual and perennial species about 135 species has been reported and out of which 35 species are found in India [2]. The high therapeutic potential of Swertia can be attributed to presence of wide and varied chemical constituents such as xanthones, iridoid glycosides (amarogentin, amaroswerin, swertiamarin, swerosides, *etc.*), Xanthones glycosides (mangiferin, *etc.*) and polyphenolic acid (protocatechuic acid, *etc.*). Overall there are approximately 419 metabolites of which 40 bioactive compounds are reported from 30 species of Swertia genus, which are responsible for the therapeutic activity [3]. *Swertia chirata* a major plant of Swertia genus is one among the 32 highly prioritized medicinal herbs from the rich biodiversity of Uttarakhand state (India) as identified by National Medicinal Plant Board, Government of India [4]. The plant is mentioned in ancient literature *viz.* Ayurveda (used as decoction for anti-pyretic, anthelminitic, antiperiodic, laxative and in asthma and leucorrhoea) [4], in Unani text it is used as tonic, chronic fever, astringent, sedative to preganant uterus, inflammation) [5] and in Chinese medicines, it is uses for the treatment of scabies, cholicystitis, hepatitis, pneumonia, dysentery and spasm). It is well documented in Indian Pharmacopeial codex, British and American Pharmacopoeia (used as tincture and infusion) [1,6]. The geographical distribution of plant is mainly from Himalayan region from Kashmir to Bhutan at an altitude of 1200 to 3000 m above sea level. Plant is also found at Khasi hills in state of Meghalaya (India) between the height of 1200-1500 m above sea level [2]. The demand of plant is very high for immunity enhancement property [4].

The bitter principle of *Swertia* genus fall in the class of irridoids glycosides, secoirridiods glycosides and biphenyl glycosides. The characterization of iridoids and secoiridoids due to the presence of iridane and secoiridane moiety of mono-

terpenoids as they cyclopentane nucleus. Details class of phytochemicals of genus can be characterize as xanthonoids, iridoids, flavonoids, secoiridoids glycosides, terpenoids, alkaloids, some lignans, lactones and some other compounds [7,8].

The screening of phytochemicals by HPLC is useful for the quantitative analysis of the phytochemicals present in the sample. Chemical fingerprint of chromatogram is used for the quality assessment of raw herbs by qualitative and quantitative analysis [9,10]. Present study is based on the phytochemical screening of four analytical markers, their quantification in extracts and fractions of *Swertia chirata* plant by HPLC-DAD method, validation of method and evaluation for their antioxidant properties using different assays. The validation of phytochemicals of *Swertia* species by HPLC is already reported [11]. The present works include the simultaneous detection of protocatechuic acid (PCA) with three other bioactive compounds amarogentin (AG), swertiamarin (SM) and mangiferin (MF). All parameters of ICH guideline have been performed (limit of detection, specificity, limit of quantification, accuracy, precision, repeatability, intermediate precision, reproducibility, linearity, range and robustness) to validate the HPLC-DAD method.

## EXPERIMENTAL

**Collection of plant:** The plant specimen was collected in the month of September 2020 from Bageshwar district, India and authenticated at botanical survey of India, Dehradun with the accession of 177. Rest of specimen were allowed to shade dry. Air dried powdered material of *Swertia chirata* plant allowed for hot extraction (reflux condensation) with aqueous and hydroalcoholic solvent. Aqueous extract (SWA) and hydroalcoholic extract (SHA) dried under reduced pressure with complete dried on water. Fractionation of hydroalcoholic extract (SHA) was done by evaporation of about 3/4th volume of solvent from the extracted medium under reduced pressure and allowed to perform the successive fractionation with hexane (SH), chloroform (SCL), ethyl acetate (SEA), butanol (SBU) and remaining (SRE). After fractionation, the extract fractions were dried and yields were also calculated.

**HPLC quantifications:** SHIMADZU Prominence-I system LC2030C 3D Japan liquid chromatography, with quaternary pump, PDA detector (model: SPD M20A), auto sampler, C18 column (dimension 250mm × 4.6 mm internal pore size 5um), Lab solution (software) was used for the analysis of samples. Amarogentin, swertiamarin, protocatechuic acid, mangiferin and trifluoroacetic acid were procured from Sigma-Aldirich, and the solvents *viz*. methanol, hexane, chloroform, butanol, *etc*. used were of HPLC grade.

