6. ANALYSIS OF RHEIN

Natural products that are gaining light in modern research are mainly synthesized from primary metabolites by one or more unique biogenetic specialized pathways and are mostly limited to plants or microorganisms. Chemicals of unusual structures resulting from several biogenetic pathways includes terpenes, steroids, phenols, flavonoids, alkaloids and antibiotics etc. They may sometimes be characteristic of a specific genus, species or strains, which largely contribute to the production of commercial products, such as, pharmaceuticals, cosmetics, dyes and so on. These bioactive compounds are environment friendly, mostly produced from renewable resources, biodegradable in nature and are produced by the plants as secondary metabolites. Many of these phytochemicals are isolated from plants. They possess immense medicinal properties and play a significant role over the synthetic pharmaceuticals. They also contribute immensely in the existence of a species in the evolutionary progress and in species-environment interaction. These compounds act as a great revenue generating system and the industrial marketing value of these bioactive chemicals are increasing in an exponential rate reaching about $115 billion by 2020 (Marakli, 2018).

S. obtusifolia (L.) Irwin and Barneby of the family Leguminosae on account of its medicinal attributes is noted to get a place in the Chinese as well as other Asian countries' traditional medicine. The plant has gained interest all over the world. The plant is rich in quite several medicinally important secondary metabolites. These chemicals are anthraquinones, xanthones, anthrones, lactones, flavonoids, polyketides and triterpenoids and tetraglucosides (Kitanaka et al., 1981, 1990; Zhang et al., 2003; Sob et al., 2008; Liu et al., 2015). Anthraquinone derivatives are most prevalent in this species and are comprised of sennosides A and B, anthrone and dianthrone glycosides.

Due to some considerable health benefits and consumer awareness the consumption of *S. obtusifolia* in various forms and its prospect in the market has been greatly stimulated (Liu et al., 2014). Anthraquinones, forming an array in its different forms in this species, are the compounds responsible for imparting colours of different shades and used in dyes and in food and pharmaceutical industries. Till date, more than 35 anthraquinones were isolated from *S. obtusifolia* (Sob et al., 2008) that include emodine, chrysophanol, physcion, aloe-emodin, etc.

Anthraquinones are found in different families of plants like Rhamnaceae, Verbenaceae, Bignoniaceae, Polygonaceae, Rubiaceae, Liliaceae, Xanthorrhoeaceae, apart from *Senna* and *Cassia* of Leguminosae as well as in bacteria and fungi (You et al., 2013; Korulkin et al., 2015; Shukla et at., 2017).

Though the knowledge of biosynthesis of the anthraquinones has increased from the past years (Leistner, 1985; Wijnsma and Verpoorte, 1986; Suzuki and Matsumoto, 1988; Inouye and Leistner, 1988; Koblitz, 1988; Van Den Berg and Labadie, 1989; Leistner, 1995), the new trends of elucidating the biosynthesis of anthraquinone pathways by different novel technologies in functional genomics with the advent of new genes and enzymes help us in gathering knowledge of regulation of anthraquinone biosynthesis both by external factors and at the molecular level (Liu et al., 2014). The anthraquinones in plants are derived from a wide variety of precursors resulting from different pathways (Leistner, 1981; Patai and Rappoport, 1988). Though several pathways were reviewed in the past for the biosynthesis of anthraquinones, all the routes for their synthesis is not fully known as yet (Leistner, 1985; Wijnsma and Verpoorte, 1986; Suzuki and Matsumoto, 1988; Shukla et al., 2017).

Among the several possible biosynthetic pathways to produce anthraquinones, the biosynthesis of anthraquinones in higher plants as well as in some bacterial and fungi populations are produced mainly by two proposed pathways: chorismate/o-succinylbenzoic acid and polyketide pathways (Han et al., 2001; Shukla et al., 2017).

Anthraquinones synthesized by polyketide pathways occurred in the families of higher plants like Leguminosae, Rhamnaceae, Polygonaceae and in Fungi (Simpson, 1987; Patai and Rappoport, 1988; Shukla et al., 2017). Anthraquinones are oxidized aromatic compounds of polyketide origin derived from the cyclization of an octaketide chains produced. Acetyl CoA or Malonyl CoA in its activated form is the source of the formation of this C2 unit from acetic acid. According to the acetate malonate theory (Simpson, 1987; Inouye and Leistner, 1988; Han et al., 2001; Shukla et al., 2017) the most probable hypothesis is the condensation of acetic acid giving polyketomethylene chain that can also be produced by the condensation of malonate fragments and acetate fragments where one Acetyl CoA is flanked by seven Malonyl CoA units. The pathway is described in Figure 6.1. Acetyl Co-A or Malonyl Co-A in its activated form is the source of the formation of this C2 unit from acetic acid. This polyketomethylene chain may be formed by the condensation of malonate and acetate fragments and can bend in various ways with the subsequent cyclization as per the number of $-CH_2-CO$ units.

The benzene nucleus forms by two different pathways:

a) via, aldol condensations to produce resorcinol derivatives (such as 2,4 dihydroxy-6-alkyl benzoic acid also called orsellinic acid).

b) via, Claisen condensations leading to acylphloroglucinol such as acetyl phloroglucinol.

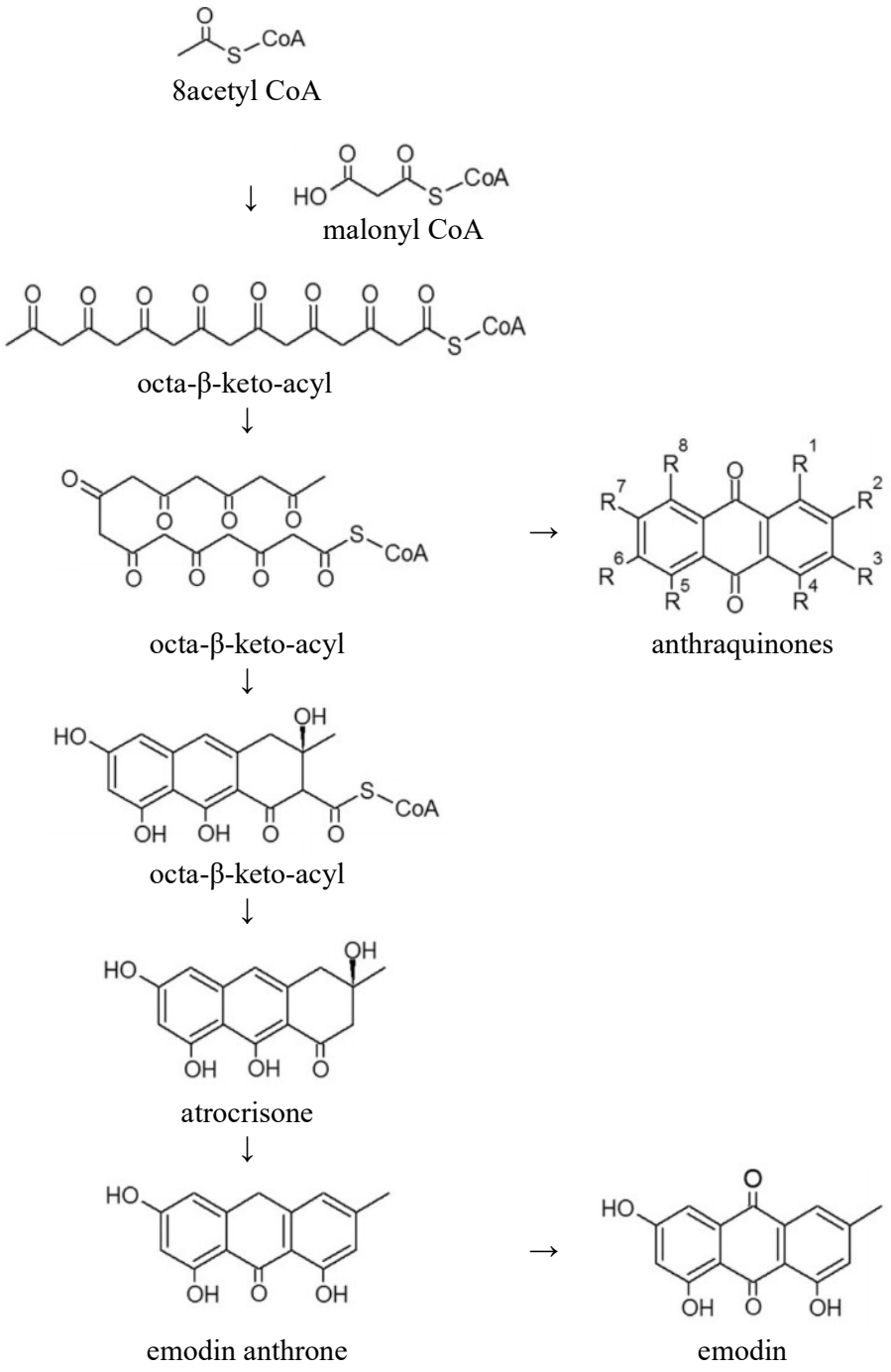

Figure 6.1 Polyketide pathway for the biosynthesis of anthraquinones

Anthraquinone derivatives such as endocrocin, chrysophanol, clavorubin and emodin are some of the naturally occurring anthraquinones, produced by this pathway (Han et al., 2001).

Anthraquinones in the genera like *Morinda*, *Galium*, *Rubia*, and *Cinchona* of family Rubiaceae, produced by Chorismate/ o-succinylbenzoic acid pathway, is called as Rubia type (Shukla et al., 2017). But according to Liu et al. (2014), the preliminary stage of the biosynthetic pathway (Figure 6.2) for producing anthraquinones from *S. obtusifolia* involving the unigenes is also derived from the combined biosynthetic reaction of isochorismate with MVA (mevalonic acid) and 2-methyl-d-erythritol 4-phosphate in MEP (malonate) pathways. The first ring (A) and second ring (B) of the anthraquinones are formed from 1,4-dihydroxy-2-naphthoc acid through isochorimmic acid and alpha ketoglutaric acid. The third ring (C) is produced from Isopentyl diphosphate and 3,3 dimethylallyl diphosphate via malonate and mevalonate pathways.

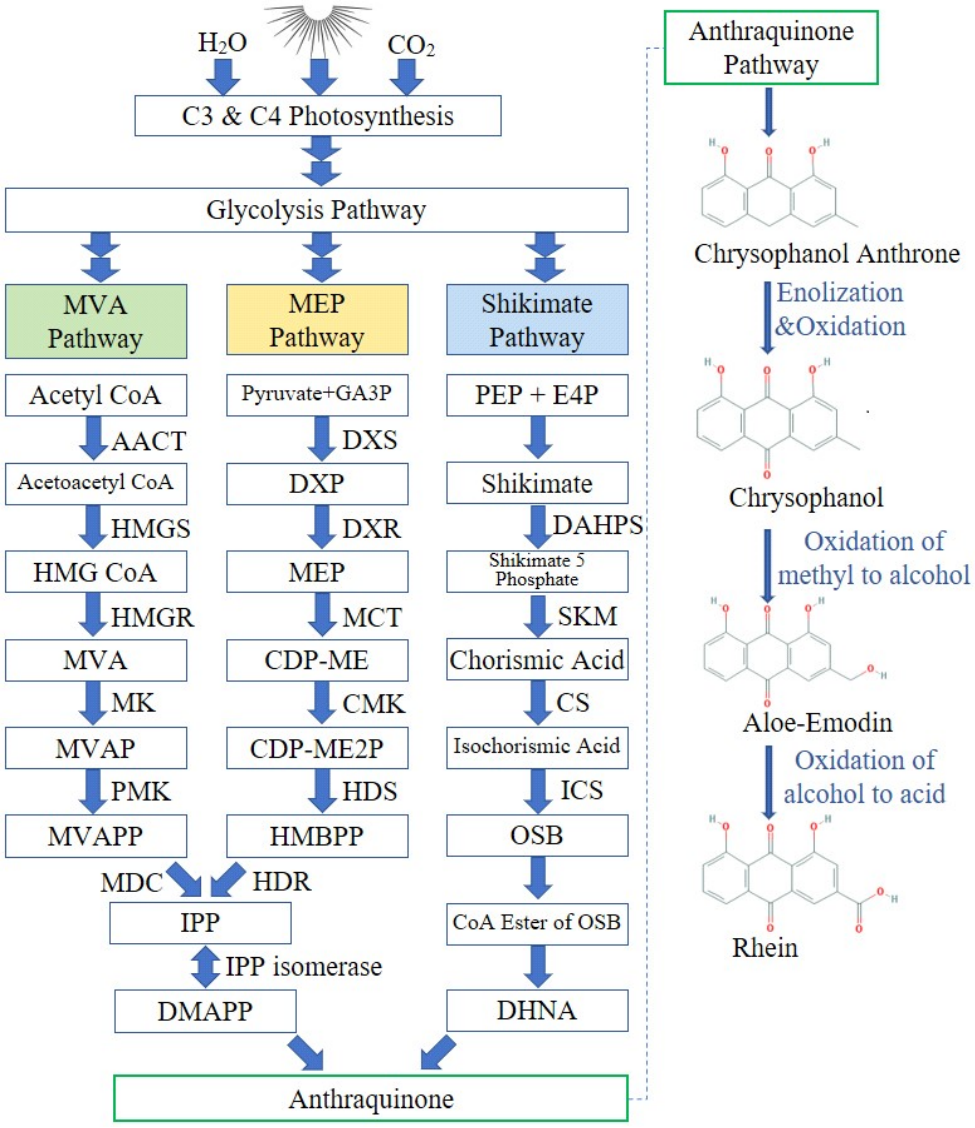

Figure 6.2 Pathway of rhein

MVA: Mevalonate; CoA: Coenzyme A; AACT, acetoacetyl-CoA thiolase; HMG CoA: 3-hydroxy-3-methylglutaryl coenzyme A; HMGS: 3-hydroxy-3-methylglutaryl-CoA synthase; HMGR: 3-hydroxy-3-methylglutaryl-CoA reductase; MK: mevalonate kinase; MVAP: mevalonate 5-phosphate; PMK: phosphomevalonate kinase; MVAPP: mevalonate 5-diphosphate; MDC: MVAPP decarboxylase; MEP: 2C-methyl-D-erythritol 4-phosphate; GA3P: glyceraldehyde 3-phosphate; DXS: 1-deoxy-D-xylulose 5-phosphate synthase; DXP: 1-Deoxy-D-xylulose 5-phosphate; DXR: DXP reductoisomerase; MEP: 2C-methyl-D-erythritol 4-phosphate; MCT: 2C-methyl-D-erythritol 4-phosphate cytidyl transferase; CDP-ME: 4-diphosphocytidyl-2-C-methylerythritol; CMK: 4-diphosphocytidyl-2C-methyl-D-erythritol kinase; CDP-ME2P: CDP-ME-2-phosphate; HDS: 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase; HMBPP: 4-hydroxy-3-methylbut-2-enyl diphosphate; HDR: 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; IPP: isopentenyl diphosphate; DMAPP: dimethylallyl diphosphate; PEP: phosphoenol pyruvate; E4P: erythrose-4-phosphate; DAHPS: 3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase; SKM: shikimic acid; CS: chorismate synthase; ICS: isochorismate synthase; OSB: o -succinylbenzoic acid; DHNA: 1,4-dihydroxy-2-naphthoic acid.

