CHAPTER 3

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Chemical composition analysis of *S. virginianum* leaf extract: Unveiling phytochemical diversity

3.1 Introduction

Identifying chemical compounds in plants has catalysed the emergence of ethnopharmacology, a contemporary field dedicated to uncovering potential therapeutic drugs from medically significant plants. This approach has proven invaluable in discovering novel drugs and active compounds from natural origins. Initially, pharmacological researchers faced significant technological challenges in the quest for new bioactive compounds, particularly in the extraction, isolation, and characterisation processes. However, despite these hurdles, researchers have adeptly navigated methodological obstacles by delving into the intricate chemistry of plants and addressing the complexities inherent in characterising plant metabolites from chemically diverse crude mixtures.

In recent years, gas and liquid chromatography–mass spectrometry techniques for untargeted phytochemical profiling have gained prominence (Allwood & Goodacre, 2010). This approach, owing to its precision, sensitivity, rapidity, and specificity, offers numerous advantages in studying and characterising the phyto-constituents of medicinal plants.

Among various extracts, freshly prepared leaf extracts (methanolic and aqueous) of *S. virginianum* (*Sv*) have shown promising antioxidant and anti-breast cancer responses without displaying toxicity towards normal cells. Previous studies have focused on *Sv*'s methanolic extract, primarily identifying phytol, hexadecyl acrylate, and p-cresl otanoat (p-cresyl octanoate) (Javaid et al., 2021). However, there remains a gap in systematic data regarding evaluating *Sv* leaf constituents using advanced phytochemical analytical techniques.

This chapter addresses this gap by conducting a systematic phytochemical profiling of promising anti-breast cancer activity exhibited by specific extracts. Using gas chromatography-mass spectrometry (GC-MS) and high-resolution liquid chromatography-mass spectrometry (HR-LCMS) techniques, we endeavour to identify and characterise the phyto-constituents present in these extracts.

3.2 Materials and method

3.2.1 Identification of metabolites of *Sv* leaf sample through GC-MS

Gas Chromatography-Mass Spectrometry (GC-MS) is a sophisticated technique for separating complexes, quantifying analytes, and identifying unknown phyto-compounds within plant extracts (Allwood & Goodacre, 2010). Gas chromatography and mass spectrometry are pivotal for assessing the composition and purity of a sample. Gas chromatography segregates mixture components based on their volatility, while mass spectrometry identifies and quantifies individual compounds. The methodological principle involves vaporising the sample and injecting it into a gas chromatograph for separation based on differing boiling points. The separated components then proceed to the mass spectrometer, where they undergo ionisation, and their mass-to-charge ratio is measured for compound identification and quantification. This amalgamation of techniques facilitates a thorough analysis of the sample's composition and purity.

In this context, GC-MS analysis has been conducted to identify metabolites present in *Sv* plant extracts, given their notable antioxidant, anti-inflammatory, and anti-proliferative activities, as elucidated in previous chapters. Gas chromatography coupled with mass spectrometry (GC-MS QP-2010, Shimadzu, Japan) was employed.

3.2.1.1 Experimental details:

A total of 20 mg of extract was utilised, adding 0.75 mL of ice-cold methanol. The extraction process occurred at 70°C for 15 minutes, followed by centrifugation for 5 minutes at 10,000 rpm. Subsequently, 750 µL of water and 325 µL of ice-cold chloroform were added to the supernatant.

After vortexing, the mixture underwent centrifugation for 10 minutes at 5000 rpm. A new tube isolated the polar phase (water/methanol) from the nonpolar fraction. The polar fraction (500 µL) was then dried using a speed-vacuum concentrator.

For derivatisation, the dried pellet was reconstituted in 60 µL of a 20 mg/mL solution of methoxyamine hydrochloride in pyridine and shaken at 37°C for 120 minutes. Following this, 130 µL of N-methyl trimethylsilyl trifluoroacetamide (MSTFA) was added, and the mixture was shaken at 37°C for 30 minutes. The derivatised samples underwent analysis with a thermal program starting at 80°C as the initial temperature with a 2-minute hold, followed by an increment of 10°C until reaching 315°C with a 1-minute hold, and finally reaching a temperature of 250°C, with a total run time of 40 minutes. Identification of compounds was based on their m/z ratios using the NIST library. (Lisec et al., 2015).