**Chromatographic conditions:** Trifluoroacetic acid (TFA) solution (1%) was prepared and taken as solvent A and filtered through 0.2 µ membrane filter. Acetonitrile:water (70:30) was taken as solvent B. Gradient programme was set as solvent B initially start with 15% and increases to 20%, for 5 min, further increased to solvent B to 70% from 5 to 30 min and allowed to hold for 35 min, and finally 15% of solvent B was allowed from next 35 to 40 min. Detection of all the analytical marker was done at 240 nm by PDA detector at ambient temperature.

**Preparation of samples:** All the extracts and fractions were dissolved in methanol (HPLC grade), and filtered with 0.2 µ membrane filter before injecting the solutions.

**Preparation of standard solution:** All four analytical markers protocatechuic acid (PCA), amarogentin (AG), swertiamarin (SM) and mangiferin (MF) were dissolved in methanol separately to prepare a stock solution. The stock solution of each analytical marker were mixed to obtain different required concentrations.

**Method validation:** Validation of method was done on the basis of ICH guidelines [12] and USP methods, which include specificity, limit of quantification (LOQ), limit of detection (LOQ), precision at limit of detection level, matrix effect, linearity, precision, accuracy, robustness and uncertainty [12].

### Antioxidant activity

**DPPH radical scavenging assay:** 1,1-Diphenyl-2-picryl hydrazyl (0.2 mM) solution was prepared in methanol as a stock solution. All the extracts and fractions were dissolved in methanol to prepare a stock solution of 10 mg/mL, serial dilution of stock solution of extracts and fractions was done on the basis to achieve $IC_{50}$ (inhibition control 50%) value of 0.1 mM DPPH with SWA, SHA, SH, SCL, SEA, SBU, SRE dilutions at 517 nm. Ascorbic acid were used as a standard [13].

**ABTS radical scavenging assay:** A 14 mM of ABTS (2,2′-azino*bis*(3-ethyl benzothiazoline-6-sulfonic acid) and 4.8 mM potassium persulphate solutions were prepared as stock solutions. Both the stock solutions were mixed equally to prepare a ABTS reagent. The reagent was allowed to stand for 16 h before use and then 1/5th dilution was made to keep the absorbance of ABTS reagent at 734 nm. All the extracts and fractions were dissolved in methanol to prepare 10 mg/mL stock solutions. Serial dilutions of stock solution of SWA, SHA, SCH, SCL, SEA, SBU, SRE were done to achieve $IC_{50}$ (inhibition control 50%) at 734 nm. Ascorbic acid were used as a standard [13,14].

**Total reducing assay:** The stock solution of 10 mg/mL of extracts and fractions were prepared in methanol serial dilution of all extracts and fractions were made on the basis to keep $IC_{50}$ value at 700 nm. A 2.5 mL of each dilutions of SWA, SHA, SCH, SCL, SEA, SBU, SRE were taken in test tube and 2.5 mL of sodium phosphate buffer (0.2 mM pH 6.6) and 2.5 mL of potassium ferricyanide (1%) were added. The solution mixture in each test tube was incubated at 50 ºC for 20 min and then 2.5 mL 10% tricholroacetic acid was added into each test test tube. Each test tube was centrifuged at 3000 rpm for 10 min and then the collected supernatant was half-fold diluted by water. Then 1 mL of 1% $FeCl_3$ was added in each dilution. The formation blue colour measured at 700 nm. Ascorbic acid was used as standard [13,14].