Rhein, a lipophilic anthraquinone, also known as cassic acid or rheic acid, obtained from natural sources of various plant species such as *Rhubarb, Rheum, Aloe, Cassia, Senna* species, occurs in free state as well as a glucoside. The molecular structure of rhein has been shown in Figure 6.3. Rein as the simplest form of anthraquinone found in this species is also used as a natural dye imparting natural colors used in dye industries and also in pharmaceutical industries.

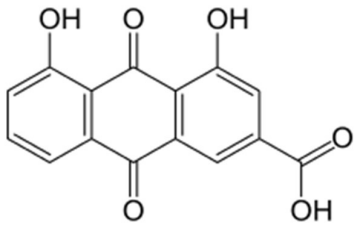

Figure 6.3 Molecular structure of Rhein

Due to its multifarious pharmaceutical uses, rhein bears much significance in the research field, as well. Rhein, with IUPAC name 4,5-dihydroxy-9,10-dioxoanthracene-2-carboxylic acid and the molecular formula $C_{15}H_8O_6$, has recently became a subject of interest because of its scores of medicinal properties which includes hepatoprotective, chondroprotective, neuroprotective, antidiabetic, antimicrobial, anticancer, antioxidant, activity (Zhou et al., 2015). It is also the precursor compound of commercial diacerein (1,8-diacyl derivative), a medicine used for treating inflammation and osteoarthritis (Dutta et al., 1998).

Rhein has pharmacological efficacy in combating various maladies like, prevention of fibrosis in liver injury, lowering of lipids in liver and reducing of inflammation in fatty liver (Sheng et al., 2011), improving of tight junction function and protection against fibrosis of kidney (Ji et al., 2005; Peng et al., 2013), maintenance of osteoblastic activity and decrease of inflammation and cartilage destruction (Cong et al., 2012). Lipid

lowering and anti-obesity activities showed body weight reduction by lowering fat and high- and low-density lipoprotein content (Sheng et al., 2012). Rhein was observed to take part in anti-cancer activity and directs cell apoptosis by ER stress, calcium and mitochondria mediated pathways (Fermand et al., 2011). It also prevents cancer cell invasion into systemic circulation by preventing angiogenesis and breakdown of the extracellular matrix and suppresses several tumors promoting signaling pathways (Lai et al., 2009). Anti-inflammatory activities of rhein include depreciation of the secretion of pro-inflammatory cytokines such as interleukin-6 and interleukin-1β (Cong et al., 2012). Antioxidant activity of rhein helps decrease plasma glucose by increasing survival of islet beta cells in type 2 diabetes mellitus, thus having antidiabetic activity (Du et al., 2011). Rhein has antimicrobial activity and inhibits arylamine N-acetyltransferase and cell growth in *Helicobacter pylori* (Chung et al., 1998) and effective against many genotypes of *Staphylococcus aureus* (Yu et al., 2008). Rhein showed anti-allergic activity by inhibiting the production of leukotrienes and releasing histamine from mast cells (Singh et al., 2012).

Rhein is, thus, a very effective medicinal compound with multifarious medicinal properties. As a result, extraction of rhein from various possible plant sources has remained a topic of interest to many researchers. Rhein was extracted from the leaves of *Cassia alata* L. (Hauptmann and Nazario, 1950), pods and leaves, rhubarb (Lemli, 1965; Habib and El-Sebakhy, 1980). Rhein was also extracted from the young plants and tissue culture of *Cassia senna* L. (Fairbairn and Shrestha, 1967; Rai et al., 1974), callus culture of *Cassia podocarpa* (Rai, 1988), leaves of *Cassia angustifolia* Vahl (Mehta and Laddha, 2009; Meier et al., 2017), pods and leaves of *Cassia fistula* Linn. (Sakulpanich and Gritsanapan, 2009; Shailajan et al., 2013), leaves of *Senna alata* (Panichayupakaranant et al., 2009), leaves of *Cassia acutifolia* (Park and Kim, 2015),

fruits and leaves of *Senna alexandrina* Mill. (Leguminosae) (Epifano et al., 2015; Abdo, 2017), seeds of *Cassia occidentalis* (Panigrahi et al., 2016; Rekha et al., 2016), leaves of *Senna tora* Linn (Fathalla, 2018), etc. Despite being a potential source of rhein, the species *S. obtusifolia* was not much explored by previous researchers. Recently, Nandi and Maiti (2018) reported the presence of rhein in the leaves of *S. obtusifolia* (L.) Irwin and Barneby.

Occurance of rhein in fruits and leaves within the same genus *Senna,* of the species *S. alexandrina* Mill. and *Aloe vera* (L.) Burm. were confirmed by Epifano et al. (2015). Determination of rhein along with other anthraquinones was carried out in *Polygoni multiflori*, a chinese herb (Zuo et al., 2008). HPLC-MS-CID analysis of various secondary metabolites and medicinal components like phenolics, phenolic glycosides, and various anthraquinones were studied for *S. obtusifolia* and *S. tora* by Harry-O'Kuru et al. (2012). Mehta and Laddha (2009) isolated rhein by the hydrolysis of sennosides and further extracted from the mixture of anthraquinones from the leaves of *Cassia angustifolia*. Rhein is also reported in *S. tora* earlier in the literature (Meena et al., 2010).

This chapter illustrates the extraction, identification, and quantification procedures of the anthraquinone rhein obtained from the leaves of *S. obtusifolia*, collected from twenty different provenances. The variations in the amount of rhein obtained from these twenty provenances were also studied. For these purposes, TLC, HPLC, and ESI MS techniques were utilized.

6.1 MATERIALS AND METHODS

6.1.1 Materials

Seeds of *S. obtusifolia* were collected from 20 different provenances (Asansol (ASN), Bandhavgarh (BDG), Bishnupur (BNP), Bolpur (BPR), Dahod (DHD), Dehradun (DHN), Devendranagar (DNG), Gobindapur (GBP), Haridwar (HDR), Jabalpur (JBP), Jaipur (JPR), Jhargram (JRM), Kalyani (KLN), Kharagpur (KPR), Lakshmikantapur (LKP), Mangalore (MGL), Nagpur (NGP), Purulia (PRL), Raipur (RPR), Tatanagar (TNG)) of India. Seeds from these 20 places were sown in a field at Panskura, a semiurban area in Purba Medinipur district, to raise plants from which data were collected to derive the relative performance. These plants were used as stock and maintained in the experimental gardens. Young and fresh leaves from the plants of 48 days of age were collected for the biochemical experiments.

6.1.2 Methods

6.1.2.1 Preparation of Sample

The healthy *S. obtusifolia* leaves obtained from the field were dried in shade and then placed in hot air oven in controlled temperature of 45°-50°C for around 5 hours or till they were devoid of any moisture. The dried leaves were then powdered to use in extraction procedure. 15 gm of that leaf powder were taken for extraction procedure for a single experiment to find out any presence of the biomolecule rhein. Four such powered leaf samples were prepared for each of the 20 provenances for the extraction and analysis of rhein.

6.1.2.2 Extraction of Rhein

The powdered leaf of *S. obtusifolia* of 15 gm was taken in 200 ml flask. Then 100 ml of 25% methanol in water was poured to it and warmed to 45°C. This mixture was

stirred with 5 ml of concentrated hydrochloric acid. Then biphasic mixture was formed from the above mixture by adding 100 ml of toluene to it. The whole mixture was refluxed for 8 hours at 100°C temperature and then brought to room temperature. The crude drugs were removed by filtering the mixture. The filtrate obtained from the mixture was consisting of aqueous and organic compounds. The organic and the aqueous layers of the filtrate were separated out from one another by gravity. Both the aqueous layers and the filtered crude drug were washed with toluene once or twice for removing the presence of free anthraquinones, if any. All these toluene extracts obtained were combined with the organic layer. This organic extract was then partitioned with 75 ml of aqueous solution of sodium hydrogen carbonate till the solution turned pink color which was then acidified with 10% solution of hydrochloric acid (ice cold) until the pH of the litmus paper turns acidic. The precipitate was then collected up in ethyl acetate (75 ml) which was then washed 3-4 times with distilled water and dried over anhydrous sodium sulphate. The ethyl acetate layer was evaporated, and a dark yellow compound was obtained. It was then taken for TLC, HPLC and ESI MS studies.

6.1.2.3 TLC Procedures

TLC was done on pre-coated silica gel G60 F254 plate (E. Merck) with methanol and 0.5% acetic acid in the ratio of 85:15 and is used as the mobile phase. The standard rhein and the rhein samples extracted from the leaves of *S. obtusifolia* were spotted in separate points along the same horizontal line on the pre-coated silica gel plates before placing in the separation chamber. This experiment was conducted for the preliminary screening of the presence of rhein in the extracted samples.

6.1.2.4 HPLC Procedures

6.1.2.4.1 Chromatographic Conditions and Instrumentation

For the detection of rhein, HPLC analyses were conducted on isocratic system on UV detector under the optimized condition with predefined instrument specifications. The model number of the isocratic system was LC-10AdVP made by Shimadzu (Asia-Pacific) Pvt Ltd. The sample was kept at 10 µl by injector system. The analytical-shim-pack CLC-OCTA DECYL SILANE (ODS-C18) (46 nm 10 × 25 cm) was used as the main column, and shim-pack G-ODS (4 nm 10 × 1 cm) was used as the guard column. The other specifications were as follows: rate of flow was 1 ml/min, time for analysis was 15 minutes, detector wavelength was 254 nm, phase was stationary, cell temperature was 40°C, maximum pressure was 400 kgf/cm^2, sampling frequency was 1.5625 Hz, slit width was 1.2 nm, and bandwidth was 4 nm. To elute rhein, the mobile phase preparation was done by mixing acetic acid (0.5%) and HPLC grade methanol in 85:15 ratio.

6.1.2.4.2 Preparation of Standards

Rhein (Analytical Standard) was used as the standard for preparing standard solutions for the analysis which was received from Sigma-Aldrich. HPLC grade methanol was used for preparing standard rhein stock solutions. Same solutions were later utilized for preparing the calibration curve. The standard stock solution was rhein was prepared (1 mg/25 ml of methanol) with HPLC grade methanol and stored at room temperature. Working solutions with appropriate concentration were mader stock solutions through serial dilutions. For the preparation of calibration curve, concentrations of 5, 10, 20, and 40 µg/ml were prepared from stock solutions in HPLC grade methanol. All the standard solutions were passed through 0.5 micron syringe filter before injecting into HPLC system.

6.1.2.4.3 Preparation of Sample Solutions

HPLC grade methanol with 500 mg/l concentration in the stock solution was prepared to dissolve the extracted rhein derivative. A membrane filter (0.2 micron) with filtration assembly was used for filtering the solution in the mobile phase. Then, for removing the air bubbles, sonication was done for 10 minutes. All the sample solutions were prepared under identical conditions.

6.1.2.4.4 ESI MS

ESI MS is a powerful and reliable tool for determining the identity, quantity of protein molecules, rhein etc. (Yates, 1988; Roepstorff, 1997; Loo, 2000; Mayr, et al., 2006; Banerjee and Mazumdar, 2012). As the compound rhein is a highly polar biomolecule with high melting and boiling points, ESI MS study (Fenn, 2003) was adopted to determine the molecular weight that confirmed the presence of rhein in the extracted leaf samples of *S. obtusifolia*. Solvents used were mixtures of water and methanol with the nanomolar extract, as the system can detect the presence of the desired compound specifically and precisely at a very low concentration. The samples were extracted similarly.

ESI MS study was performed with the instrument Waters Xevo G2-XS-QTOF. The flow was kept at 5 µl/minute and positive ion mode ESI MS TOF application was done in which the spraying nozzle was kept at positive potential. The heated capillary temperature was set at 250°C. The optimized parameters setting was as follows: spray voltage was 2.54 kV; nitrogen gas flow was 598 liter/hour, time of fly (TOF) was 7602.

6.2 RESULTS AND DISCUSSION

The results obtained from TLC analysis, HPLC analysis and ESI MS studies are highlighted here.

6.2.1 TLC Analysis

The extracts isolated from the leaves showed positive response in thin layer chromatographic analysis. The standard and the extracted samples both applied in separate points showing bands along the same horizontal line which matches with the standard rhein after separation when viewed under short wavelength UV light and showed pink color with Borntrager's reagent, thus confirming the presence of rhein in the extracted samples on a preliminary basis.

6.2.2 HPLC Analysis

6.2.2.1 Chromatogram of Standard Rhein

The chromatograms obtained from the standard solutions are depicted in Figure (6.4 - 6.7). The retention time and the peak absorbance area at 254 nm were plotted. The average retention peak time of the standard rhein was found in the range of 6.942-7.195 minutes.

6.2.2.2 Calibration Curve

HPLC chromatogram provides information for both the identification of molecular presence and quantification of the concentration of the desired compound (rhein) of that solution. In respect of finding the quantity of rhein, chromatograms were generated from four different concentrations of standard rhein concentrations such as 5, 10, 20 and 40 µg/ml and the chromatograms are shown in Figure (6.4 – 6.7), respectively. The plotting of peak absorbance area against the concentration of standard rhein is showing

a linear trend (Figure 6.8). The regression line, coefficient of determination and correlation coefficient are also included in it.