3.2.2 Identification of metabolites of *Sv* leaf sample through High-Resolution Liquid Chromatography and Mass Spectrometry (HR-LCMS/MS) Analysis

LC-MS is a hybrid analytical technique that combines liquid chromatography and mass spectrometry. Liquid chromatography separates a mixture of metabolites from a plant extract based on characteristics such as charge, hydrophobicity, polarity, and size. At the same time, mass spectrometry aids in identifying each separated component and provides spectral information (Allwood & Goodacre, 2010). Identification is typically conducted using available libraries or databases.

In an LC-MS/MS setup, two mass spectrometry detectors are connected to an HPLC instrument. This analytical technique offers high selectivity, specificity, and sensitivity, providing mass and structural information about unknown metabolites within the plant extract. While conventional mass spectrometry methods offer information regarding nominal mass, high-resolution mass spectrometry techniques can discern minute differences in mass between two

metabolites, leading to highly accurate mass determination and precise identification of metabolites.

3.2.2.1 Experimental details

For the HR-LC/MS analysis of the MF, the sample was outsourced to the Sophisticated Analytical Instrument Facility (SAIF) at the Indian Institute of Technology, Bombay (IIT Bombay), India. The facility provided access to an Agilent Technologies 6550-iFunnel Q-TOF LC/MS system in California, United States of America. Before analysis, the freshly prepared *Sv* leaf aqueous and methanolic extracts were reconstituted in deionised water and HPLC-grade methanol, respectively. Subsequently, the samples were filtered through a 0.22 µm membrane filter (Millipore). The HR-LC-MS/MS analysis was conducted using the Agilent Technologies 6550-iFunnel Q-TOF LC/MS system, which comprised a hip sampler, a binary pump, a column component, and a Q-TOF with an electrospray ionisation source. Chromatographic separation of phytochemicals in both extracts was achieved using ultra-high-performance liquid chromatography (UHPLC) with a Hypersil gold column (C18X 2.1 mm-3 Micron). The mobile phase consisted of a binary combination of 0.1% formic acid in water (A) and 90% acetonitrile, 10% water, and 0.1% formic acid (B), with a flow rate of 0.300 mL/min, an injection volume of 5 µL, and a pressure of 1200.00 bar.

The gradient solvent system employed was as follows: 0–1 min of 95% (A) and 5% (B); 1–20 min of 100% solvent (B); 20–25 min of 100% solvent (B); 25–26 min of 95% (A) and 5% (B); and 26–30 min of 95% (A) and 5% (B). MS extracts (leaf aqueous and leaf hydro-alcoholic) were analysed in dual ion modes (positive and negative) using a 1290 Infinity UHPLC System coupled with 6550 iFunnel Q-TOF. The Agilent iFunnel technology generated ions via the electrospray technique. It focused on Agilent Jet Stream technology with a hexabore capillary sampling array and dual-stage ion funnel for increased ion sampling and transmission as an ion source.

The Q-TOF Mass Spectrometer segment of the instrument was set at a capillary tension of 3500 V, a gas flow rate of 13 L/min at a temperature of 250°C, a sheath gas flow rate of 11 L/min

at a temperature of 300°C, and a 35-psi nebuliser gas flow pressure. Data acquisition and mass spectrometry evaluation were performed using the Agilent Metlin database.

3.3 Result

3.3.1 Phytochemicals evaluated by Gas chromatography-Mass spectrometry

In the Gas Chromatography-Mass Spectrometry (GC-MS) analysis, the aqueous extracts of *Sv* leaf revealed the presence of fourteen carbohydrates, five amino acids, and three fatty acids. In contrast, the hydro-alcoholic extracts of *Sv* leaf exhibited thirty-five carbohydrates, two amino acids, and two fatty acids. Both extracts contained three phenolics, Shikimic acid and Arbutin. Additionally, various sugar alcohols and sugar acids were identified in both extracts. Notably, Arbutin, known for its antioxidant, antimicrobial, and anti-inflammatory properties, possesses potential anticancer properties against various cancers with low acute or chronic toxicity, as published studies indicate (Nahar et al.,2022 and Bhalla et al.,2022). However, no other phyto-compounds of specific medicinal importance were observed in the GC-MS results (Table 3.1.1).