**Ferric reducing antioxidant potential (FRAP) assay:** FRAP reagent was prepared by mixing of 25 mL of acetate buffer (30 mM, pH 3.6) with 2.5 mL of TPTZ [2,4,6-*Tirs*-(2-pyridyl)-*s*-triazine] solution (10 mM in 40 mM hydrochloric acid) followed by the addition of 2.5 mL freshly prepared ferric chloride solution (20 mM). The solution was allowed to incubate at 37 ºC for 15 min before use. Ascorbic acid were taken as standard was serially diluted to obtain a uniform graph against

FRAP reagent at 593 nm. All the extracts and fractions were dissolved in methanol to prepare the stock solutions of 10 mg/mL. The concentration dilutions of extracts and fractions were adjusted to obtain a significant absorbance for the determination of FRAP value equivalent to ascorbic acid from standard graphs [13,14].

**Total antioxidant capacity assay (phsophomolybdate assay):** Phosphomolybdate reagent (solution of 4 mM ammonium molybdate, 28 mM sodium phosphate and 0.6 M sulphuric acid) was prepared. Ascorbic acid was used as standard and dissolved in methanol to prepared 10 mg/mL stock solution. A 1 mg/mL were used as working solution to obtain a uniform graph against phosphomolybdate reagent at 695 nm. All extracts and fractions were dissolved in methanol to prepare a stock solution of 10 mg/mL. The concentration dilutions of extracts and fractions were adjusted to obtain a significant absorbance for the determination of total antioxidant capacity (TAC) value equivalent to ascorbic acid from standard graphs [13,14].

## RESULTS AND DISCUSSION

**Extracts and fractions of *Swertia chirata* plant yield:** Total yield of SWA and SHA extracts was found to be 11.093 ± 0.369% and 13.680%, respectively. The SHA extract showed a higher yield as compared with SWA hence given higher preference as compared with SWA extract. Serial fractionation of SHA extract was done and final yield with *n*-hexane was found to be 0.468 ± 0.018% (SH), with chloroform 1.232 ± 0.042% (SCL), with ethylacetate 2.473 ± 0.594% (SEA), with butanol 2.643 ± 0.662% (SBU) and the remaining material 4.378± 0.098% (SRE) were achieved.

**HPLC quantifications:** Screening and quantification of four analytical marker compounds in extracts and fractions were done by HPLC-DAD, and the chromatogram of the standard, extracts and fractions are shown in Fig 1. The SWA and SHA extract showed the presence of all four analytical marker compounds (PCA, SM, MF and AG) (Table-1). The SHA consists higher extent of PCA (0.081. ± 0.002), SM (0.953 ± 0.015%), MF (1.031± 0.008%), whereas in SWA, AG (0.158 ± 0.001%) content was found higher than SHA (0.104 ± 0.005). In between fractions, PCA and AG were not found in SH, similarly, PCA was absent in SBU. Among all the fractions, SCL showed higher PCA (1.225 ± 0.036%), In the same way, SM (0.440 ± 0.007%) and MF (3.693 ± 0.033%) in SBU, and AG (3.640 ± 0.146%) in SEA. Remaining material after fractionation (SRE) did not show any presence of analytical marker compounds as observed in the SRE chromatogram (Fig. 1).