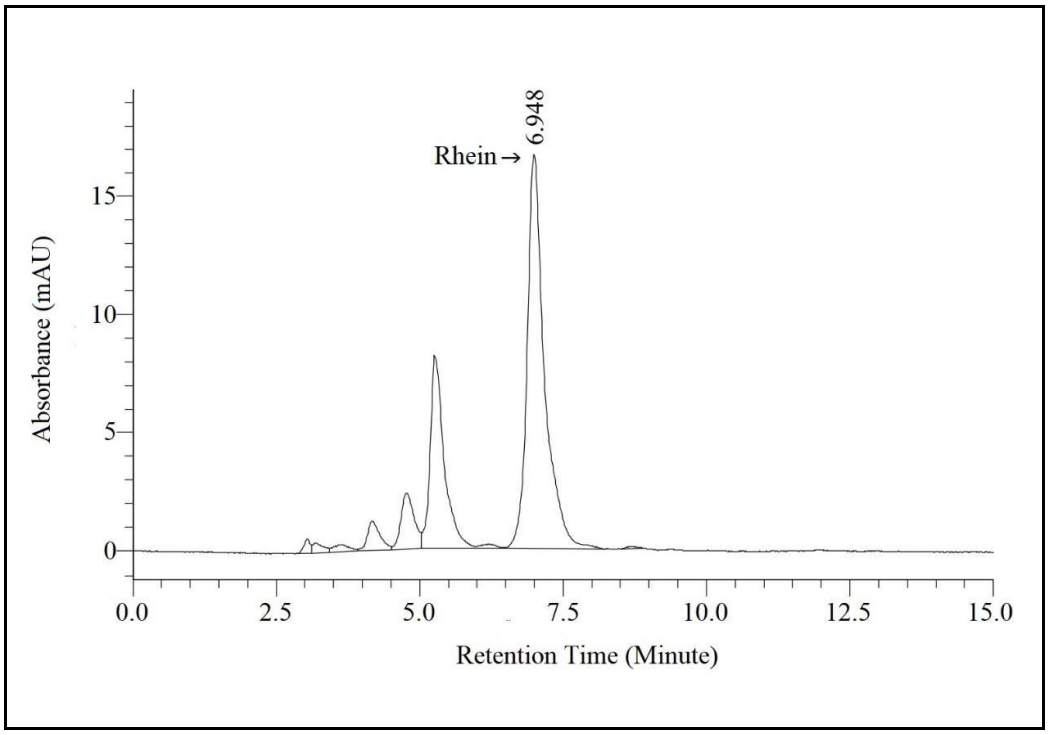

Figure 6.4 Chromatogram of standard rhein solution of concentration of 5 µg/ml

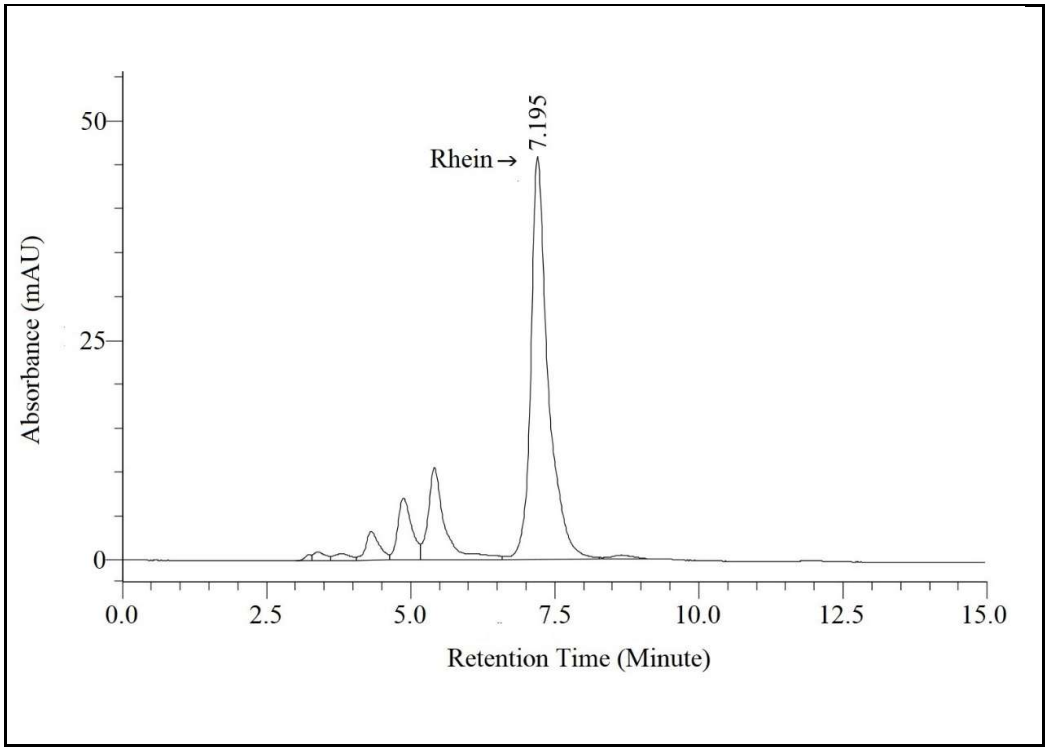

Figure 6.5 Chromatogram of standard rhein solution of concentration of 10 µg/ml

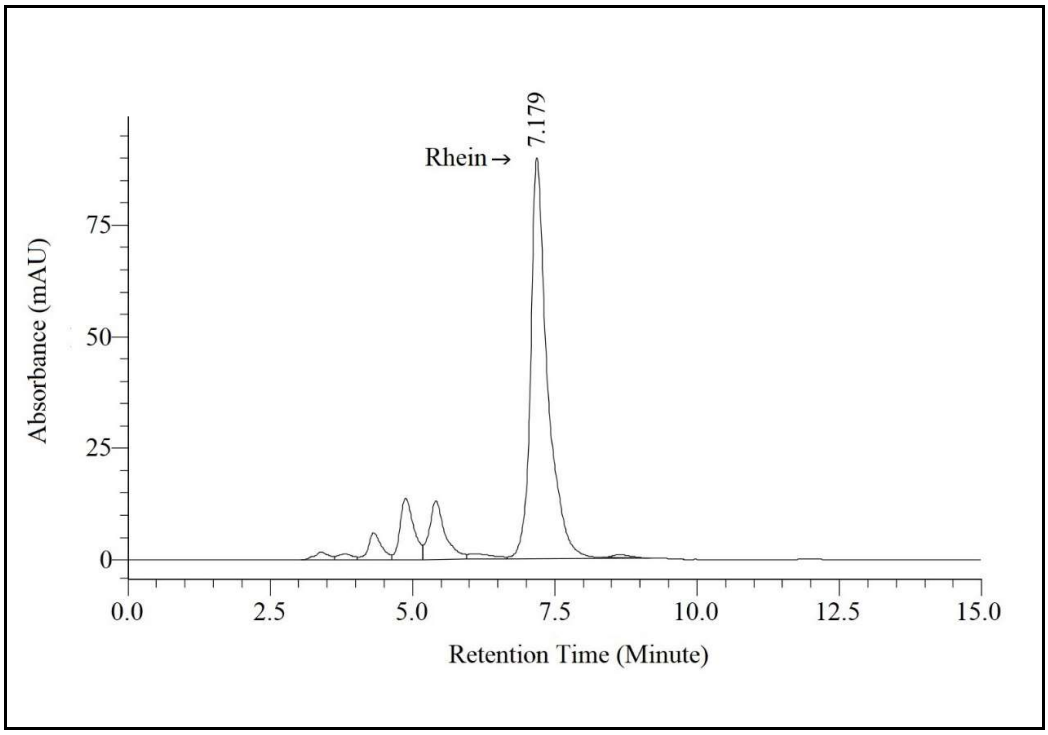

Figure 6.6 Chromatogram of standard rhein solution of concentration of 20 µg/ml

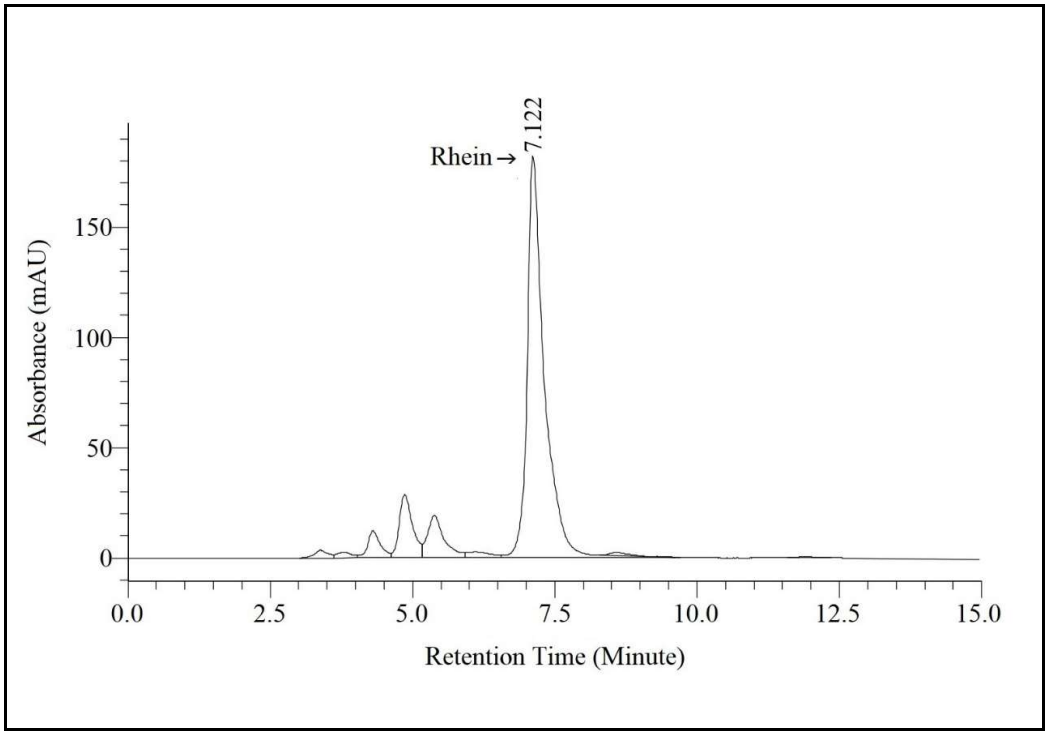

Figure 6.7 Chromatogram of standard rhein solution of concentration of 40 µg/ml

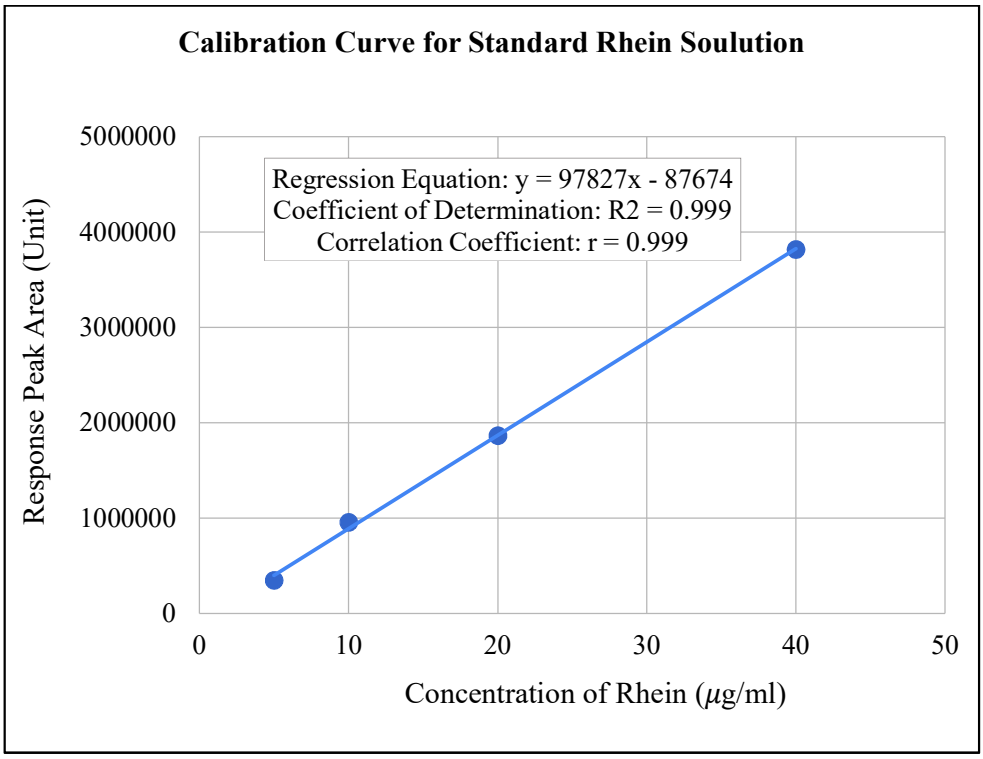

Figure 6.8 Calibration curve generated from chromatogram of standard rhein solution

The relationship between the solution concentration levels (µg/ml) and the corresponding response areas (mAU × Time) has been explored with the befitting straight line (Figure 6.8). The absorbances were measured for different serial dilutions such as 40, 20, 10 and 5 µg/ml. The regression was obtained as y = 97827 x - 87674, together with the value of coefficient of determination as 0.999. This indicates an excellent fit between solution concentration levels and response peak areas. Figure (6.9 - 6.28) confirmed the presence of rhein in all the chromatograms of the extracted sample solutions injected in the HPLC column. The high coefficient of determination ensures a better quantification of rhein with a higher confidence level for all the chromatograms obtained from the leaf samples of *S. obtusifolia* of different provenances shown in Figure (6.9 - 6.28).

6.2.2.3 Identification and Quantification of Rhein

The chromatograms of the extracted rhein from the leaf samples of *S obtusifolia* collected from different provenances were depicted in Figure (6.9 - 6.28) showing optimized mobile phase consisting of 0.5% acetic acid and methanol in 85:15 ratio, measured at 254 nm, eluted rhein with a retention time of around 7 minutes in all the samples of the species which coincides with the same precision and accuracy with that of the standard rhein. The extracted leaf samples of *S. obtusifolia* from different provenances determines the good separation of rhein. The chromatograms also revealed the determination of the amount of rhein present in each sample. For example, the chromatogram of the sample of the accession RPR (Raipur) provenance (Figure 6.27) showed a sharp peak at around 7 minutes that confirmed the presence of rhein. The quantity of rhein was calculated according to the area under the curve corresponding to the peak. For each sample, extraction and chromatogram were performed four times. Mean, SD and LSD calculations were done based on the sample size of four performances. Ample variation in the quantity of rhein for different leaf samples of *S. obtusifolia* was observed using the HPLC procedure. The sample of the accession RPR (Raipur) contained 31.94 µg rhein per gm of dried leaf that is the highest among all the samples. On the other hand, the sample of the accession LKP (Lakhsmikantapur) contained only 0.36 µg rhein per gm of dried leaf and that was found as the lowest amount among all the samples.