Table 3.1.1 : List of Metabolites found in GC-MS analysis of *Sv* leaf extracts

	Sv Leaf Aqueous extract	*Sv* Leaf methanolic Extract
Compound Nature	**Name of the compound**	
Carbohydrates (Monosaccharides, Disaccharides, Oligosaccharide, Polysaccharides, Sugar alcohol, Sugar acid)	D-(+)-Arabitol L-(-)-Sorbose D-(-)-Fructopyranose D-(+)-Glucosamine D-Fructose D-(+)-Talose D-Mannitol Glucopyranose Ribonic acid	meso-Erythritol L-Threonic acid D-(+)-Ribonol Ribitol L-(-)-Arabitol Adonitol D-Mannitol

	Myo-Inositol D-(+)-Galactose Glucopyranose D-(+)-Cellobiose Sucrose Maltose D-(+)-Turanose Lactose	D-Psicofuranose D-(-)-Tagatopyranose D-(-)-Tagatose D-Psicose D-(-)-Fructopyranose L-(-)-Sorbose D-Mannitol D-Sorbitol Arabinofuranose D-Glucose beta.-D-Galactofuranose D-Galactose Myo-Inositol D-(+)-Galactose Melibiose D-Mannopyranose D-(+)-Talopyranose Sucrose D-Trehalose 3-.alpha.-Mannobiose

		2-.alpha.-Mannobiose D-(+)-Turanose Maltose D-Glucopyranose Galactinol D-Lactose Sucrose Palatinose
Amino acids	L-Valine L-Proline	L-Valine L-Alanine L-Leucine L-Proline Glycine
Fatty Acids	Malic acid Stearic acid	Malic acid Palmitic Acid Stearic acid
Phenolics	Quinic acid Chlorogenic acid 3-O-Coumaroyl-D-quinic acid	2,6-Bis(tert-butyl) phenol Quinic acid Chlorogenic acid
Precursor of Phenolics and Alkaloid	Shikimic acid	Shikimic acid
Glycoside	-	Arbutin

3.3.2 Phytocompounds evaluated by HR-LCMS/MS

In the HR-LC/MS/MS analysis, more than thirty phyto-compounds were identified in both the aqueous and hydro-alcoholic extracts of *Sv* leaf. Figures 3.3.1 and 3.3.2 depict the chromatograms obtained for *Sv* leaf extracts. In the *Sv* aqueous leaf extract, the analysis revealed the presence of one flavonoid, one terpenoid, two amino acid derivatives, two coumarones, two glycosides, and eight alkaloids. Conversely, the *Sv* hydro-alcoholic extract contained six flavonoids, two terpenoids, two amino acid derivatives, two carbohydrate derivatives, three polyphenols, three glycosides, and three alkaloids. Tables 3.3.2 and 3.3.3 lists the compounds identified in each extract, their respective class, retention time (RT), chemical formula, molecular weight, and m/z ratio.

An *in silico* molecular docking study was selected from the identified compounds to investigate the anticancer activity of the given extracts.

