Chapter 3

Extraction, Purification, and Characterization of Polyphenols from Faba Beans

3.1. Introduction

Faba bean (*Vicia faba* L.) is rich in nutrients such as phenolics, proteins, complex carbohydrates, dietary fiber, choline, lecithin, minerals and other secondary metabolites (Abu-Reidah et al., 2014; Zhou et al., 2013). Recently, many advanced techniques have been helped in purification and isolation of bioactive compounds from plants (Joana Gil-Chávez et al., 2013). The appropriate method for screening of phytochemicals can select the plant materials for bioactivity such as antidiabetic antioxidant, antibacterial, or cytotoxicity (Spainhour, 2010). The sequential extraction process had been used to isolate and purify the active compounds that are responsible for the bioactivity. Column chromatographic techniques were used for the extraction and purification of the bioactive compounds. Modern techniques like HR-LCMS accelerate the process of purification of the bioactive molecule or chemo-profiling of plant materials (Kale et al., 2018). The techniques like TLC, column chromatography, FTIR, HPLC, HR-LCMS, mass spectroscopy were used extraction, purification and characterization of polyphenols from faba beans. Chapter 3 mainly describes the extraction, chemo-profiling, and characterization of polyphenols from faba beans by different techniques such as TLC, FTIR, HR-LCMS, mass spectroscopy.

3.2. Experimental

3.2.1. Chemicals and reagents

Polyphenols standards were obtained from Himedia laboratories (Himedia). All the solvents like methanol, acetone, ethanol and chloroform and other reagents were analytical grade and water used was distilled.

3.2.2. Seed material

Experiments were carried out on seed extracts of faba bean (*Vicia faba* L.) which was of Vikrant variety procured from National Bureau of Plant Genetic Resources (NBPGR) New Delhi, India. Faba bean **(***Vicia faba* ***L.*****)** is normally cultivated in a subtropical and temperate region and it comes under the fourth most important legume crop in the world, next to dry beans, dry peas and chickpea(Singh et al., 2013). Generally, faba bean (*Vicia faba* L.) is utilized as a legume.

3.2.3. Preparation of seed extract

Tap water, distilled water, and 70% ethanol were used stepwise for removal of all contaminant including dust particles from faba bean seed (*Vicia faba* L.). It was dried in an oven at 50^0C for 24 hrs and crushed to fine powder. Finely powdered bean sample (40 g) was dissolved in 100 ml of solvents for sequential extraction process from nonpolar to polar one (hexane, chloroform, methanol, ethanol, acetone, and water) and kept for 48 hrs under shaking at 130 rpm and temperature (40^0C). Extract samples were filtered after every step and placed into rotary vacuum evaporator according to the boiling point till all the solvent got evaporated. Dried concentrated extracts of polar and nonpolar solvents were stored in a refrigerator at 4ºC and further, it was used for purification, characterization and therapeutic application purpose.

3.2.4. Phytochemical screening

Phytochemical compositions of the seeds extract were determined using the methods described by Trease and Evans, 2002 (Evans, 2002). Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow colored precipitate indicates the presence of alkaloids. Extracts were diluted with distilled water up to 10 ml and mixed properly. Formation of foam indicates the presence of saponins. 0.25 gm of the extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicated the presence of saponins. Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black color indicated the presence of phenols or tannins. 1% gelatin solution containing sodium chloride was added to the extract. Formation of white precipitate showed the presence of tannins. Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow color, which later became colorless on the addition of dilute acid, indicated the presence of flavonoids. 5 ml (1 mg/ml) of the fraction with few drops of chloroform was added and then 3 ml of concentrated H_2SO_4 was added in the mixture. Change of reddish brown color revealed the presence of terpenoids. The extracts were treated with few drops of conc. Nitric acid. Formation of yellow color marked the presence of proteins. The Test solution was mixed with few drops of Benedict's reagent (alkaline solution containing cupric citrate complex) and boiled in a water bath. The formation of reddish brown precipitate to showed a positive result for the presence of carbohydrate.

3.2.5. Thin layer chromatography

The identification of polyphenols in the various seed extracts was performed with the separation analysis (Thin layer chromatography). Chloroform, ethyl acetate, and formic acid were taken as mobile phase in the ratio of 5:4:1 in 50 ml distilled water

(Medić-Šarić et al., 2004). Spots on TLC plate was developed with 1% ferric chloride solution

3.2.6. Fourier transform infrared spectroscopy (FTIR)

Alpha ATR-FTIR, Bruker, USA was used for the FTIR analysis. Samples were scanned in the wave number range of 400–4000 cm^{-1}. FTIR study revealed that the chemical bonds absorbed radiation in the middle infrared (MIR) region between 400 and 4000 cm^{-1}, and each functional group of a molecule had specific absorption frequencies in the IR spectrum. Fourier transform infrared spectra (FTIR) can generally use to determine the structure of biological composition and structure of the molecular functional group. It can be determined by analyzing the position, width, and intensity of acquired spectra in a complex biological system based on individual algorithms.