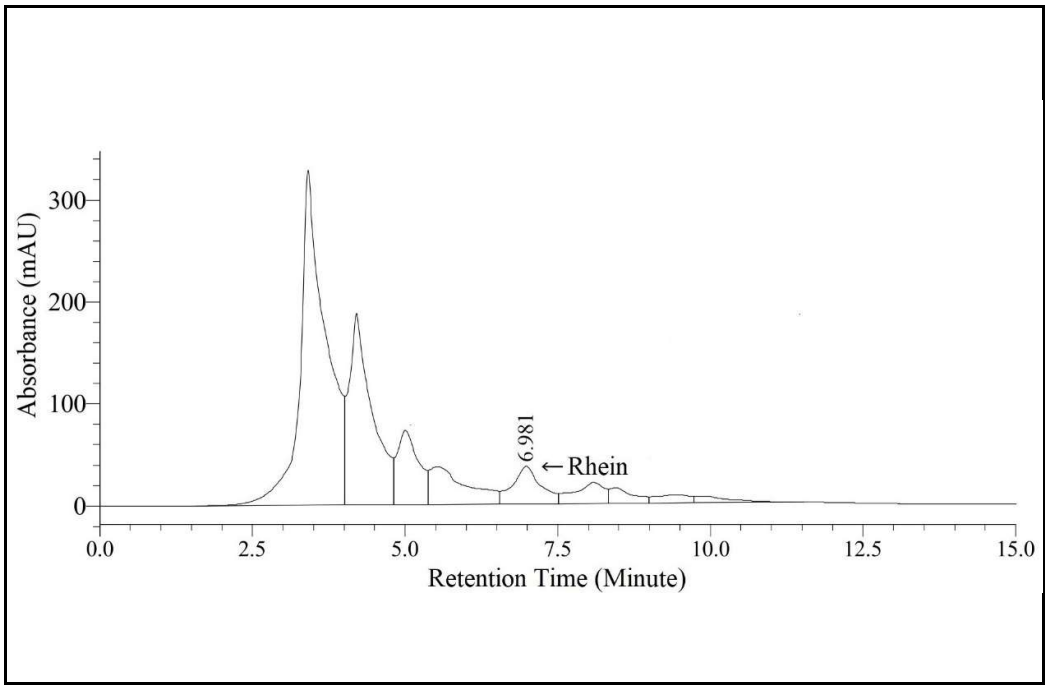

Figure 6.9 Chromatogram of extracted rhein solution from the leaves of the plants of the accession ASN (Asansol).

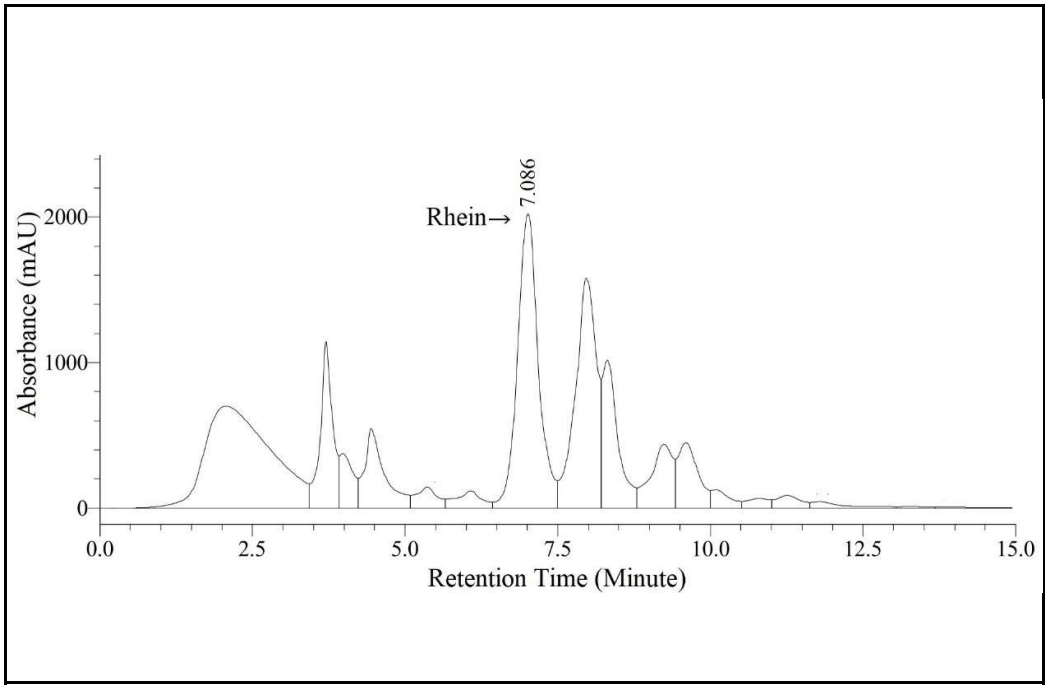

Figure 6.10 Chromatogram of extracted rhein solution from the leaves of the plants of the accession BDG (Bandhavgarh)

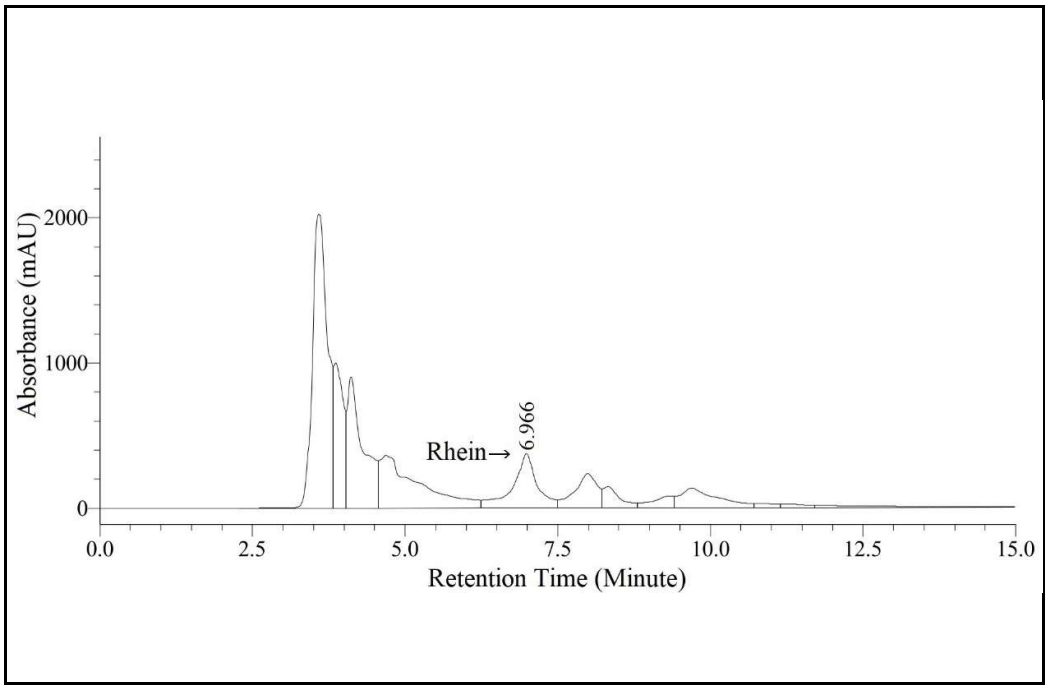

Figure 6.11 Chromatogram of extracted rhein solution from the leaves of the plants of the accession BNP (Bishnupur)

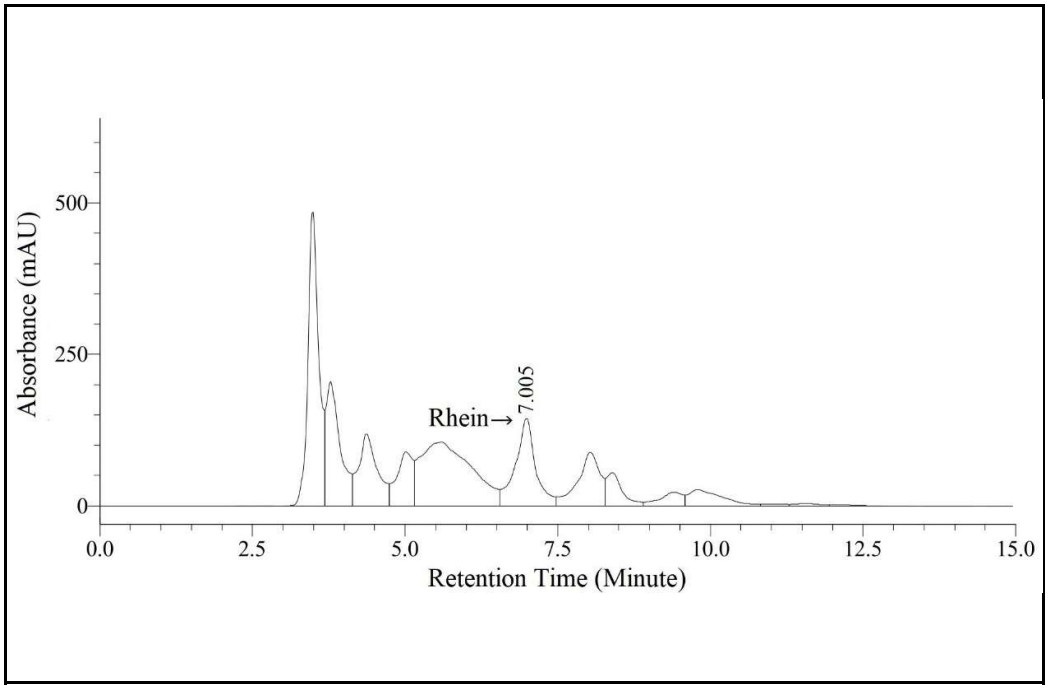

Figure 6.12 Chromatogram of extracted rhein solution from the leaves of the plants of the accession BPR (Bolpur)

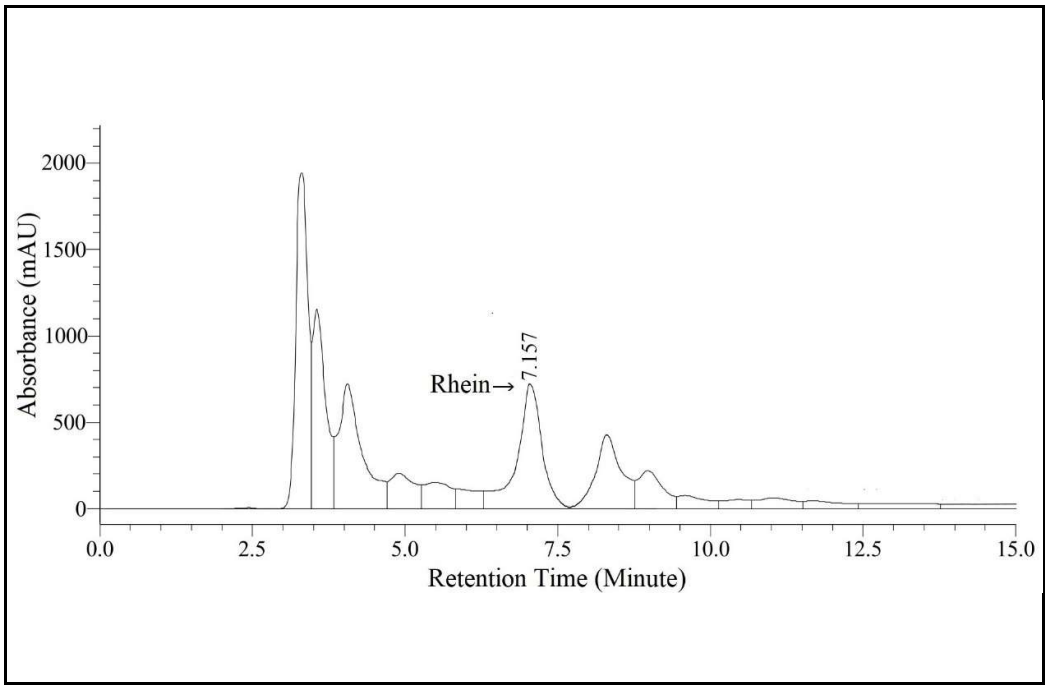

Figure 6.13 Chromatogram of extracted rhein solution from the leaves of the plants of the accession DHD (Dahod)

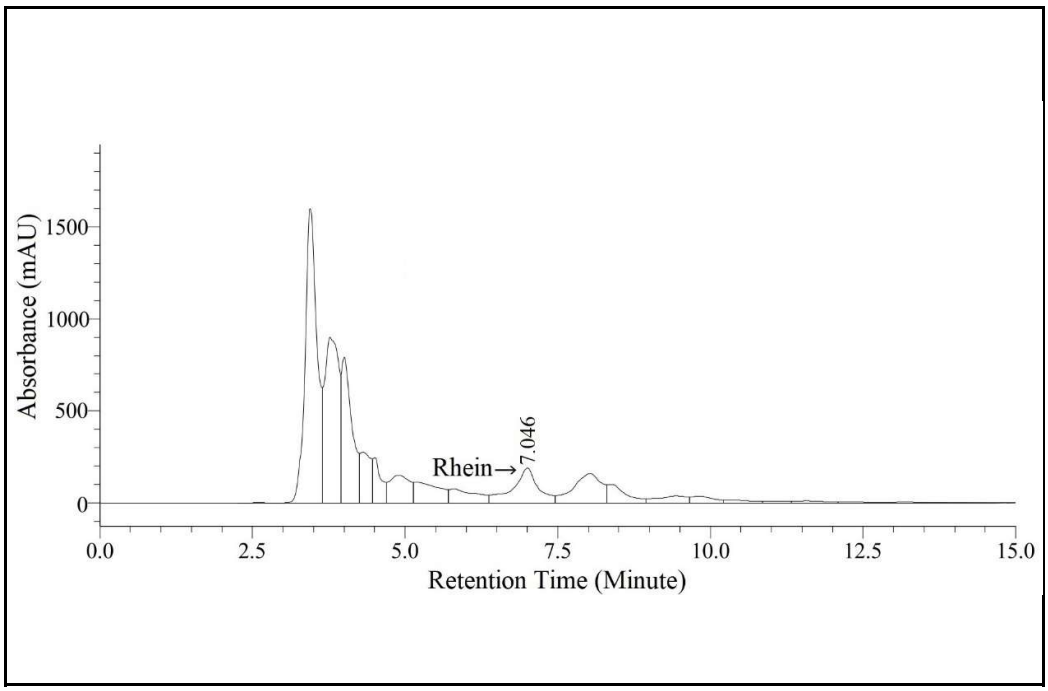

Figure 6.14 Chromatogram of extracted rhein solution from the leaves of the plants of the accession DHN (Dehradun)