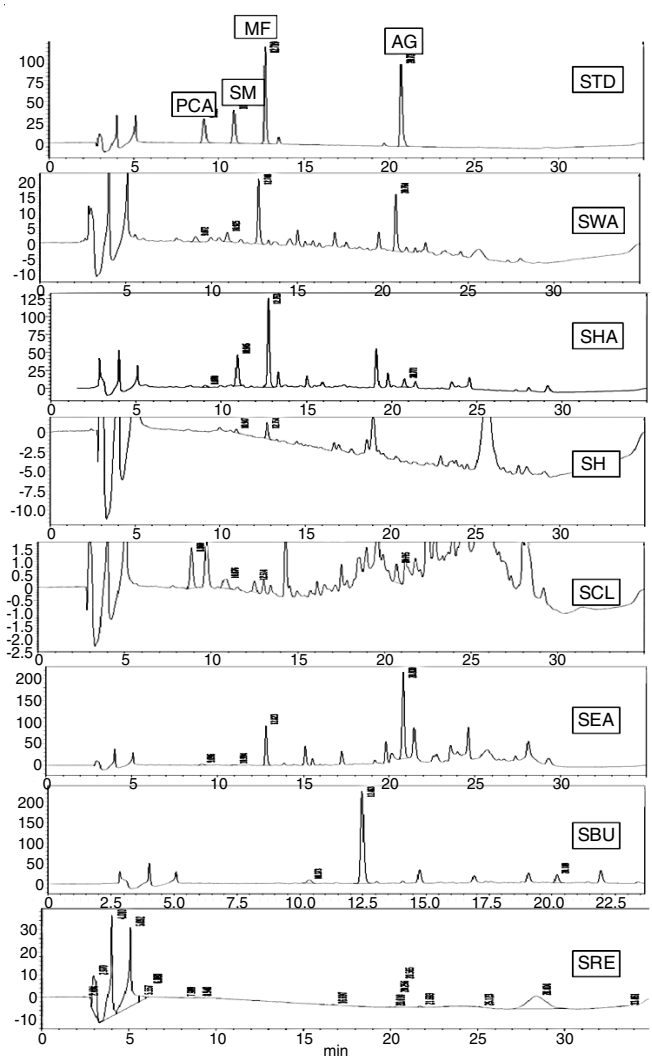


Fig. 1. HPLC chromatogram of standard (STD), SWA extract, SHA extract, SH fraction, SCL fraction, SEA fraction, SBU fraction and the remaining (SRE) material

### Method validation

**Specificity:** The specificity were passed by determining the percentage RSD (relative standard deviation), tailing factor, USP plate count of individual marker compounds as mentioned in Table-2.

TABLE-1
CONTENT OF PCA, SM, MF AND AG IN EXTRACTS AND FRACTIONS BY HPLC-DAD DETECTION

| Extracts | PCA | SM | MF | AG |
|---|---|---|---|---|
| SWA | 0.062 ± 0.002 | 0.084 ± 0.006 | 0.182 ± 0.004 | 0.158 ± 0.001 |
| SHA | 0.081 ± 0.002 | 0.953 ± 0.015 | 1.031 ± 0.008 | 0.104 ± 0.005 |
| Fractions obtained from hydroalcoholic extract | | | | |
| SH | Absent | 0.074 ± 0.001 | 0.126 ± 0.001 | Absent |
| SCL | 1.225 ± 0.036 | 0.362 ± 0.039 | 0.118 ± 0.001 | 0.182 ± 0.008 |
| SEA | 0.254 ± 0.042 | 0.044 ± 0.005 | 1.443 ± 0.010 | 3.640 ± 0.146 |
| SBU | Absent | 0.440 ± 0.007 | 3.693 ± 0.033 | 0.322 ± 0.013 |
| SRE | Absent | Absent | Absent | Absent |

TABLE-2
SPECIFICITY OF PCA, SM, MF AND AG PERFORMED IN TERMS OF RSD, USP PLATE COUNTS AND TAILING FACTOR

| Compound name | Conc. (ppm) | Average RT | %RSD | Average plate count (USP) | %RSD | Average tailing factor | %RSD | Range |
|---|---|---|---|---|---|---|---|---|
| PCA | 10 | 8.160 | 0.151 | 17167 | 0.983 | 1.135 | 0.910 | 0.80-2.0 |
| SM | 10 | 10.468 | 0.092 | 38612 | 0.635 | 1.146 | 0.242 | 0.80-2.0 |
| MF | 10 | 11.712 | 0.063 | 69509 | 1.225 | 1.203 | 0.180 | 0.80-2.0 |
| AG | 10 | 19.803 | 0.012 | 183713 | 0.932 | 1.141 | 0.078 | 0.80-2.0 |

**Linearity:** A significant linearity were obtained between the solutions of ranges of 0.1 to 5 µg/mL of concentration (100 to 2 ng of injecting concentration). As per standard limit of 0.99, the present method shows a good linearity (Table-3).

**Sensitivity:** Limit of detection (LOD) and limit of quantification (LOQ) of all the four analytical marker compounds were range between 5 to 0.01 µg/mL. The S/N ratio was shown as an appropriate range between 5 to 0.1 µg/mL concentration, which were found suitable for all four analytical marker compounds for the LOD and LOQ detection (Table-4).