3.2.7. Chemo-profiling of phenolics compounds by RP-HPLC–ESI-Q-TOF-MS

Chemo-profiling of faba bean seed extract was done by Reversed-Phase High-Performance Liquid Chromatography coupled to Electrospray Ionization Quadrupole Time-of-Flight Mass Spectrometry (RP-HPLC–ESI-Q-TOF-MS). Instrument model specification detail was 1290 Infinity UHPLC System, B1260 infinity Nano HPLC with Chipcube, 6550 iFunnel Q-TOFs (Aligent technologies, USA).This instrument was equipped with an Agilent C18-Hypersil gold column (100 x 2.1mm, 3 μm). Acidified water (0.1% formic acid, v/v) and 90 % acetonitrile +10% water +0.1% formic acid was taken as mobile phase. The flow rate was maintained at 0.3 mL min^{-1}. Separation was achieved at 45 °C with a multi-step linear gradient elution program in which phase B changed from 0 to 5% in 2 min, from 5 to 95% in 25 min, and from 95 to 5% in 5 min. The UV-Vis spectra were recorded from wavelength (190 to 400 nm). Parameters for analysis were set using negative ion mode with spectra acquired over a mass range from

m/z (125 to 1200). The optimum values of the ESI-MS parameters were: nozzle voltage, +10kV; drying gas temperature, 190 °C; drying gas flow, 9.0 L/min; nubilizing gas pressure, 35 psi, scan rate 1 (spectra/second) and gas flow rate 13L/min.

3.2.8. Column chromatography

Gradient fractionation of seed extract (from the acetone seed extract) was performed by eluting the column (silica gel,280-300 mesh) with toluene: ethyl acetate: methanol (4:3:3) and fractions were collected. Further, it was dried and concentrated by lypholizer or rotary vacuum evaporator. Major polyphenols fraction has designated as D1 (05) (gallic-acid), D2 (08) (catechin) from fractions no. 5 and fraction no.8 respectively.

3.2.9. HPLC study condition

C18 column and the Waters HPLC apparatus with photodiode array detector was used for method development. Millipore filtration assembly was employed for mobile phase filtration. The sample (aqueous extract) and polyphenols standard(a mixture of gallic acid, ellagic acid, catechin, and epicatechin) were filtered by syringe filter (0.45 mm). A mixture of acetonitrile (A), H_2O (B), (0.1% acetic acid) in a ratio of (60:40 v/v) was filtered (0.22mm) for mobile phase development and, degassed by ultrasonicator. The flow rate was maintained at 1 ml/min along with isocratic.

3.2.10. Mass spectrometry

Mass spectrometry with the data-dependent mode of MS^n is a fast and reliable method to confirm the structures of unknown compounds. Mass spectra were obtained with an LTQ linear ion trap tandem mass spectrometer (Thermo Electron, San Jose, CA, USA), equipped with an atmospheric pressure ionization interface. The spectra were observed in direct infusion mass (MS, MS /MS, MS^n) with ESI negative ionization mode. Mass ranging was varying from 50 to 2000 m/e. Data-dependent experiments were

performed under automatic gain control conditions. This technique is generally useful for information of the molecular weight of compounds. This technique has been applied in the identification of drug metabolites in complex biological matrices (Anari et al., 2004) and identification of polyphenols (Lee et al., 2005).

3.3. Results and Discussion

3.3.1. Preliminary Phytochemical Screening of *Vicia faba* Crude Seed Extract

Qualitative phytochemical screening was done using the method described by Indian Ayurvedic Pharmacopeia (Mukherjee and Wahile, 2006).

Table 3.1: Phytochemical composition of faba beans in different solvents

Phytochemicals	Hexane	Chloroform	Methanol	Acetone	Ethanol	Ethyl acetate	Water
Alkaloids	–	+	+	+	+	+	+
Tannins	–	+	+ +	+	+ ++	+	+
Saponins	–	–	-	-	-	-	+
Phenolics compounds	–	+	+ ++	+ +	+	+	+
Flavonoids	+	+	+	+	+	+	+
Terpenoids	–	+ +	–	+	+	–	–
Glycosides	-	-	+	+	+	+	+
Carbohydrates	+	+	+	+	+	+	+
Proteins	–	+	+	+	+	+	+
Reducing sugar	+	+	+	+	+	+	+

On the basis of phytochemical screening showed the presence of tannins, alkaloids, flavonoids, coumarins, terpenoids, and steroids **(Table 3.1)**. Generally, the

polar nature of phytochemicals was present in the polar solvent. Based on phytochemical screening, it was found that polar solvents detected the presence of polyphenols in faba bean seed extract. Suggested literature also validates the above findings(Bravo, 1998).