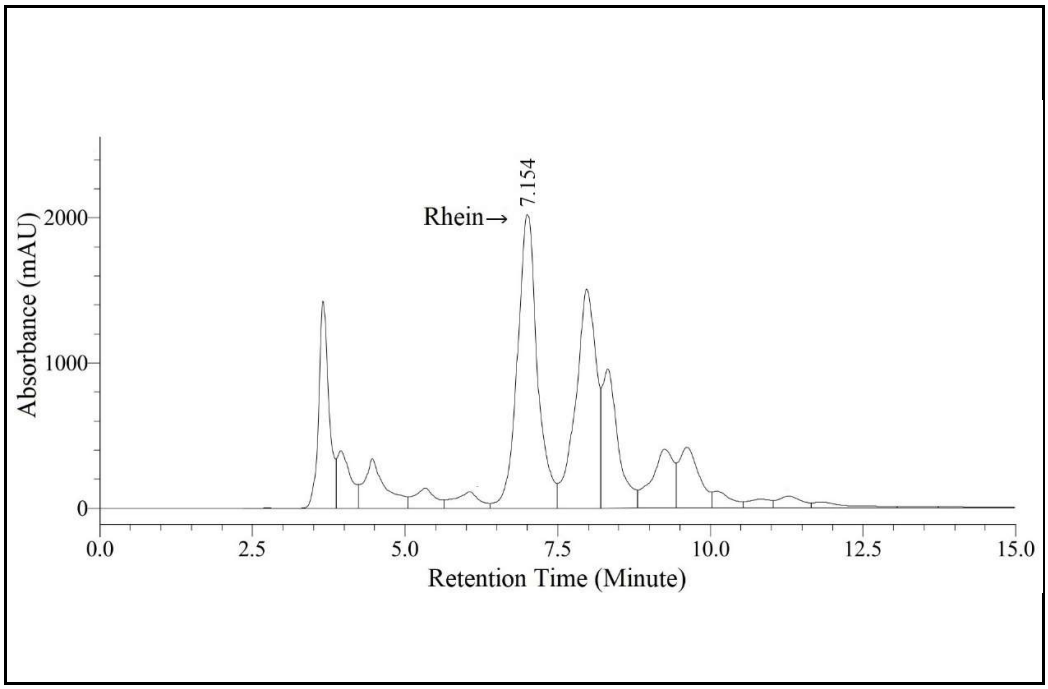

Figure 6.15 Chromatogram of extracted rhein solution from the leaves of the plants of the accession DNG (Devendranagar)

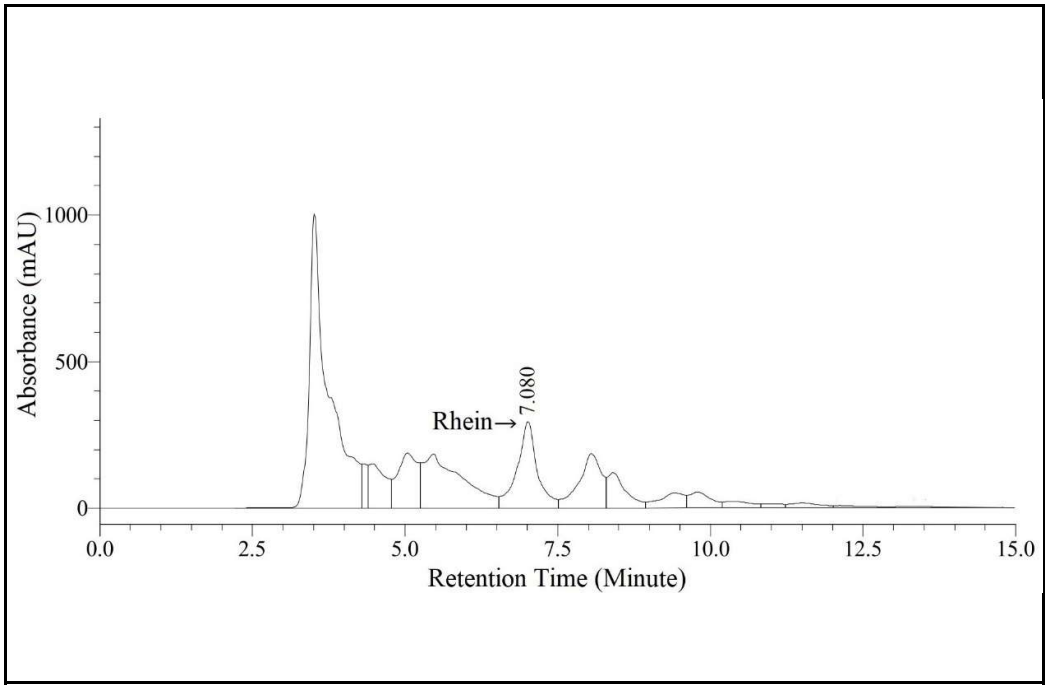

Figure 6.16 Chromatogram of extracted rhein solution from the leaves of the plants of the accession GBP (Gobindapur)

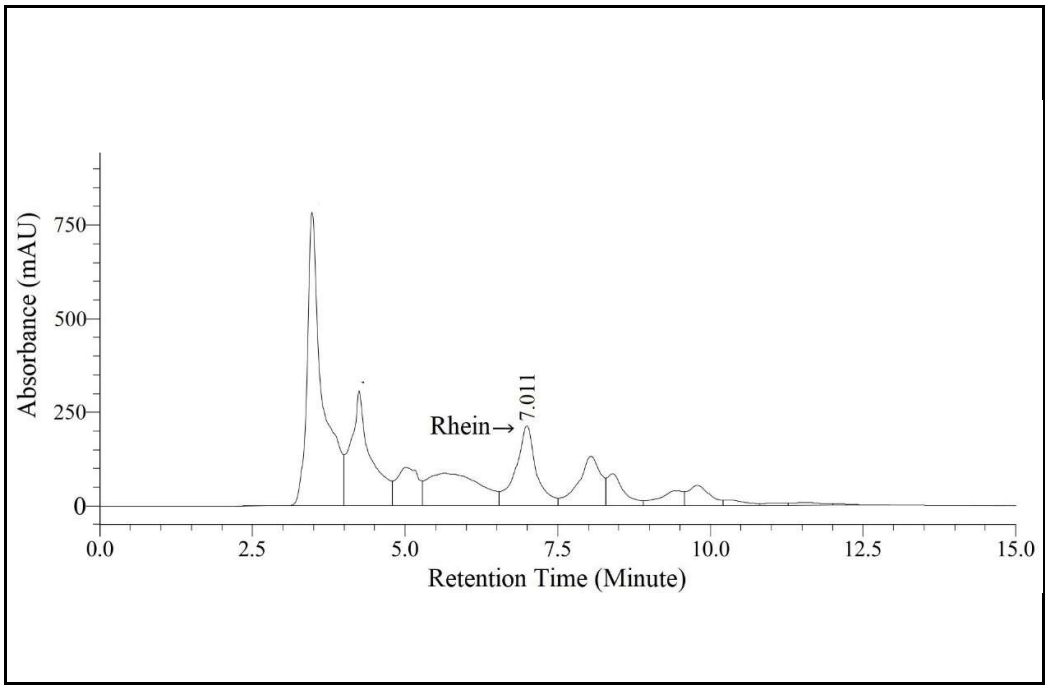

Figure 6.17 Chromatogram of extracted rhein solution from the leaves of the plants of the accession HDR (Haridwar)

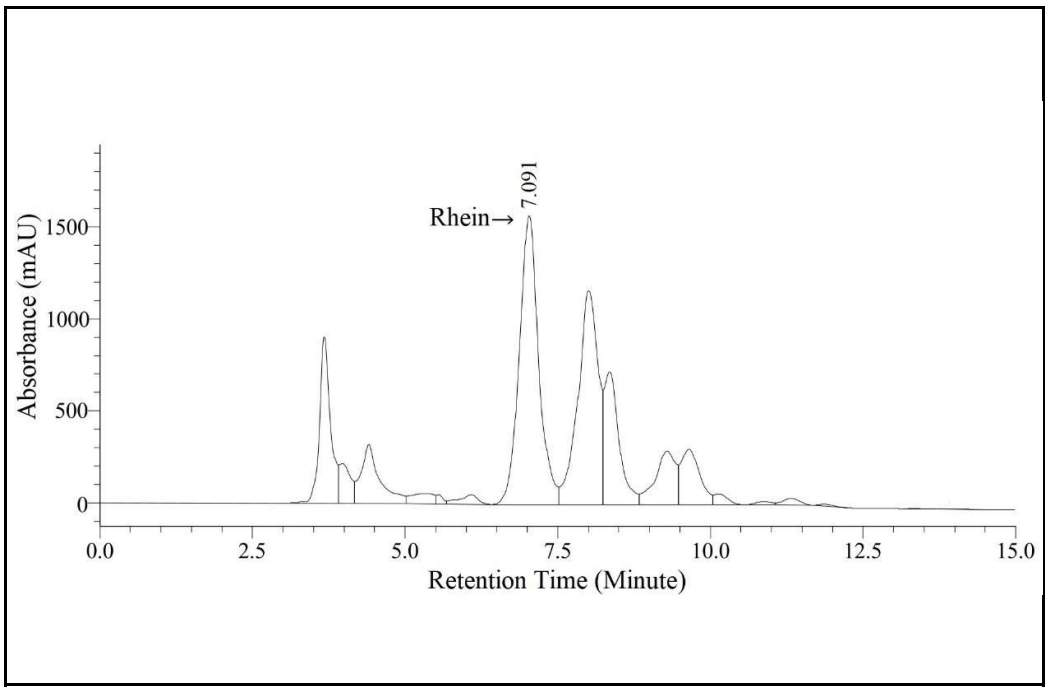

Figure 6.18 Chromatogram of extracted rhein solution from the leaves of the plants of the accession JBP (Jabalpur)

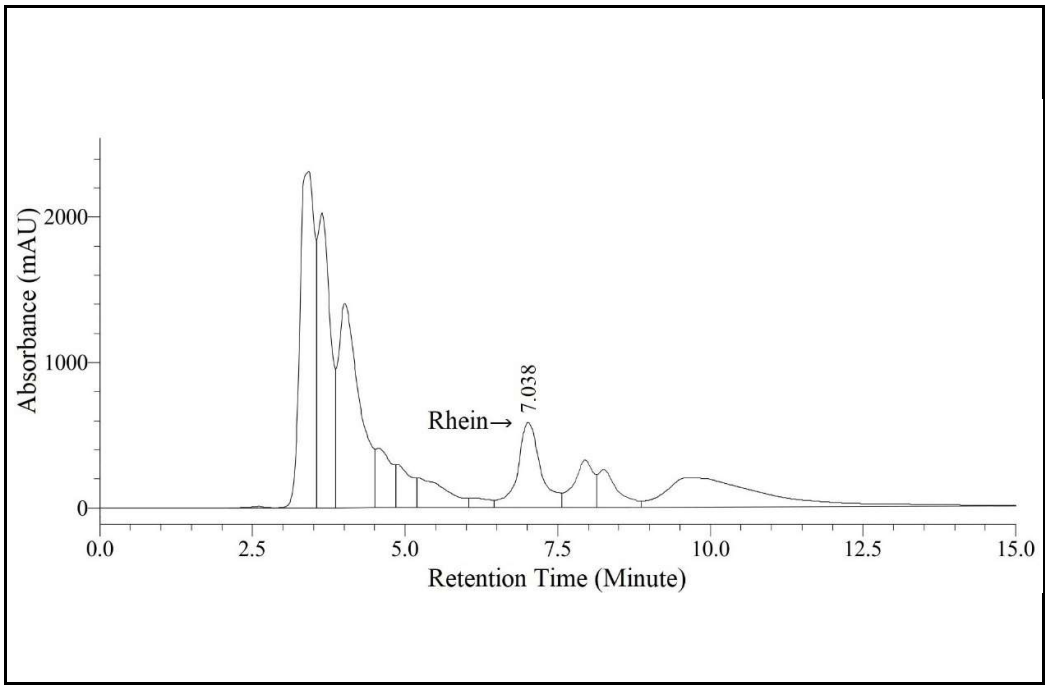

Figure 6.19 Chromatogram of extracted rhein solution from the leaves of the plants of the accession JPR (Jaipur)