**Precision:** All the four compounds were performed at two different concentrations (4 and 10 µg/mL) and each sample was repeated six times in a day for intraday precision and repeated six times for three consecutive days for interday precision. The RSD results of retention time of compounds were found in the permissible limit (Table-5).

**Accuracy:** Four different concentrations between 50 to 150% in spikes were taken for the recovery analysis. A significant recovery were found within the standard limit of 80 to 120% (Table-6). PCA showed a 102.62% recovery with 1.91%

TABLE-3
LINEARITY OF IN TERMS OF CORRELATION REGRESSION COEFFICIENT ($R^2$) OF RETENTION TIME, SLOPE OF CURVE AND INTERSECT OF PCA, SM, MF AND AG MARKERS COMPOUNDS

| Compound name | Linearity range (µg/mL) | Injection volume conc. (ng) | $R^2$ | Slope | Intercept |
|---|---|---|---|---|---|
| PCA | 0.1-5 | 2-100 | 0.9994 | 50834 | -894.85 |
| SM | 0.1-5 | 2-100 | 0.9990 | 28325 | 2685.4 |
| MF | 0.1-5 | 2-100 | 0.9950 | 88958 | 2302.7 |
| AG | 0.1-5 | 2-100 | 0.9995 | 35374 | 223.44 |

TABLE-4
SENSITIVITY ANALYSIS OF HPLC-DAD IN TERMS OF LIMIT OF DETECTION (LOD), LIMIT OF QUANTIFICATION OF PCA, SM, MF AND AG OBTAINED BY S/N RESPONSE OF HPLC SOFTWARE

| Compound name | Linearity range (µg/mL) | Injection volume conc. (ng) | $R^2$ (conc. *vs.* S/N ratio) | Regression equation | LOD | LOQ |
|---|---|---|---|---|---|---|
| PCA | 0.1-5 | 2-100 | 0.9917 | y = 2.3554x ± 0.5445 | 1.042 | 4.014 |
| SM | 0.1-5 | 2-100 | 0.9923 | y = 1.15965x ± 0.4956 | 1.569 | 5.953 |
| MF | 0.1-5 | 2-100 | 0.9978 | y = 6.5253x ± 0.9376 | 0.316 | 1.389 |
| AG | 0.1-5 | 2-100 | 0.9978 | y = 2.3961x ± 0.3368 | 1.111 | 4.033 |

TABLE-5
INTRA-DAY AND INTERMEDIATE PRECISION ANALYSIS OF PCA, SM, MF AND AG

| Compound name | Concentration (µg/mL) | Intra-day precision | | Inter-day precision | | Limit (%RSD) |
|---|---|---|---|---|---|---|
| | | Mean area | %RSD | Mean area | %RSD | NMT |
| PCA | 4 | 198395 | 0.667 | 19288 | 1.083 | 10 |
| | 10 | 499469 | 0.077 | 499455 | 0.030 | 10 |
| SM | 4 | 106212 | 0.344 | 105468 | 0.725 | 10 |
| | 10 | 278226 | 0.090 | 279126 | 0.358 | 10 |
| MF | 4 | 323158 | 0.633 | 319972 | 0.862 | 10 |
| | 10 | 907246 | 0.203 | 907886 | 0.062 | 10 |
| AG | 4 | 139690 | 0.630 | 139651 | 0.631 | 10 |
| | 10 | 343136 | 0.369 | 344112 | 0.252 | 10 |

TABLE-6
ACCURACY IN TERM OF RECOVERY OF PCA, SM, MF AND AG WAS OBTAINED FROM SPIKE SOLUTION

| Sample name | Concentration (%) | Recovery (%) | %RSD | Limit (% recovery) |
|---|---|---|---|---|
| PCA | 50, 100, 120, 150 | 102.62 | 1.91 | 80 to 120 |
| SM | 50, 100, 120, 150 | 102.62 | 1.46 | 80 to 120 |
| MF | 50, 100, 120, 150 | 102.85 | 1.91 | 80 to 120 |
| AG | 50, 100, 120, 150 | 102.59 | 1.41 | 80 to 120 |

RSD, SM showed 102.62% recovery with 1.46% RSD, MF 102.85% recovery with 1.91% RSD and AG 102.59% recovery with 1.41% RSD.