3.3.2. Investigation of phenolic compounds by thin layer chromatography

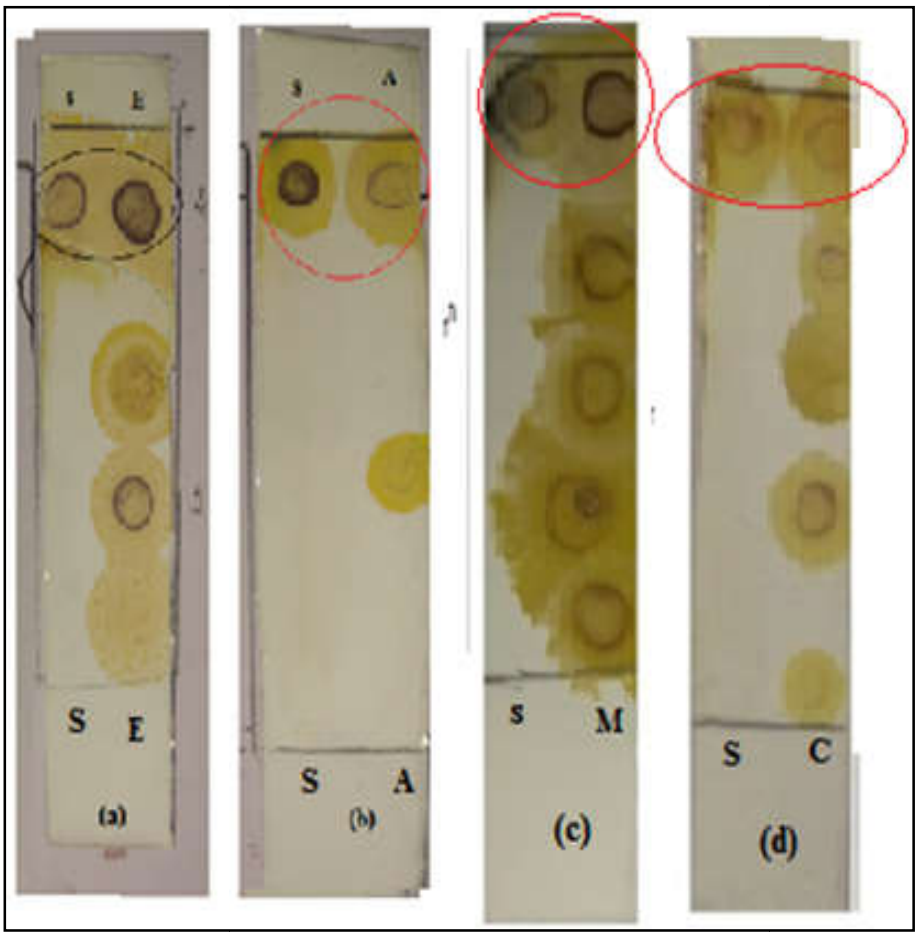

Figure 3.1. TLC separation of phenolics compounds in different seed extract such as ethanol (a),acetone (b),methanol (c) and (d) chloroform seed extract (d) standard (s) gallic acid in the chloroform , ethyl acetate and formic acid as mobile phase in the ratio of 5:4:1

Figure 3.1 showed the Rf value as 0.29, 0.33, 0.35 and 0.38 with ethanol, acetone, methanol, chloroform solvent seed extract respectively.

Their Rf value was found to be similar to the gallic-acid standard. Rf values of phenolics fractions of pea and broad bean and others were in coherence with Rf values of faba bean extracts (Vijayabaskar and Shiyamala, 2012) (AMAROWICZ et al., 1996)

3.3.3. FTIR analysis of faba bean seed extract

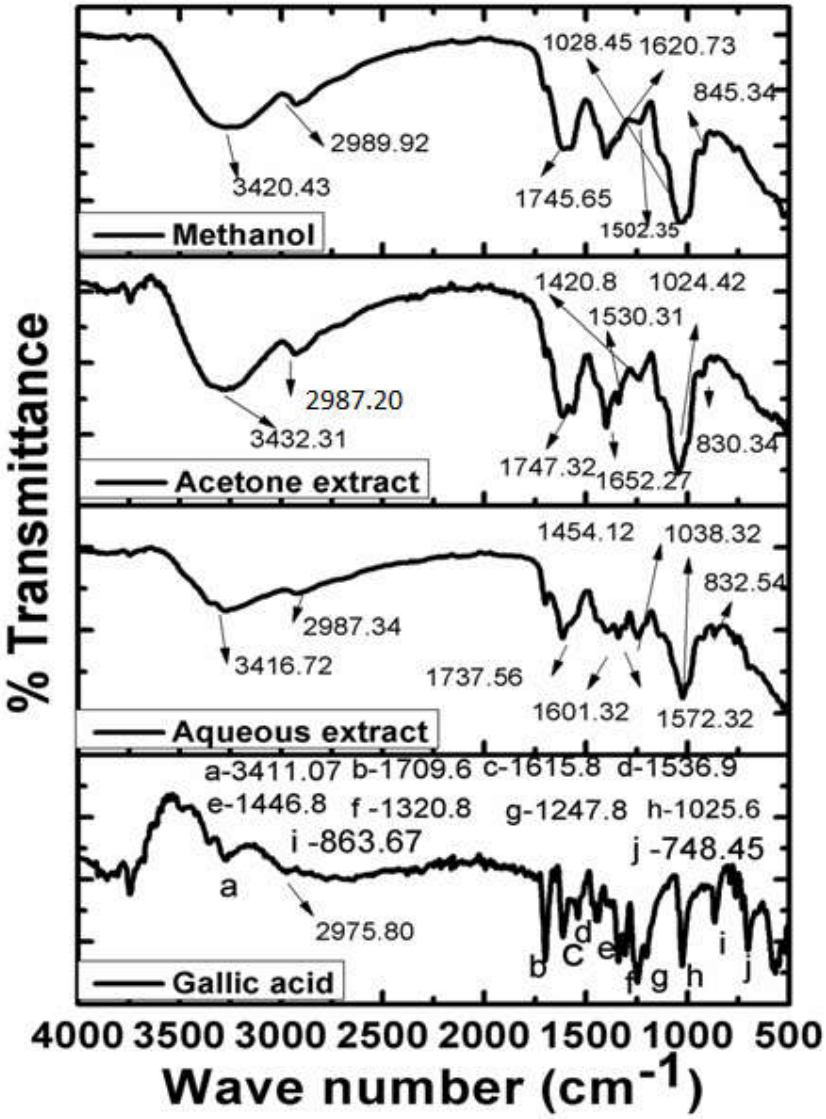

Figure 3.2. FTIR analysis of *Vicia faba* seed extract) in different solvents (acetone, methanol, aqueous) with compare to gallic acid.

FTIR analysis generally tells about the functional group present in an inorganic or organic molecule.Polyphenols have characteristic functional features such as band at wave

number 3200-3320 cm-^{1} (stretching vibration of a phenolic hydroxyl group (-OH)) ,2900-3000 cm^{-1} (stretching of aromatic (C-H) group),1632-1654 cm^{-1} (aromatic ketone group),1020 cm^{-1} (ether (C-O) group) ,1300-1400 cm^{-1} (methyl group) and 1100-1118 cm^{-1}(Du et al., 2009).