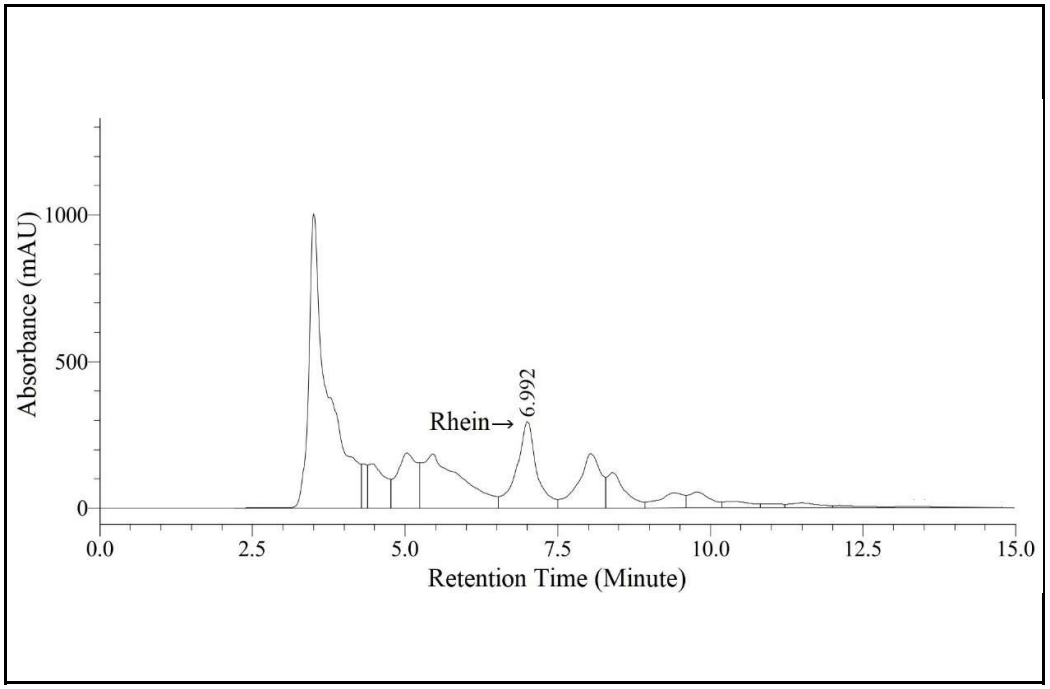

Figure 6.20 Chromatogram of extracted rhein solution from the leaves of the plants of the accession JRM (Jhargram)

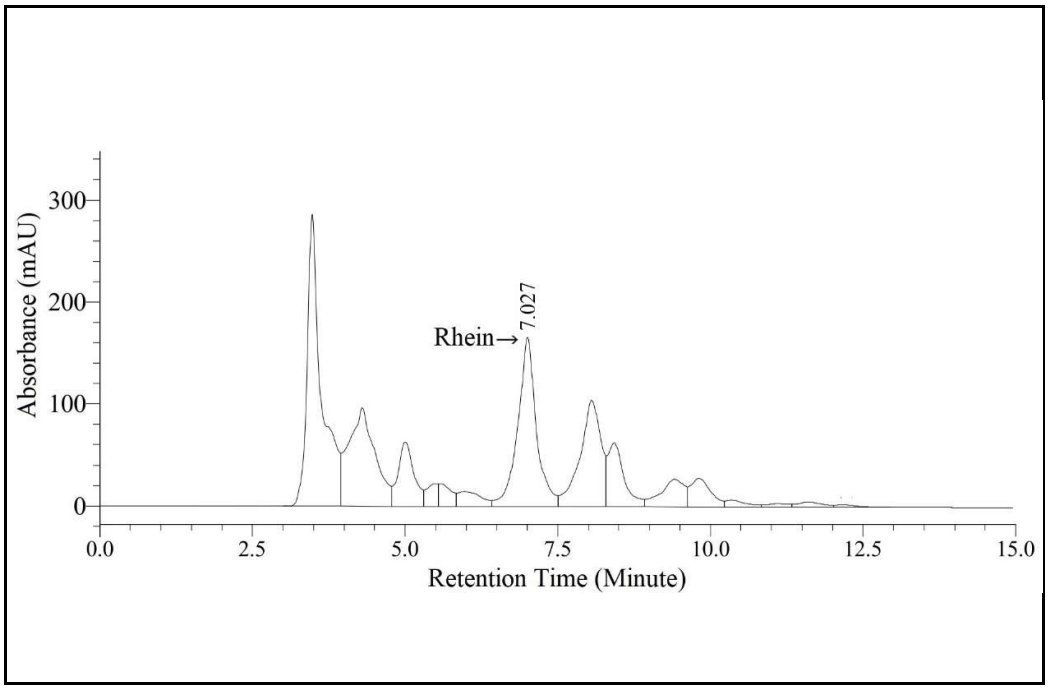

Figure 6.21 Chromatogram of extracted rhein solution from the leaves of the plants of the accession KLN (Kalyani)

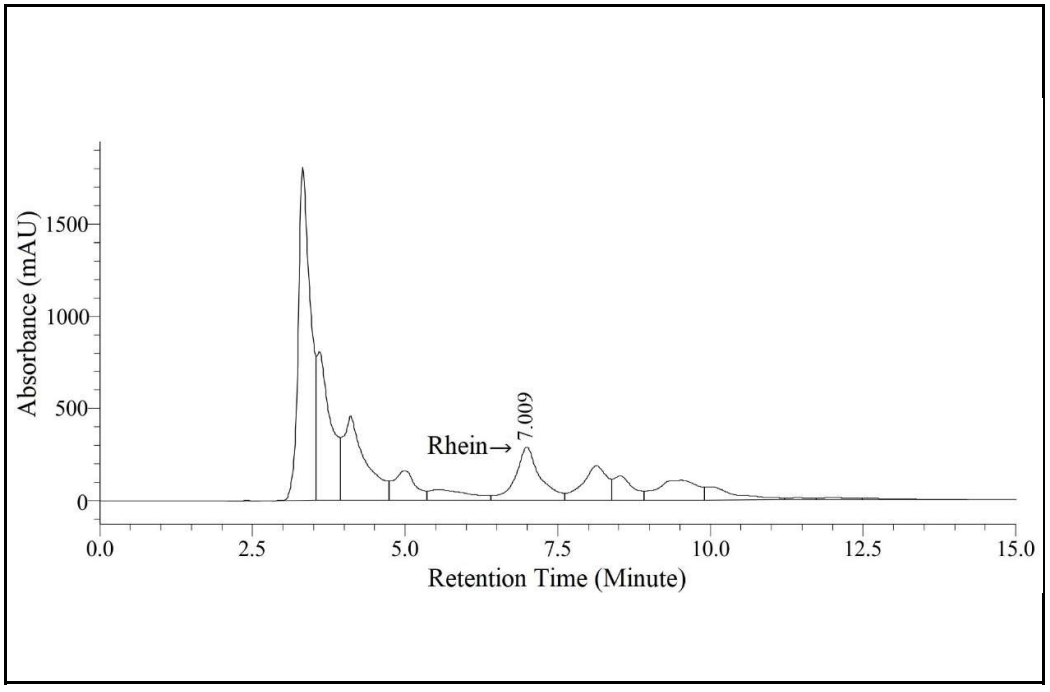

Figure 6.22 Chromatogram of extracted rhein solution from the leaves of the plants of the accession KPR (Kharagpur)

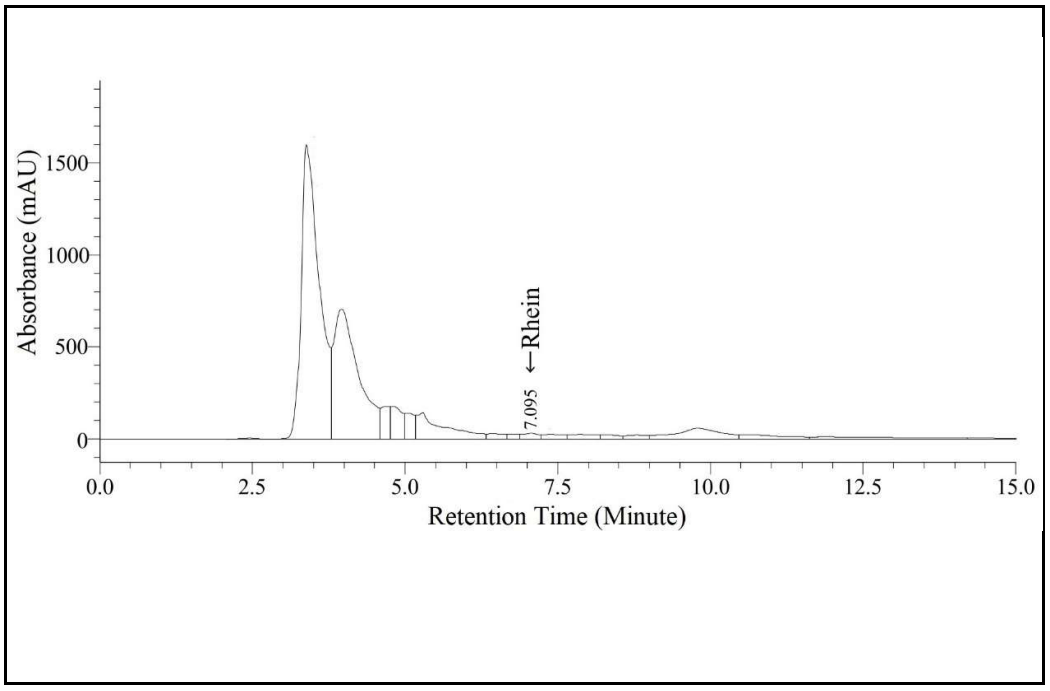

Figure 6.23 Chromatogram of extracted rhein solution from the leaves of the plants of the accession (Lakshmikantapur)

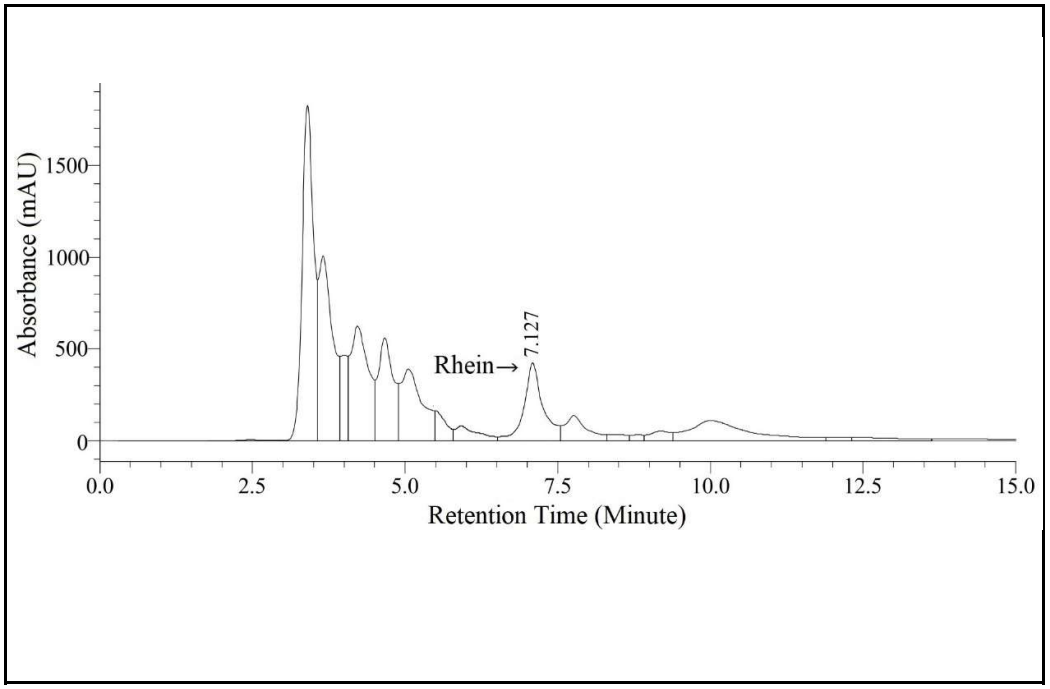

Figure 6.24 Chromatogram of extracted rhein solution from the leaves of the plants of the accession MGL (Mangalore)

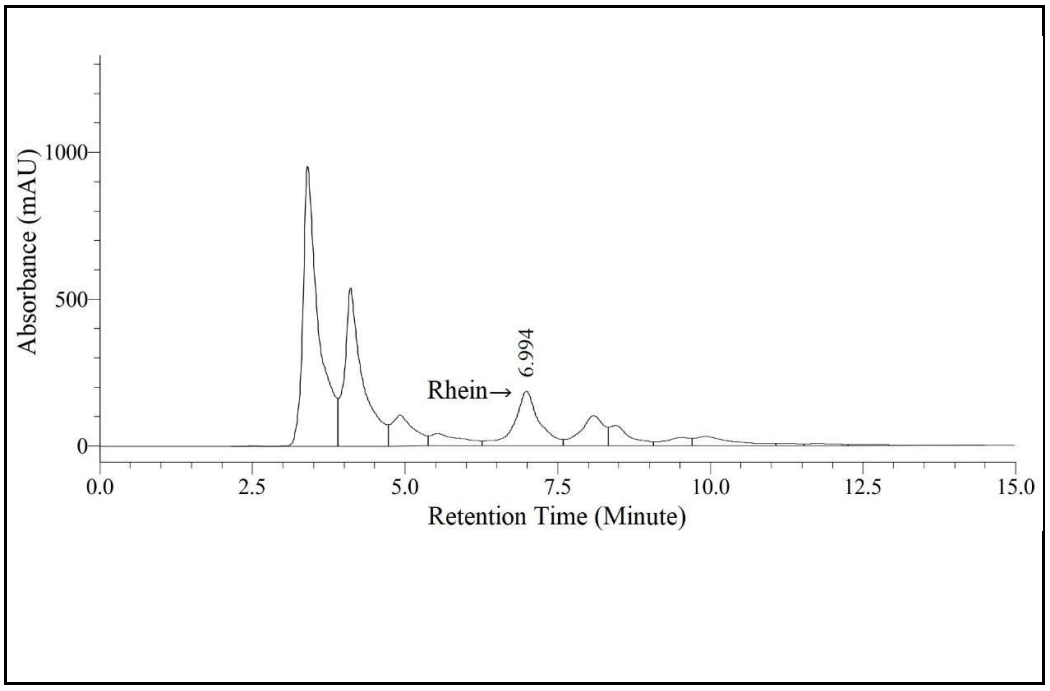

Figure 6.25 Chromatogram of extracted rhein solution from the leaves of the plants of the accession NGP (Nagpur)