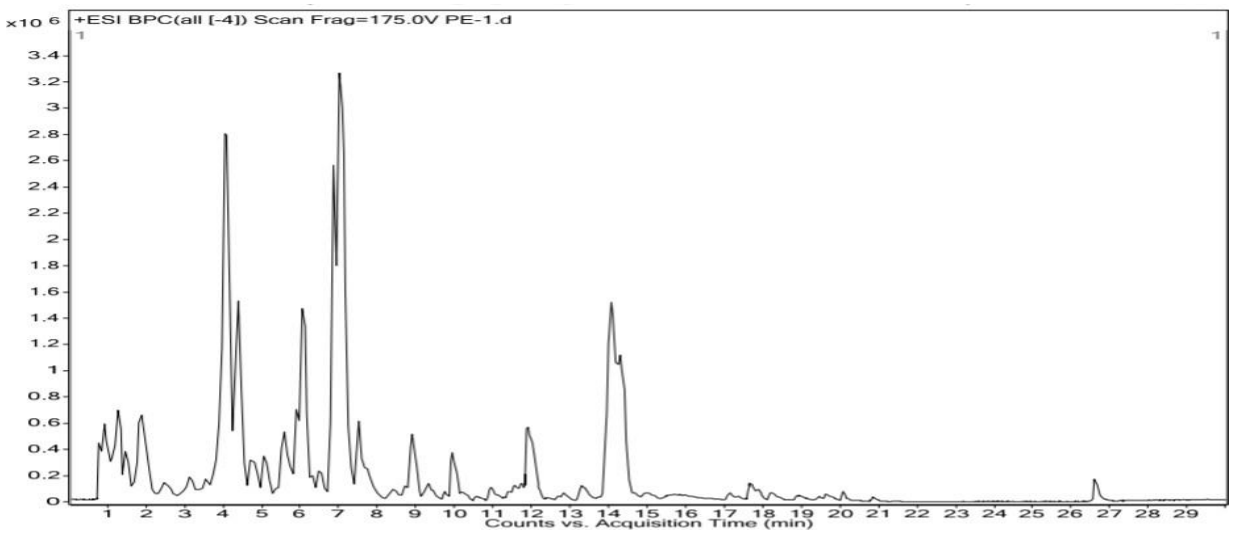

Figure 3.3.1: HR LC-MS/MS chromatogram of *Sv* leaf aqueous extract

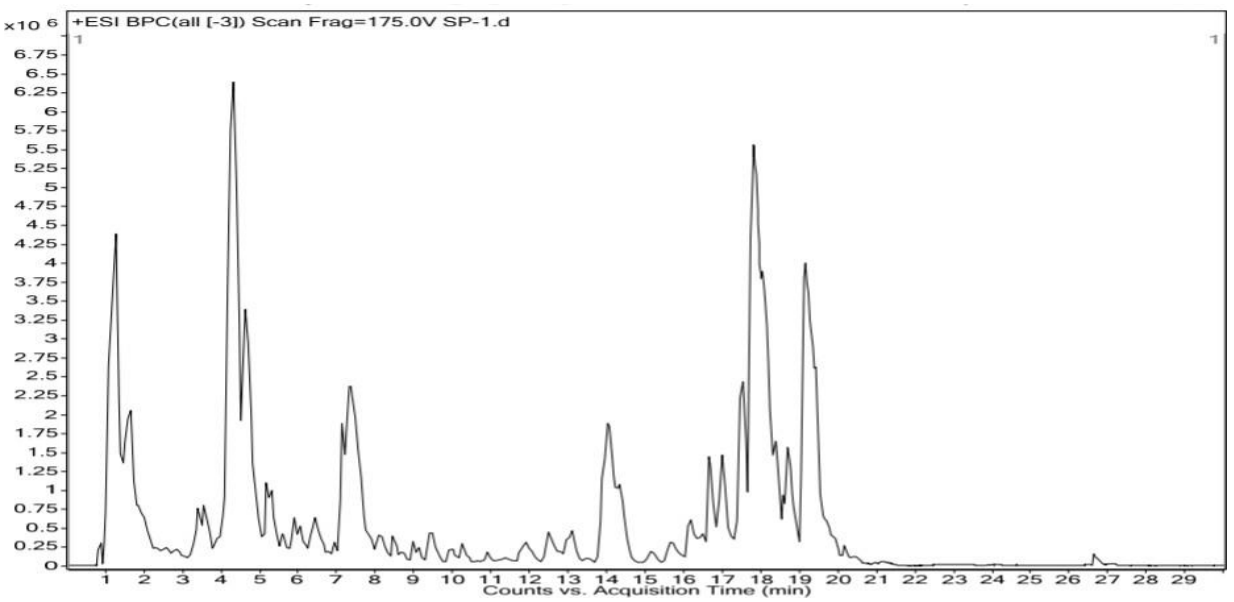

Figure 3.3.2: HR LC-MS/MS chromatogram of *Sv* leaf methanolic extract

Table 3.3.2: **Identified phyto-compounds in *Sv* leaf aqueous extract**					
Name	**Class**	**Formula**	**RT**	**m/z**	**Mass**
8-Hydroxy-3-chlorodibenzofuran	Coumarone	C12 H7 Cl O2	0.803	241.0064	218.017
O-Demethylfonsecin	naphthopyranones	C14 H12 O6	0.806	299.0489	276.057
4-Methoxybenzyl O-(2-sulfoglucoside)	glycosyl compounds	C14 H20 O10S	1.076	381.0823	380.075
4-Fluoro-L-threonine	fluoroamino acid	C4 H8 F N O3	1.159	138.0568	137.049
Isoamyl nitrite	o-nitroso compounds	C5 H11 N O2	1.172	118.0877	117.080
N-(1-Deoxy-1-fructosyl)leucine	Amino acid derivative	C12 H23 N O7	1.404	294.1574	293.149
Methyl N-methylanthranilate	benzoate ester	C9 H11 N O2	1.779	166.0881	165.080
16-Ketoestradiol	Steroids	C18 H22 O3	2.504	309.147	286.157
4-Hydroxycoumarin	Coumarin	C9 H6 O3	2.998	163.0409	162.033
Indoleacrylic acid	unsaturated monocarboxylic acid	C11 H9 N O2	3.236	188.0725	187.065
Tobramycin	Glycoside	C18 H37 N5O9	3.988	468.2623	467.256
Sulfamethopyrazine	sulfonamide	C11 H12 N4 O3 S	5.07	303.0526	280.063