FTIR analysis **(Figure 3.2)** showed the presence of bands in the 3400-3500 cm^{-1} region are assigned to different OH functional groups (from carboxyl or phenols)(gallic acid and seed extract) and those between 2800 and 2985 cm^{-1} belong to stretching vibrations of the aliphatic and aromatic –CH (gallic acid and seed extracts). The peaks at 1707 cm^{-1} and 1246 cm^{-1} indicated the presence of carboxylic groups. The aromatic character of the compound was confirmed by the absorption bands at 1600-1640 cm^{-1}, along with the intense absorption at 820–878 cm^{-1}(gallic acid and seed extract). The band at 1020-1150 represent c-o stretch (gallic acid and seed extract). The bands between 1200 and 1270 cm^{-1} represent the C-O deformation vibrations of phenols and carboxyl group (gallic acid and seed extract). Similar findings were also made by other researchers the tannic acid contains some aromatic esters which show signal characteristics bands of carbonyl groups: C=O stretching vibration at 1730-1705 cm $^{-1}$ and C-O at 1100-1300 cm^{-} 1 (Silverstein et al., 1974), (Stuart, 2005).

3.3.4. Identification and characterization of polyphenols by RP-HPLC–ESI-Q-TOF-MS

Major peaks were observed at retention time 1.6959 minute, 2.632 minutes, 4.872 minutes, 5.695 minute and 6.67 minutes in negative ESI associated HPLC with acetone seed extract (**Figure 3.3**).

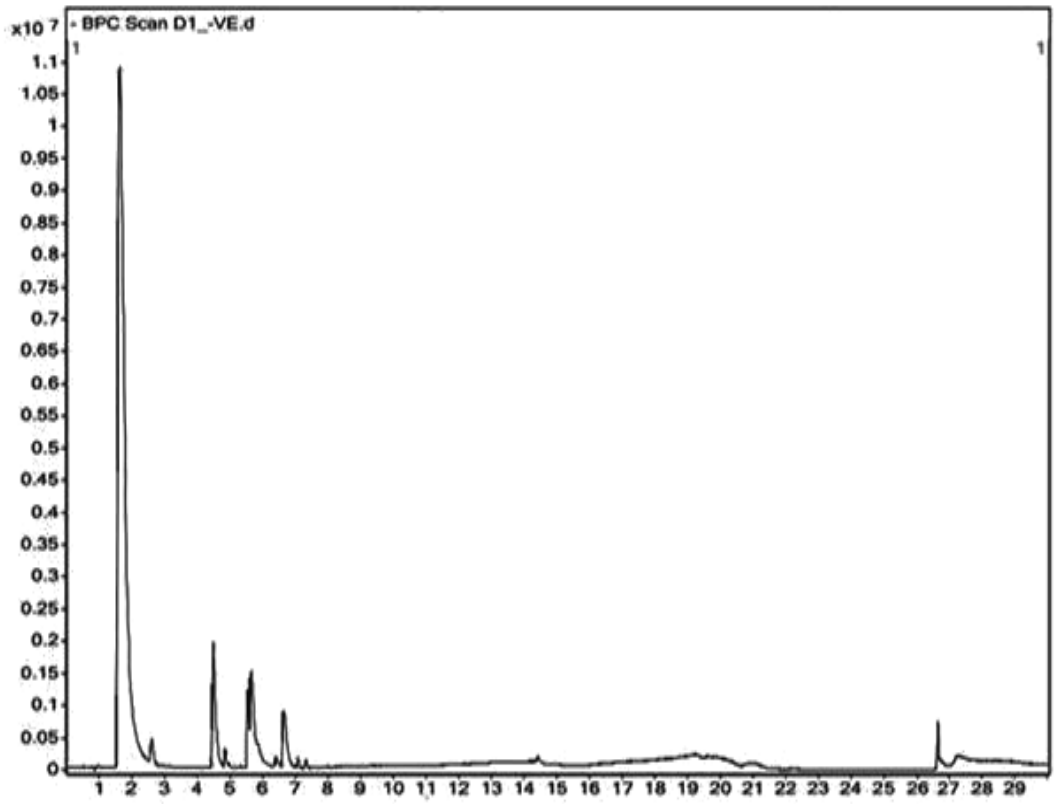

Figure 3.3. Negative ESI associated HPLC chromatogram represents four major peaks at RT 1.6959 minute (gallic acid), 2.632 minutes ((epigallocatechin (ellagic acid)),4.872 minute(catechin),5.696 minute (epigallocatechin gallate) and 6.677 minutes (epicatechin monogallate) with acetonic seed extract of *Vicia faba* beans.

Polyphenols were found at retention time 1.659 minutes, 2.632 minutes ,4.872 minutes, 5.695 minutes and 6.67 minutes r that were corresponded to gallic acid (m/z[M−H]=169.0124,$C_7H_6O_5$),epigallocatechin(m/z[M−H=305.0644,$C_{15}H_{14}O_7$),catechin (m/z[M−H]=289.0656,$C_{15}H_{14}O_6$), ellagic acid derivatives epigallocatechin gallate (m/z[M−H]=457.0578,$C_{22}H_{18}O_{11}$) and epicatechin monogallate (m/z[M−H] =441.081 ,C_{22} H_{18} O_{10}) (**Figure 3.4**). Similar findings were also made by other researchers (Abu-Reidah et al., 2015; Karar and Kuhnert, 2015; Mishra et al., 2018; Sarabhai et al., 2013).