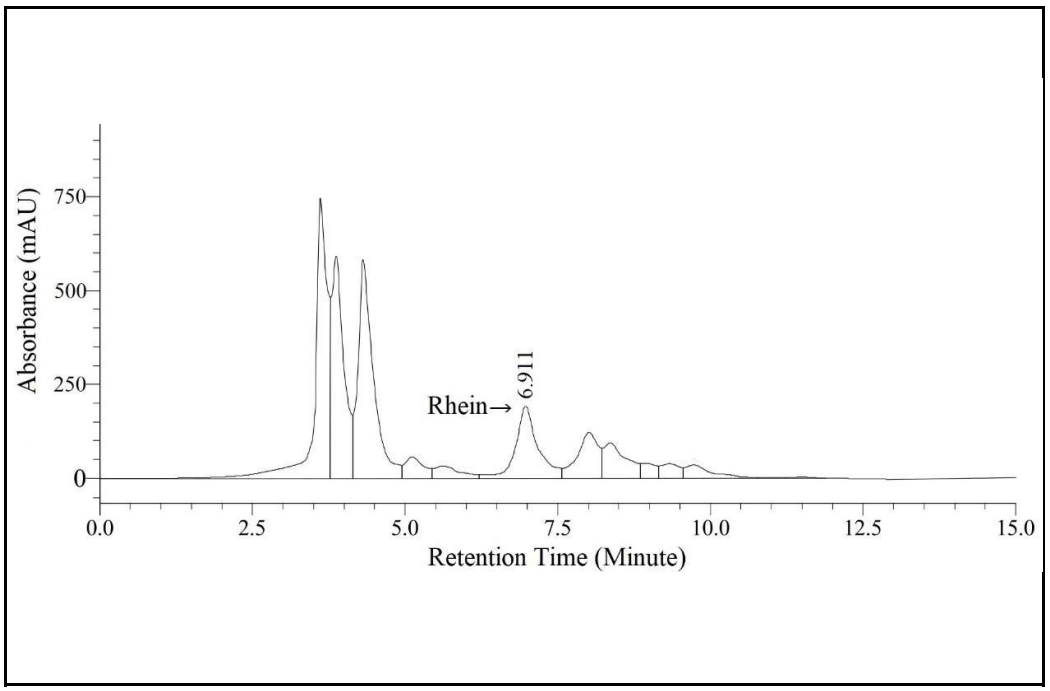

Figure 6.26 Chromatogram of extracted rhein solution from the leaves of the plants of the accession PRL(Purulia)

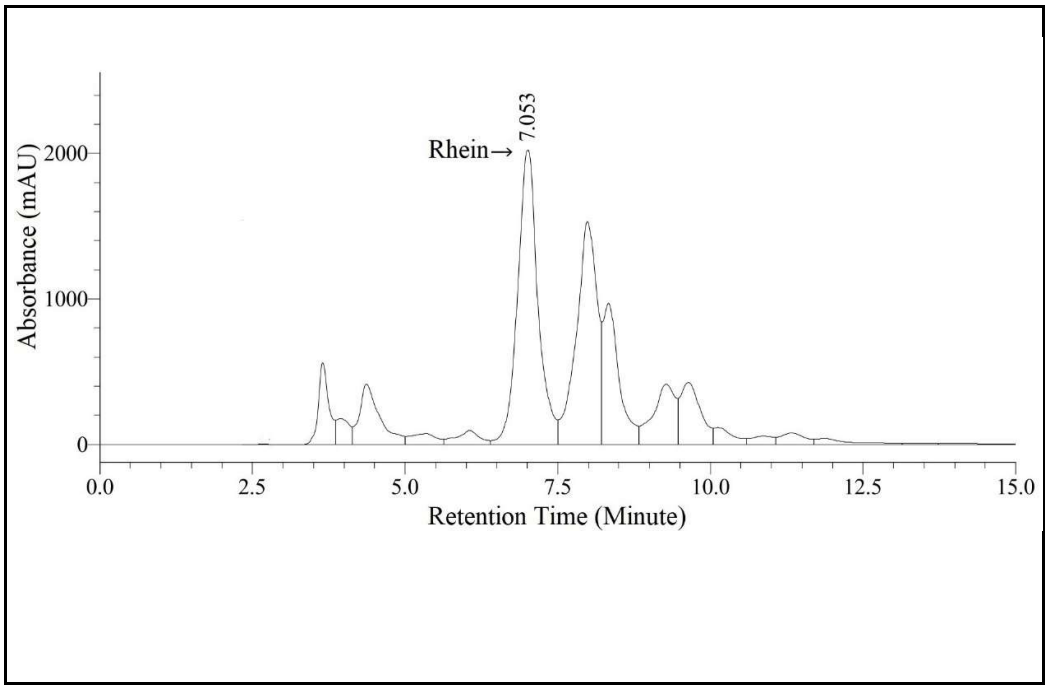

Figure 6.27 Chromatogram of extracted rhein solution from the leaves of the plants of the accession RPR (Raipur)

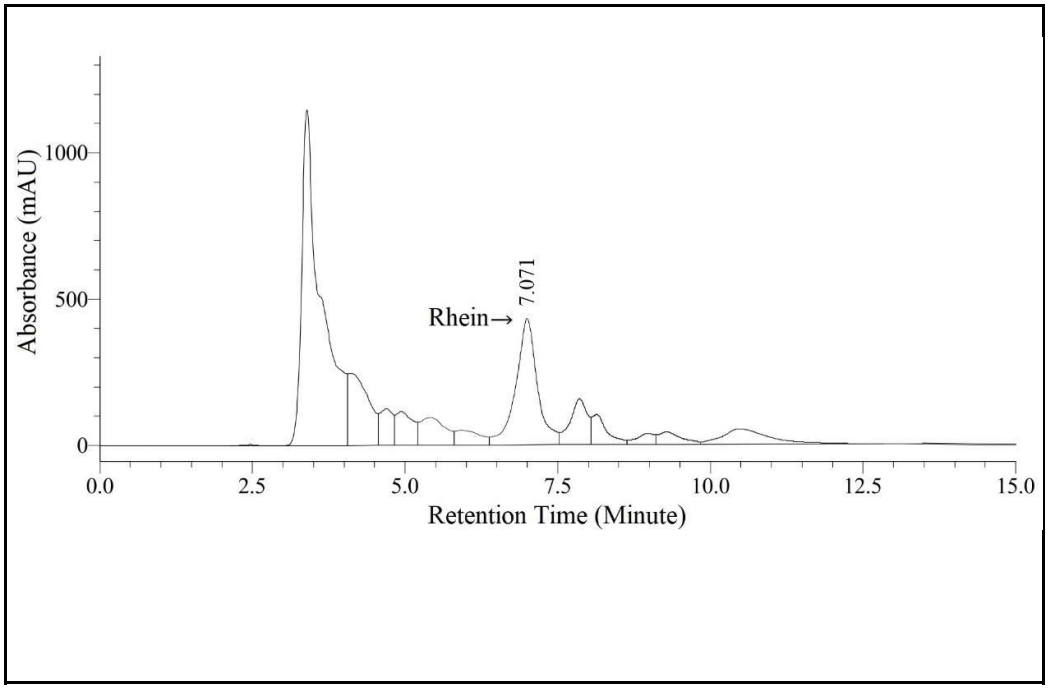

Figure 6.28 Chromatogram of extracted rhein solution from the leaves of the plants of the accession TNG (Tatanagar)

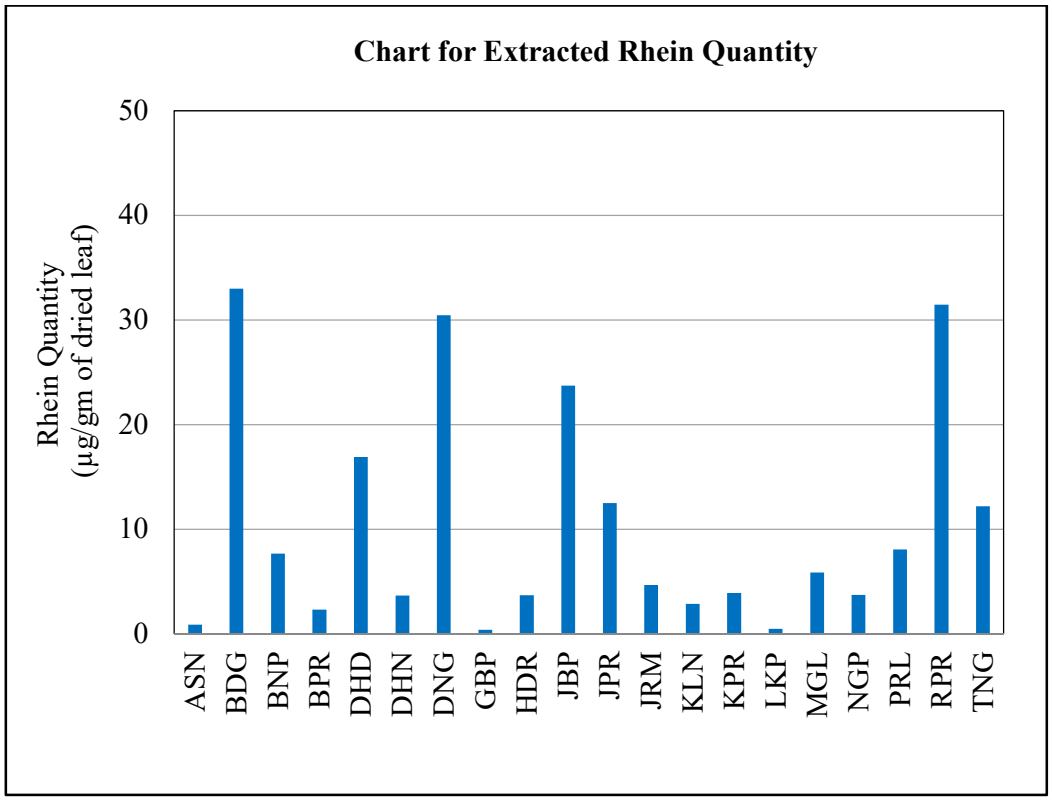

Figure 6.29 Chart for extracted rhein quantity in microgram per gram of dried leaf against the accessions.

The quantity of rhein in µg/mg (microgram per gram of dried leaf samples) of *S. obtusifolia* obtained from different provenances are shown in Figure 6.29 and the average peak area, average concentration (µg/ml), and SE of rhein quantity are summarized in Table 6.1

Table 6.1 Derivation of extracted rhein solution concentration from the calibration curve with rhein quantity per gram of dried leaf and it standard error.

Accession	Average Peak Area*	Average Rhein Concentration(µg/ml)	Rhein Quantity (µg/gm of dried leaf)	SE (Rhein Quantity)
ASN	1125306	12.40	0.83	0.03
BDG	46648143	477.74	31.85	1.58
BNP	11048512	113.84	7.59	0.59
BPR	3222603	33.84	2.26	0.12
DHD	23307782	239.15	15.94	1.01
DHN	4777839	49.74	3.32	0.24
DNG	43341761	443.94	29.60	1.09
GBP	442775	5.42	0.36	0.02
HDR	5067150	52.69	3.51	0.19
JBP	32985119	338.07	22.54	1.84
JPR	16635590	170.95	11.40	0.94
JRM	6258904	64.88	4.33	0.28
KLN	4011201	41.90	2.79	0.15
KPR	5242512	54.49	3.63	0.27
LKP	572481	6.75	0.45	0.02
MGL	8105183	83.75	5.58	0.27
NGP	5079238	52.82	3.52	0.22
PRL	11004677	113.39	7.56	0.44
RPR	46787482	479.16	31.94	2.03
TNG	17064350	175.33	11.69	0.75

Table 6.2 Differences between means and significance of pairwise comparisons of rhein quantity. The means are given in Table 6.1.

Acce-ssion	ASN	BDG	BNP	BPR	DHD	DHN	DNG	GBP	HDR	JBP	JPR	JRM	KLN	KPR	LKP	MGL	NGP	PRL	RPR
BDG	**31.02**																		
BNP	**6.76**	**24.26**																	
BPR	1.43	**29.59**	**5.33**																
DHD	**15.11**	**15.91**	**8.35**	**13.68**															
DHN	**2.49**	**28.53**	**4.27**	1.06	**12.62**														
DNG	**28.77**	2.25	**22.01**	**27.34**	**13.66**	**26.28**													
GBP	0.47	**31.49**	**7.23**	1.90	**15.58**	**2.96**	**29.24**												
HDR	**2.68**	**28.34**	**4.08**	1.25	**12.43**	0.19	**26.09**	**3.15**											
JBP	**21.71**	**9.31**	**14.95**	**20.28**	**6.60**	**19.22**	**7.06**	**22.18**	**19.03**										
JPR	**10.57**	**20.45**	**3.81**	**9.14**	**4.54**	**8.08**	**18.20**	**11.04**	**7.89**	**11.14**									
JRM	**3.50**	**27.52**	**3.26**	2.07	**11.61**	1.01	**25.27**	**3.97**	0.82	**18.21**	**7.07**								
KLN	1.96	**29.06**	**4.80**	0.53	**13.15**	0.53	**26.81**	**2.43**	0.72	**19.75**	**8.61**	1.54							
KPR	**2.80**	**28.22**	**3.96**	1.37	**12.31**	0.31	**25.97**	**3.27**	0.12	**18.91**	**7.77**	0.70	0.84						
LKP	0.38	**31.40**	**7.14**	1.81	**15.49**	**2.87**	**29.15**	0.09	**3.06**	**22.09**	**10.95**	**3.88**	2.34	**3.18**					
MGL	**4.75**	**26.27**	2.01	3.32	**10.36**	2.26	**24.02**	**5.22**	2.07	**16.96**	**5.82**	1.25	**2.79**	1.95	**5.13**				
NGP	**2.69**	**28.33**	**4.07**	1.26	**12.42**	0.20	**26.08**	**3.16**	0.01	**19.02**	**7.88**	0.81	0.73	0.11	**3.07**	2.06			
PRL	**6.73**	**24.29**	0.03	**5.30**	**8.38**	**4.24**	**22.04**	**7.20**	**4.05**	**14.98**	**3.84**	**3.23**	**4.77**	**3.93**	**7.11**	1.98	**4.04**		
RPR	**31.11**	0.09	**24.35**	**29.68**	**16.00**	**28.62**	2.34	**31.58**	**28.43**	**9.40**	**20.54**	**27.61**	**29.15**	**28.31**	**31.49**	**26.36**	**28.42**	**24.38**	
TNG	**10.86**	**20.16**	**4.10**	**9.43**	**4.25**	**8.37**	**17.91**	**11.33**	**8.18**	**10.85**	0.29	**7.36**	**8.90**	**8.06**	**11.24**	**6.11**	**8.17**	**4.13**	**20.25**

Table 6.2 shows the differences between mean values and significance of pairwise comparison of rhein quantity of different *S. obtusifolia* samples. The results were obtained using the Fisher's LSD method at 5% significance level. For the sample size of 4, the LSD value was obtained as 2.418. If the calculated LSD values is more than 2.418, then the difference is significant between the sample pairs in terms of rhein quantity. For example, the difference of mean values between the pair of samples of the accessions BDG (Bandhavgarh) and ASN (Asansol) was 31.02 (µg/gm of dried leaf), which is much higher than threshold value 2.418. This implies that *S. obtusifolia* of the accessions BDG (Bandhavgarh) and ASN (Asansol) are significantly different in terms of rhein quantity contained in the leaves. This was also evidenced from the measured amount of rhein of the said samples. The rhein quantity in the leaves of *S. obtusifolia* samples of the accession BDG (Bandhavgarh) was 31.85 (µg/gm of dried leaf) and that of the accession ASN (Asansol) was 0.83 (µg/gm of dried leaf). Also, the difference of mean values between the sample pairs of the accessions GBP (Gobindapur) and ASN (Asansol) was 0.47, which is less than 2.418. The respective rhein quantities were 0.36 (µg/gm of dried leaf) and 0.83 (µg/gm of dried leaf). Consequently, these two *S. obtusifolia* samples are not significantly different in terms of rhein quantity. The remaining values for difference between two samples in the table can be explained in a similar manner. The differences highlighted in bold text (Table 6.2) indicate that the respective pair of *S. obtusifolia* samples have significant differences in terms of rhein quantity.