**Robustness:** The capacity of analytical method to remain unchanged with some deliberate changes in flow rate (1.1 and 0.9 mL/min). Solvent A (0.11% TFA and 0.09% TFA) and solvent B (acetonitrile:water 77:23 and acetonitrile:water 67:37), each change parameter were repeated six time to determine the %RSD, USP plate count and tailing factor. With solvent A variability 0.259% to 0.464% RSD, 15794 to 167727 USP plate counts, 1.101 to 1.225 tailing factor and solvent B variablity 0.137 to 1.056% RSD, 1.143 to 1.305 tailing factor were observed (Table-7). The %RSD of retention time, USP plate counts and tailing factor suggested that robustness parameter test was passed in the validation.

**Antioxidant assay**

**DPPH assay:** In this assay, standard $IC_{50}$ of ascorbic acid was found to be 4.164 µg/mL. For the tested samples, the $IC_{50}$ of SHA extract was found to be 0.063 ± 0.010 mg/mL, which was higher than SWA 1.154 ± 0.106 mg/mL. Among the SHA fractions, SEA showed the highest DPPH reducing activity at 0.008 ± 0.002 mg/mL followed by SCL 0.019 ± 0.002, SBU 0.050 ± 0.002 and SH 0.097 ± 0.060 and lowest reducing activity was found in SRE $IC_{50}$ 9.194 ± 2.984, respectively.

**ABTS assay:** Using ABTS assay, the $IC_{50}$ of ascorbic acid was found to be 5.597 µg/mL. The $IC_{50}$ value of SHA extract was 0.243 ± 0.030 mg/mL, which is also found higher activity than SWA 6.499 ± 0.567 mg/mL. Among the fractions, SEA showed the highest ABTS reducing activity at 0.025 ± 0.001 mg/mL, followed by SBU 0.157 ± 0.002, SH 0.158 ± 0.024 and SCL 0.162 ± 0.008 and the lowest ABTS were found to be in SRE 31.00 ± 2.215. Hence, ABTS activity was found in SHA extract than SWA extract, and SEA fraction shows higher activity than other fractions.

**Total reducing assay:** Antioxidant assay by TRA were performed with standard ascorbic acid, extracts and fractions.

TABLE-7
ROBUSTNESS EXPRESSED IN TERMS OF FLOW RATE VARIABILITY, SOLVENT A CONCENTRATION VARIABILITY, SOLVENT B RATION VARIABILITY FOR PCA, SM, MF AND AG MARKERS COMPOUNDS