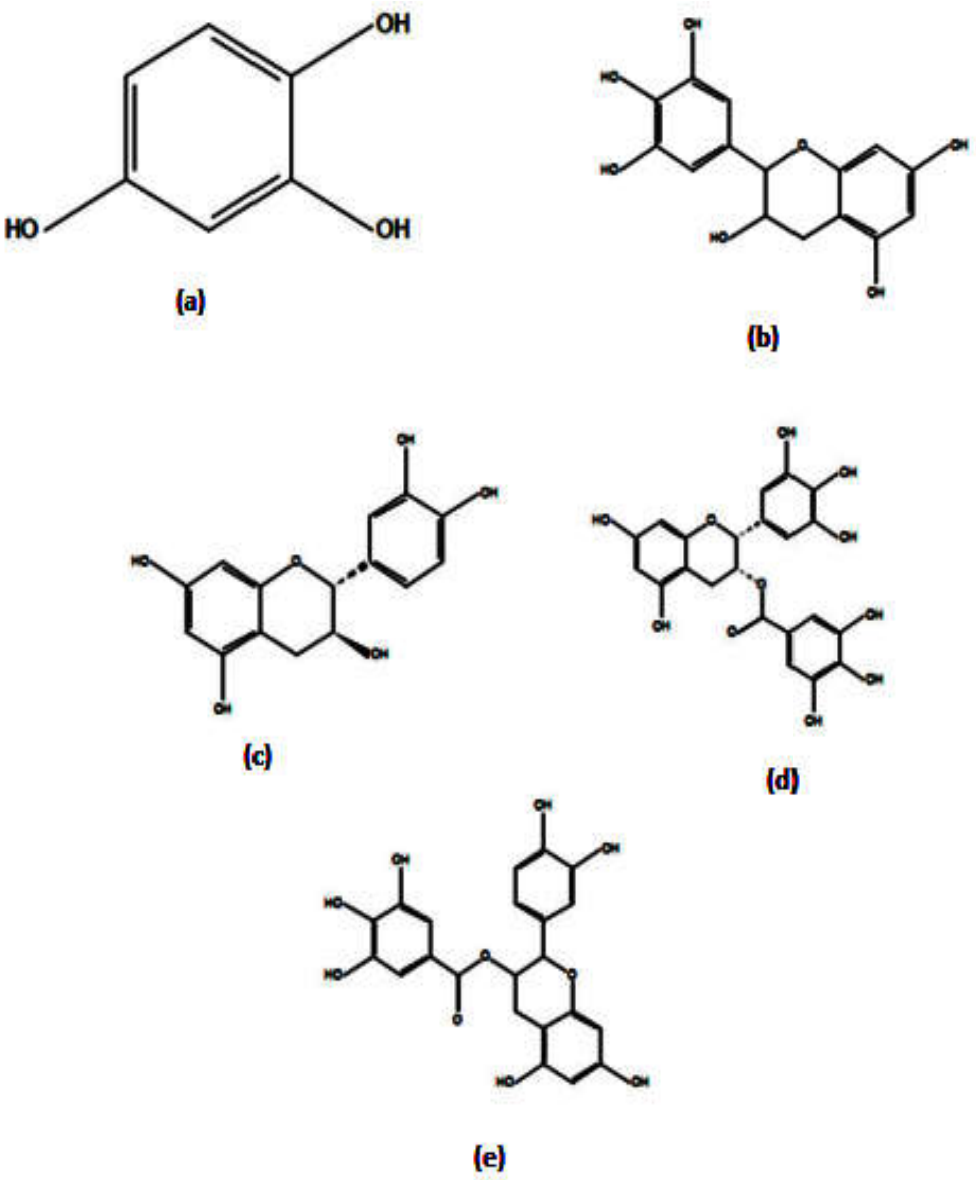

Figure 3.5. Molecular structure predicted by HR-LCMS (a) gallic acid (b) epigallocatechin (ellagic acid) (c) catechin (d) epigallocatechin gallate (e) epigallocatechin monogallate in an acetonic fraction of seed extract.

The major compounds in the faba bean extract were estimated by HPLC–ESI-Q-TOF-MS using the negative mode as given in (**Table 3.2**) and molecular structure of polyphenols was shown **(Figure 3.5)** and predicted by HRLC-MS.

Table 3.2: Major compound generated by HPLC–ESI-Q-TOF-MS (HR-LCMS) in acetone seed extract.

Compound label	RT	Masss	DB Formula	DB Diff (ppm)	(DB)
Hydroxyhydroquinone	1.659	126.03	$C_6 H_6 O_3$	13.19	3
Gallic acid	1.669	170.0197	$C_7 H_6 O_5$	10.69	2
Epigallocatechin	2.632	306.0719	$C_{15} H_{14} O_7$	6.77	1
Glutathione, oxidized	4.513	612.1449	$C_{20} H_{32} N_6 O_{12} S_2$	11.55	1
Epigallocatechin	4.518	306.0717	$C_{15} H_{14} O_7$	7.48	1
Catechin	4.872	290.0768	$C_{20} H_{32} N_6 O_{12} S_2$	7.26	3
Epigallocatechin gallate	5.695	458.083	$C_{22}H_{18} O_{11}$	4.25	1
Epigallocatechin monogallate	6.677	442.0882	$C_{22} H_{18} O_{10}$	4.1	1
Sphingosine	26.645	282.279	$C_{18}H_{37}N O_2$	3.22	1