2-Methylchrysene	carbopolycyclic	C19 H14	5.688	265.0995	242.110
Fabianine	Alkaloid	C14 H21 N O	5.69	220.1716	219.164
Solasonine	Steroidal alkaloids	C45 H73 NO16	5.766	884.5059	883.498
Koryoginsenoside R1	triterpenoids	C46 H76 O15	6.095	868.5113	867.503
beta-Solamarine	Steroidal alkaloids	C45 H73 NO15	6.131	868.511	867.503
Psychotridine	polyindoline alkaloid	C55 H62 N10	6.391	884.5056	861.516
Laurelliptine	quinoline alkaloids	C18 H19 N O4	7.435	314.1411	313.134
beta-Solanine	steroidal alkaloid	C39 H63 NO11	7.439	722.4522	721.444
5alpha-Tomatidan-3-one	steroidal alkaloids	C27 H43 N O2	8.949	414.3395	413.332
Veratramine	piperidine alkaloid	C27 H39 N O2	9.974	410.3082	409.300
Aconine	Aconitum alkaloids	C25 H41 N O9	5.3	500.2788	499.276
Kaempferol	Flavonoid	C15 H10 O6	5.529	287.0575	286.050
Piperidolate	diarylmethane.	C21 H25 N O2	9.403	324.198	323.190

Table 3.3.3: Identified phyto-compounds in *Sv* leaf methanolic extract					
Name	**Class of compound**	**Formula**	**RT**	**m/z**	**Mass**
Chlorogenic acid	ester of caffeic acid and-quinic acid	C16 H18 O9	3.995	353.0884	354.095
Quinic acid	a cyclo hexane carboxylic acid	C7 H12 O6	4.195	191.0562	192.063
Nafoxidine	a nonsteroidal selective estrogen receptor modulator or partial antiestrogen	C29 H31 N O2	4.39	470.2314	425.233
p-Coumaroyl quinic acid	Poly phenols,phenolics	C16 H18 O8	4.905	337.0941	338.101
Gambiriin A3	It is a catachin, flavanoid	C30 H28 O12	5.11	335.0785	580.160
Jubanine A	cyclopeptide alkaloids	C40 H49 N5 O6	5.396	693.3546	694.361
Vicinin 2	flavonoid-8-o-	C27 H30 O17	5.463	625.1447	626.151

	glycosides.				
Allivicin	flavonoid-3-o-glycosides.	C27 H30 O16	5.612	609.1497	610.156
N-Acetoxymethylflindersine	pyranoquinolines	C17 H17 NO4	5.764	298.1098	299.117
Myricitrin	Flavonoid	C21 H20 O12	6.245	463.0906	464.097
1,4-Di-O-caffeoylquinic acid	as quinic acids and derivatives.	C25 H24 O12	6.245	515.1222	516.129
Mammeisin	prenylated neoflavonoids	C25 H26 O5	6.331	405.1709	406.175
Isomaltulose	disaccharide carbohydrate composed of glucose and fructose.	C12 H22O11	1.052	341.1084	342.115
Brompheniramine	is an antihistamine drug of the propylamine class.	C16 H19 BrN2	1.109	377.0854	318.071
Fucosyllactose	oligosaccharide	C18 H32 O15	1.11	533.1725	488.174
L-Malic acid	dicarboxylic acid	C4 H6 O5	1.183	133.0137	134.021
4-Coumaroyl-2-	coumaric acids and	C13 H18 N2O3	2.793	249.1242	250.131