| Parameters | | | Working parameter | | | – Changes | | + Changes | |
|---|---|---|---|---|---|---|---|---|---|
| Flow | | | 1 mL/min | | | 0.9 mL/min | | 1.1 mL/min | |
| Solvent A concentration TFA (trifuloroacetic acid) | | | 0.1% TFA | | | 0.09% TFA | | 0.11% TFA | |
| Solvent B concentration (acetonitrile:water) | | | 70:30 | | | 67:37 | | 77:23 | |
| Flow | Variability | Compounds name | Area | Area %RSD | Limit NMT (%) | Plate counts (USP) | NLT | Tailing factor | Range |
| Flow (mL/min) | 0.09 | PCA | 3101146 | 0.187 | 10 | 16064 | 1800 | 1.215 | 0.8-2.0 |
| | | SM | 1705261 | 0.305 | 10 | 39794 | 1800 | 1.139 | 0.8-2.0 |
| | | MF | 5267706 | 0.353 | 10 | 75293 | 1800 | 1.184 | 0.8-2.0 |
| | | AG | 2195773 | 0.429 | 10 | 167727 | 1800 | 1.104 | 0.8-2.0 |
| | 1 | PCA | 2641308 | 1.157 | 10 | 18907 | 1800 | 1.184 | 0.8-2.0 |
| | | SM | 1441355 | 1.841 | 10 | 35070 | 1800 | 1.162 | 0.8-2.0 |
| | | MF | 4447413 | 1.056 | 10 | 63374 | 1800 | 1.274 | 0.8-2.0 |
| | | AG | 1847368 | 0.984 | 10 | 157552 | 1800 | 1.147 | 0.8-2.0 |
| | 1.1 | PCA | 1199858 | 0.139 | 10 | 22220 | 1800 | 1.256 | 0.8-2.0 |
| | | SM | 714685 | 0.305 | 10 | 46476 | 1800 | 1.219 | 0.8-2.0 |
| | | MF | 2322348 | 1.056 | 10 | 72418 | 1800 | 1.327 | 0.8-2.0 |
| | | AG | 1742173 | 0.204 | 10 | 166592 | 1800 | 1.173 | 0.8-2.0 |
| Solvent A (conc. of TFA) | 0.09 | PCA | 1562179 | 0.262 | 10 | 16064 | 1800 | 1.215 | 0.8-2.0 |
| | | SM | 863651 | 0.302 | 10 | 39794 | 1800 | 1.139 | 0.8-2.0 |
| | | MF | 2767538 | 0.259 | 10 | 75293 | 1800 | 1.184 | 0.8-2.0 |
| | | AG | 1127306 | 0.335 | 10 | 167727 | 1800 | 1.104 | 0.8-2.0 |
| | 0.1 | PCA | 1302366 | 0.252 | 10 | 17074 | 1800 | 1.167 | 0.8-2.0 |
| | | SM | 716896 | 0.358 | 10 | 42182 | 1800 | 1.160 | 0.8-2.0 |
| | | MF | 2316205 | 0.209 | 10 | 77026 | 1800 | 1.263 | 0.8-2.0 |
| | | AG | 920504 | 1.187 | 10 | 185000 | 1800 | 1.144 | 0.8-2.0 |
| | 0.11 | PCA | 1574045 | 0.301 | 10 | 15794 | 1800 | 1.225 | 0.8-2.0 |
| | | SM | 867755 | 0.298 | 10 | 38821 | 1800 | 1.146 | 0.8-2.0 |
| | | MF | 2775963 | 0.413 | 10 | 75275 | 1800 | 1.182 | 0.8-2.0 |
| | | AG | 1129790 | 0.464 | 10 | 166761 | 1800 | 1.101 | 0.8-2.0 |
| Solvent B (acetonitrile: water) | 63:37 | PCA | 1571508 | 0.617 | 10 | 18362 | 1800 | 1.153 | 0.8-2.0 |
| | | SM | 854738 | 0.584 | 10 | 54362 | 1800 | 1.188 | 0.8-2.0 |
| | | MF | 2673752 | 1.056 | 10 | 84929 | 1800 | 1.305 | 0.8-2.0 |
| | | AG | 1119493 | 0.542 | 10 | 189468 | 1800 | 1.173 | 0.8-2.0 |
| | 70:30 | PCA | 1782994 | 1.579 | 10 | 12328 | 1800 | 1.149 | 0.8-2.0 |
| | | SM | 979786 | 1.429 | 10 | 22528 | 1800 | 1.103 | 0.8-2.0 |
| | | MF | 3105847 | 1.407 | 10 | 42494 | 1800 | 1.177 | 0.8-2.0 |
| | | AG | 1398623 | 1.486 | 10 | 103208 | 1800 | 1.462 | 0.8-2.0 |
| | 77:23 | PCA | 1670677 | 0.761 | 10 | 15827 | 1800 | 1.230 | 0.8-2.0 |
| | | SM | 914045 | 0.137 | 10 | 25756 | 1800 | 1.177 | 0.8-2.0 |
| | | MF | 2938784 | 0.615 | 10 | 47629 | 1800 | 1.239 | 0.8-2.0 |
| | | AG | 1185690 | 0.444 | 10 | 155547 | 1800 | 1.143 | 0.8-2.0 |

The intensity of blue colour formation in terms of $IC_{50}$ of ascorbic acid were found at 156.68 µg/mL. In the test sample, the SHA $IC_{50}$ was 3.554 ± 1.783 mg/mL, which is higher than SWA 6.432 ± 3.217 mg/mL. Among all the fractions, SBU showed the highest TRA reducing activity 0.413 ± 0.309 mg/mL, followed by SCL 0.974 ± 0.585, SEA 1.230 ± 0.615 and SH 4.443 ± 3.866 and no reducing activity was found in SRE fraction.