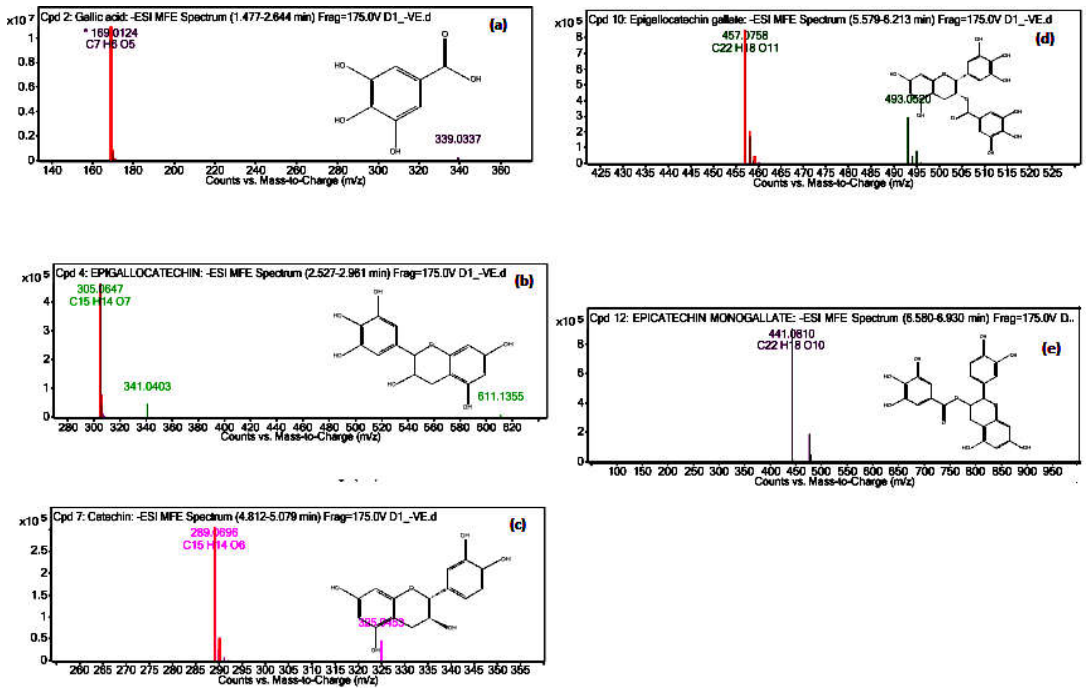

Figure 3.4. Negative ion electrospray tandem mass spectra represents polyphenols such as (a) gallic acid polyphenols such as (m/z[M−H]=169.0124,$C_7H_6O_5$(b)epigallocatechin(m/z [M−H=305.0644,$C_{15}H_{14}O_7$),(c)catechin(m/z[M−H]=289.0656,$C_{15}H_{14}O_6$)(d) epigallocatechin gallate (m/z [M−H]=457.0578, $C_{22}H_{18}O_{11}$) (e) epigallocatechin monogallate (m/z [M−H] =441.081 ,C_{22} $H_{18}O_{10}$) in acetonic extract of seed.

3.3.5. HPLC analysis of acetone seed extract after column chromatography

HPLC was done for confirmation of polyphenols in acetone seed extract fractions. Polyphenols standard (gallic acid, ellagic acid, catechin, and epicatechin) was used as a standard molecule with its retention time (7.25 minute, 8.78 minutes,12.35 minute, and 11.59 minutes) and acetone seed extract fraction showed a similar retention time **(Figure 3.6**). HPLC data showed that the phenolic compound was present in the extract.

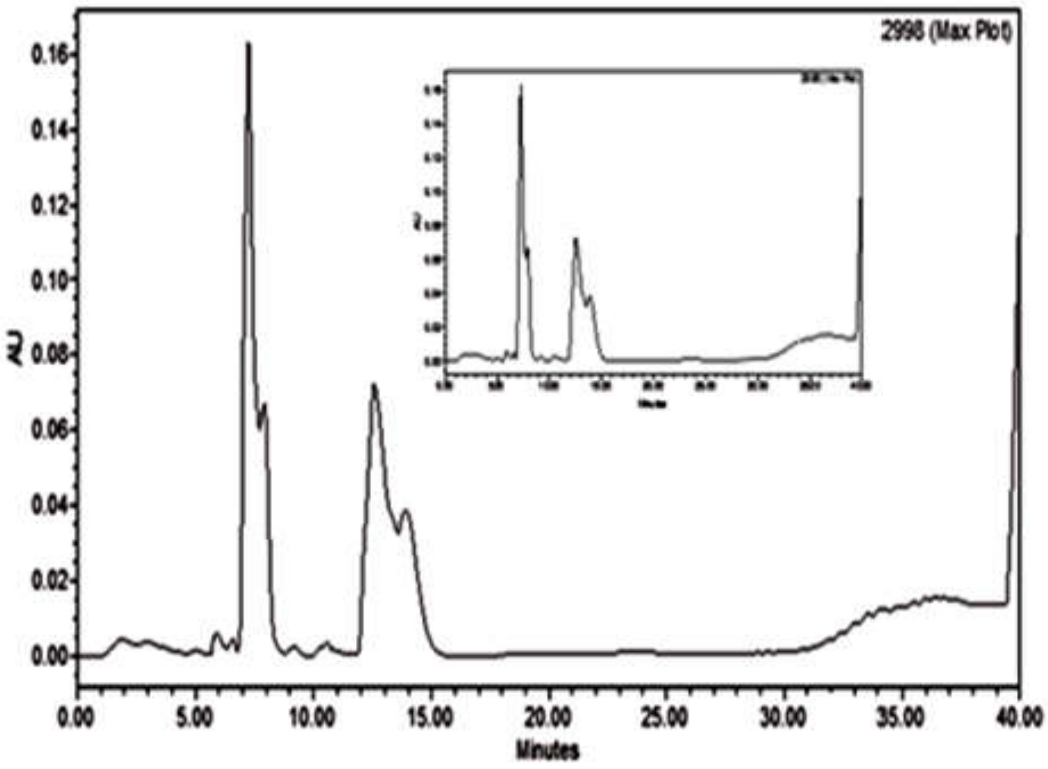

Figure 3.6. HPLC analysis of acetone seed extract fraction and polyphenols standard