Based upon the extracted rhein quantity (µg/gm of dried leaf), *S. obtusifolia* samples from twenty provenances have been clustered into few groups as presented in Figure 6.30. The samples of the accessions RPR (Raipur), BDG (Bandhavgarh), DNG (Devendranagar)

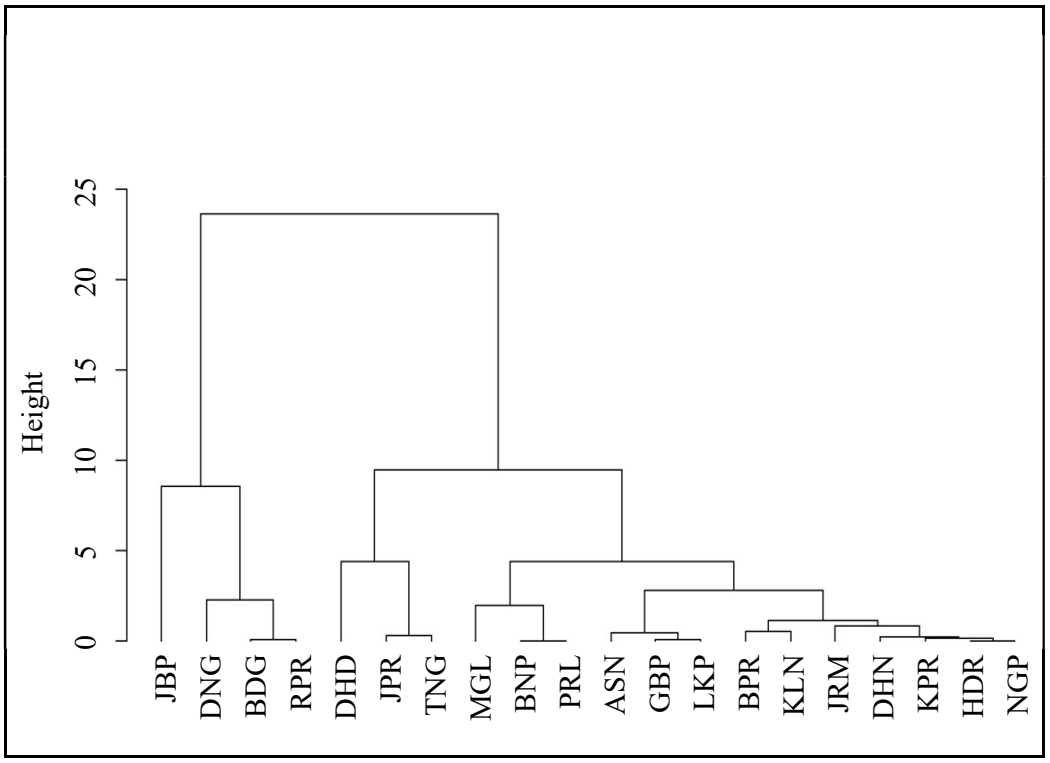

Figure 6.30 Clustering of *S. obtusifolia* of 20 accessions based on the quantity of rhein extracted from dried leaf

and JBL (Jabalpur) reveal a higher percentage of rhein. Within this group, the quantity of rhein in the samples of the accessions RPR (Raipur) and BDG (Bandhavgarh) are nearly equal. The respective quantities of rhein are 31.94 (µg/gm of dried leaf) and 31.85 (µg/gm of dried leaf). The samples of the accessions DNG (Devendranagar) and JBL (Jabalpur) contain slightly lesser quantity of rhein. They were quantified as 29.06 (µg/gm of dried leaf) and 22.54 (µg/gm of dried leaf), respectively.

The samples of the accessions DHD (Dahod), TNG (Tatanagar) and JPR (Jaipur) belong to the next cluster. The respective quantities of rhein are 15.94 (µg/gm of dried leaf), 11.69 (µg/gm of dried leaf) and 11.40 (µg/gm of dried leaf). The samples of the accessions BNP (Bishnupur), PRL (Purulia), and MGL (Mangalore) show similar characteristics in terms of content of rhein in the leaves of *S. obtusifolia*. Accessions

BNP (Bishnupur) and (PRL) Purulia are at par as the quantity of rhein in these two samples are almost equal. The respective quantities are 7.59 (µg/gm of dried leaf) and 7.58 (µg/gm of dried leaf). On the other hand, the sample of the accession MGL (Mangalore) contains a lesser quantity of rhein. The corresponding quantity is 5.58 (µg/gm of dried leaf).

The samples of the accessions BPL (Bolpur), KLN (Kalyani), JRM (Jhargram), HDH (Dehradun), KPR (Kharagpur), HDR (Haridwar) and NGP (Nagpur) belong to the third cluster. Four members of this cluster – HDH (Dehradun), HDR (Haridwar), NGP (Nagpur) and KPR (Kharagpur) have shown similarity with respect to the extracted quantity of rhein. The respective quantities of rhein are 3.32 (µg/gm of dried leaf), 3.51 (µg/gm of dried leaf), 3.52 (µg/gm of dried leaf) and 3.63 (µg/gm of dried leaf). The sample of the accession JRM (Jhargram) contains slightly higher quantity of rhein which is 4.33 (µg/gm of dried leaf). The other two samples of the accession KLN (Kalyani) and BPR (Bolpur) contain the second lowest and lowest quantity of rhein respectively, among the seven members of the group. The respective quantities are 2.79 (µg/gm of dried leaf) and 2.26 (µg/gm of dried leaf).

The remaining three samples for the accessions ASN (Asansol), GBP (Gobindapur) and LKP (Lakshmikantapur) contain an almost insignificant quantity of rhein and hence, belong to a separate cluster. The corresponding rhein quantities are 0.83 (µg/gm of dried leaf), 0.45 (µg/gm of dried leaf) and 0.36 (µg/gm of dried leaf).

6.2.2.4 Confirmation of Rhein in ESI MS spectrum

The ESI MS spectrum obtained from the leaf extract of *S. obtusifolia* is shown in Figure 6.31. A sharp peak at 284.0228 was detected in the spectrum which is very close to the

molecular weight of rhein. The molecular weight of rhein is 284. This spectrum strongly supports the presence of rhein in the considered samples.

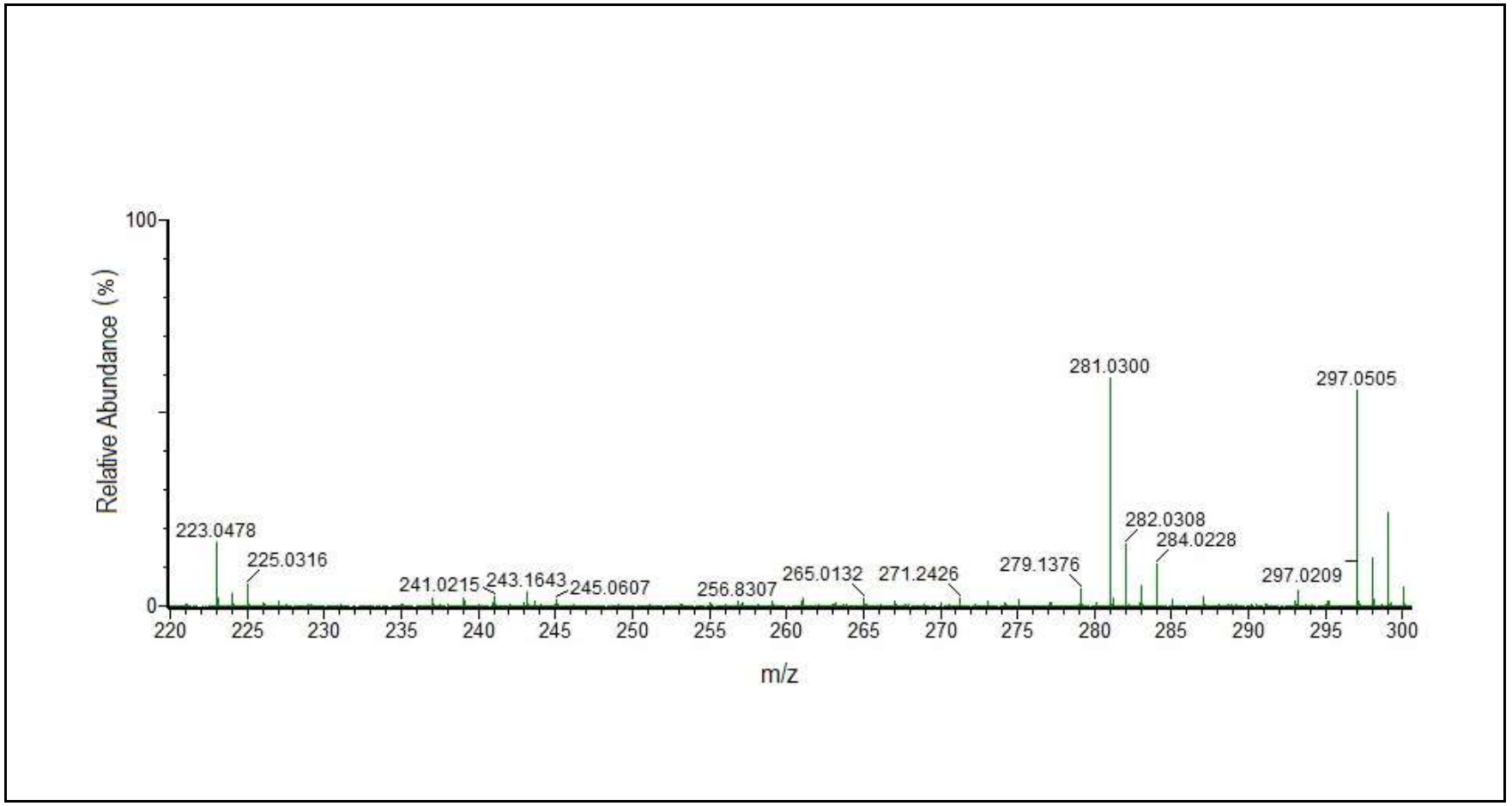

Figure 6.31 ESI MS spectrum of *S. obtusifolia* sample solution

6.2.3 Discussions

Characterization and quantification of rhein from the leaves of twenty provenances of *S. obtusifolia* with the aid of TLC, HPLC and ESI MS analyses have been dealt with in this chapter. TLC analysis of the standards, as well as the samples helped primarily in confirming the presence of rhein in the species as shown in earlier work as well (You et al. 2013). HPLC experiments were performed to identify and estimate the amount of rhein in *Senna alata* (Panichayupakaranant et al., 2009). Comparisons and the study of variations of sennosides in the pod, leaf, and flower of two types of *Senna alexandrina* Mill. from five locations in Ethiopia were studied reflecting the intraspecific diversity among the species (Abdo, 2017). Similarly, rhein was extracted, isolated, and quantified from the twenty different provenances for *S. obtusifolia.*

The presence of rhein was reported in seeds of *S. obtusifolia* by Yang et al., (2015). However, the detailed study of isolation, exaction and quantification of rhein was lacking. The presence of rhein in the leaf samples of *S. obtusifolia* in all the twenty provenances collected was first reported in the study of Nandi and Maiti, (2018). The study also established a subtle variation in the amount of rhein within the considered twenty samples. Difference in the quantity of rhein in the samples, ranging from 0.36 (µg/gm of dried leaf) to 31.94 (µg/gm of dried leaf), indicates a considerable extent of intraspecific variation among twenty provenances of the species. A reasonably good amount of rhein, approximately 32 µg per gram of dried leaf tissue was recorded for the accessions DNG (Devendranagar), BDG (Bandhavgarh) and RPR (Raipur). The leaf samples of the accessions RPR (Raipur), BDG (Bandhavgarh), DNG (Devendranagar) and JBP (Jabalpur) contain comparatively higher quantity of rhein and are placed in the same samples bucket.

Rhein content in the leaf extracts of *Cassia fistula* collected from warm summer areas such as central and north east areas of Thailand showed higher quantity of rhein than the samples from southern parts (Sakulpanich and Gritsanapan, 2009). Similar trend has also been witnessed here, as the provenances from the warmer areas showed higher quantity of rhein in comparison to the provenances of relatively lower range of temperature during summer.

The ESI MS study finally confirmed the presence of rhein from the extracted leaf samples of the species by determining the molecular weight of the sample from the spectrum. Similar studies regarding the ESI MS studies have been performed by the researchers like Ye et al., (2007) and Loo et al., (2000). LSD values of rhein calculated from the species showed a significant variation of rhein among different pairs of samples. The dendrogram constructed here is based on the quantity of rhein present in the leaf samples highlighting the relative distance between the provenances, based on this biochemical trait. The *S. obtusifolia* samples of the accessions such as RPR (Raipur), BDG (Bandhavgarh), DNG (Devendranagar) and JBP (Jabalpur) containing the higher quantity of rhein were grouped together. Similarly, the samples of the accessions such as ASN (Asansol), BPR (Bolpur), KLN (Kalyani), LKP (Lakhsmikantapur), and GBP (Gobindapur) containing lesser quantity of rhein were grouped separately. In this respect, they are characteristically similar and closely related to each other in terms of quantity of rhein.