**FRAP assay:** A FRAP values in µmol/mL equivalent to ascorbic acid of all extracts and fractions were determined through the equation obtained from the standard curve of ascorbic acid against FRAP reagent. The SHA possessed a higher FRAP value 18.830 ± 1.242 than SWA 3.280 ± 0.462. Similarly, SH showed a highest FRAP value 28.922 ± 3.153 followed by SEA 19.547 ± 2.119, SCL 15.664 ± 1.945 and SBU 15.195 ± 0.911, while SRE showed a very low FRAP value 0.049 ± 0.003 as compared to others fractions.

**Total antioxidant capacity (phosphomolybdate assay):** The TAC values of all the extracts and fractions were determined through line equation obtained by the standard ascorbic acid against phosphomolybdate reagent. The TAC values of SWA, SHA, SH, SCL, SEA, SBU, SRE were determined in terms of equvalent to ascobic acid. In this assay also, SHA extract possessed a higher TAC value 1.035 ± 0.006 (equivalent to ascorbic acid) than SWA extract 0.132 ± 0.132. Similarly, SH shows the highest TAC value 3.263 ± 0.325 followed by SEA 2.750 ± 0.466, SCL 1.945 ± 0.226 and SBU 1.728 ± 0.258, while SRE exhibited a very low TAC value 0.132 ± 0.013 as compared to others fractions.

Phytochemical analysis shows that presence of all the analytical markers in both water (SWA) and hydroalcoholic (SHA) extracts. Among these four analytical markers, protocatechuic acid (PCA), swertiamarin (SM) and mangiferin (MF) were found higher in SHA extract wheresas amarogentin (AG) content was found higher only in SWA extract. The extract yields were also found higher in SHA in comparison to SWA.

Among all the four fractions (SH, SCL, SEA, SBU) and remianing after fractionation (SRE) of SHA, SH exhibited a lowest yield in presence of SM and MF, but possessed the highest activity against FRAP and TAC assay, whereas SEA showed the second highest yield and also showed a good activity against DPPH, ABTS and second highest activity against FRAP and TAC after SH.

## Conclusion

All four analytical markers compounds *viz*. protocatechuic acid (PCA), swertiamarin (SM), mangiferin (MF) and amarogentin (AG) have been quantified and validated in each extracts and fractions by HPLC-DAD method. The hydroalcoholic extract (SHA) showed higher activity as compared with water extract (SWA) against the five different antioxidant assays. Fractions shows different response of the ethyl acetate fraction (SEA) shows most potent activity against DPPH, ABTS which is very near to standard ($IC_{50}$ = 4.144 µg/mL DPPH, $IC_{50}$ = 5.597 µg/mL ABTS). Thus, SEA fractions can be a choice in case of free radicals inflammation, against FRAP and TAC assays while the *n*-hexane (SH) showed a higher FRAP, TAC with equivalent to standards (ascorbic acid µmol/mL) value as compared to other fractions. The SEA fraction also show seconds highest FRAP and TAC value with significant yield, hence, in case of electron donor antioxidant assay, SH fraction can be used in TRA assay. The butanol fraction (butanol) showed a mild antioxidant activity when compared to standard (standard $IC_{50}$ = 156.68 µg/mL). The remaining fraction (SRE) of SHA extract exhibited the highest yield (4.378 ± 0.098%) followed with SBU, SEA, SCL extracts. A very poor yield of *n*-hexane (SH) extract (0.468 ± 0.018%) was obtained when compared with the yield of SEA fraction, thus SEA fraction also can used as second but more prominent as a choice for FRAP and TAC due to the higher yield (2.473 ± 0.5794%). Hence comparatively choice between fractions, SEA can be better choice for selection as antioxidant, except in case TRA assay were SBU fractions can be a better choice. In extracts, SHA is best choice as compared to SWA extracts because of higher yield and higher activity against all the studied antioxidant assays.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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