3.3.6. Mass spectrometry

Mass spectroscopy generally tells about the molecular weight of any compounds. The protonated and fragmented ions of MS/MS of the m/z 169 (fraction 5) ion showed fragment ion at m/z 125.1. Abundant [M+H]+ ions as the base peaks were formed in fraction 8. Their fragmentation pattern and molecular weight of fraction 5 and 8 showed the presence of gallic acid and catechin in faba been seed extract(Lee et al., 2010). Mass spectrum of fraction 6 and fraction 8 was represented in **Figure 3.7** and **Figure 3.8**. The molecular structure of gallic acid and catechin were predicted in **Figure 3.9.**

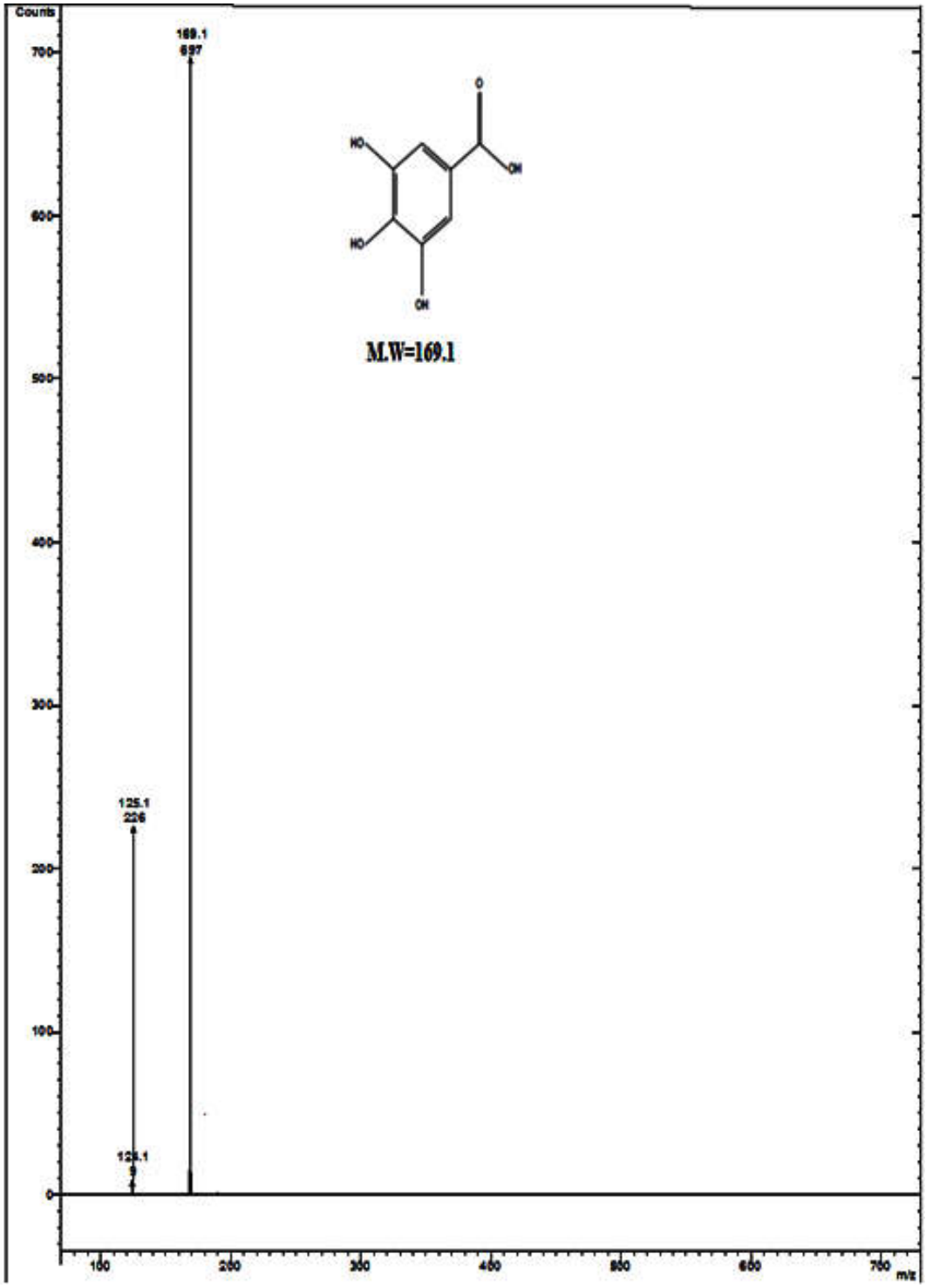

Figure 3.7. Mass spectra of fraction 5 (acetone seed extract) represent fragmentation pattern of gallic-acid (169.1 ⟶ 125.1) in negative ESI mode.

School of Biochemical Engineering, IIT(BHU) Varanasi

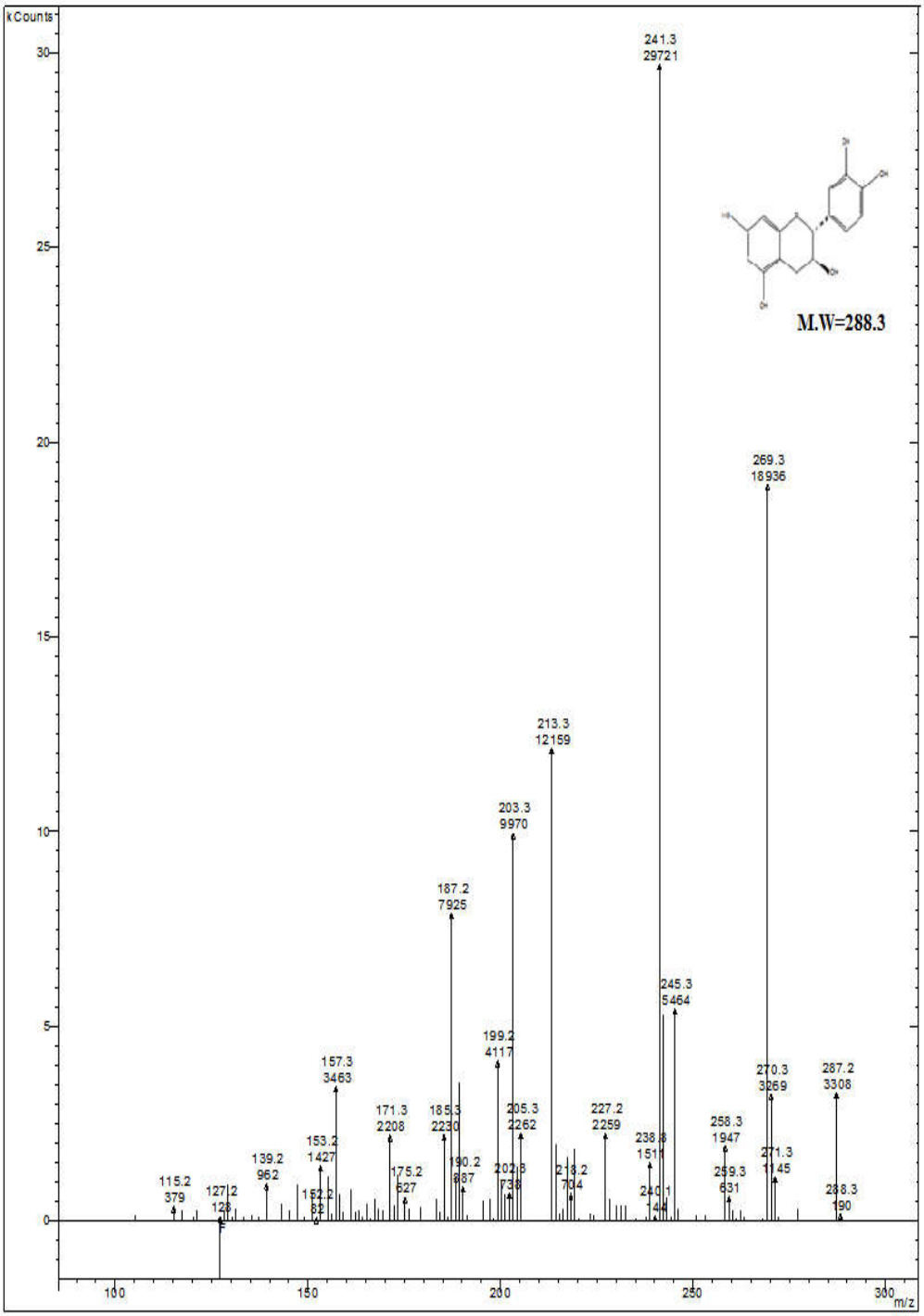

Figure 3.8. Mass spectra of fraction 8 as molecular weight represents fragmentation pattern of catechin (288.3→269.3→246.3→241.1→213.3→203→187.2→115.2) in negative ESI mode.

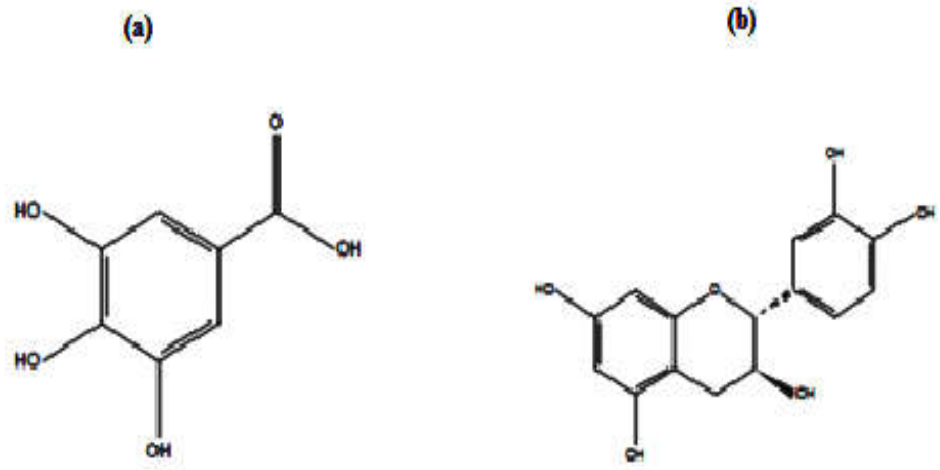

Figure 3.9. Structure of (a) gallic acid (b) catechin

3.3. Conclusion

Extraction and purification of polyphenols from faba bean depend upon nature and type of solvents. Solvents with increasing polarities were used to obtain extracts with different metabolite profiles.TLC, FTIR, phytochemical screening detected the presence of a phenolic compound in seed extract. LC/MS Mass spectrometry provides abundant information for the structural elucidation of the compounds like gallic acid, catechin, epicatechin, ellagic acid. Mass spectroscopy of fraction detected gallic acid and catechin. HPLC analysis separately instead of LC/MS also validate their standard that matched with seed extract peak.