hydroxyputrescine	derivatives.				
Pedaliin	flavonoid o-glycosides.	C22 H22O12	6.415	477.1064	478.113
Aspulvinone H	cyclobutyrolactone lignan	C27 H28 O5	6.806	431.187	432.194
Ferulic acid	Ferulic acid is a hydroxycinnamic acid	C10 H10 O4	7.032	193.0514	194.058
Corchorifatty acid F	lineolic acids and derivatives.	C18 H32 O5	8.778	327.2194	328.226
TR-Saponin C	glycosylated derivatives of triterpene sapogenins	C54 H82O21	9.018	1065.511	1066.51
Avermectin B1b aglycone	Avermectin B1b aglycone is a member ofpyrans.	C33 H46 O8	14.711	615.3183	570.317
Geranylfarnesyl diphosphate	Precursor for Sesterterpenoid	C25 H44 O7P2	16.424	577.2714	518.256
Harderoporphyrin	porphyrins	C35 H36 N4O6	17.419	607.2585	608.265
Ritterazine A	Natural cytotxic steroidal alkaloids	C54 H76 N2O10	20.305	971.5559	912.5399

1-O-Sinapoylglucose	Glycoside. It derives from a hydroxycinnamic acid.	C17 H22O10	3.794	385.1142	386.1213
Quercetin	Flavonoid	C15 H10 O7	5.965	303.0477	302.0404
Desglucomusennin	triterpenoid.	C45 H72O16	7.261	868.5012	867.4939
Inundatine	sesquiterpenoid.	C16 H23 NO2	6.69	262.1781	261.1709

3.4 Discussion

In the current study, the analysis of *Sv* leaf extracts using GC-MS and HR-LC-MS/MS techniques has revealed the presence of various secondary metabolites.

A previous investigation by Venkatesh (2014) evaluated the bioactive compounds in Solanum villosum leaf extract using GC-MS. The ethanol leaf extracts were found to contain twelve bioactive components, including Methyl 11,14,17-Eicosatrienoate (29.59%), 4-(3,5-Di-Tert-Butyl-4-Hydroxy Phenyl) Butyl Acrylate (12.04%), N-Hexadecanoic acid (9.41%), Phytol (8.54%), 2H-1-Benzopyran-6-ol, DL-Alpha tocopherol (5.70%), Gamma-tocopherol (3.55%), 3,4-Dihydro-3,5,7,8-Tetra methyl-2-(4,8,12-Trimethyl Tridecyl)-Acetate (3.55%), 3,7,11,15-Tetramethyl-2-Hexadecen-1-ol (3.38%), Cyclotrisiloxane, Hexamethyl (2.12%), Trimethyl (4,1,3,3-Tetramethyl butyl)Phenoxy) silane (2.12%), and Octasiloxane (1.97%). Methyl 11,14,17-Eicosatrienoate exhibited anti-inflammatory properties, while phytol showed antioxidant and anticancer activities (Islam, 2016). Additionally, tocopherols such as DL-Alpha and Gamma-tocopherol suggest potential cardiovascular benefits (Bruno, 2019).

Chirumamilla et al. (2022) investigated the bioactive chemicals in leaf and root extracts of Solanum khasianum, identifying heptadecane 9-hexyl (43.65%) and stigmasterol (23.18%) as major potent compounds. Moreover, another study by Chirumamilla et al. (2023) analysed the phytochemical components of Solanum khasianum Clarke's stem and fruit extract via GC-MS analysis. The stem extract contained 18 compounds, including sucrose (18.74%) and tert-hexadecanethiol (17.24%), while the fruit extract contained 29 compounds. The root extracts contained Dodecanoic acid, 3-hydroxy (33.53%), and 9,12-Octadecadienoic acid (17.87%). Saraswathi et al. (2021) conducted a study on Solanum virginianum fruit extracts, identifying prime components in the aqueous extract as Methyl tetra decanoate, 1-octadecene, 9-methyl-10,12-hexadecadien-1-ol acetate, 9-hexadecenoic acid, methyl ester, (Z)-, 2-hexadecenoic acid, 2,3-dimethyl-, methyl ester, (E)-, 9-eicosene, (E)-, methyl eicosa- 5,8,11,14,17-

pentaenoate, 1-tricosene, and 3-eicosene, (E)-. The ethanolic extract contained mE-2-octenyl tiglate, methyl tetradecanoate, flavone, 9-hexadecenoic acid, hexadecenoic acid, methyl ester, and 9-octadecenoic acid (Z)-, methyl ester.

These findings underscore the diverse array of bioactive compounds present in Solanum species, suggesting their potential therapeutic applications across various health conditions.

This study conducted the systemic HR-LC-MS/MS analysis of secondary metabolites in *Sv* leaf extracts for the first time. The alkaloids identified in both *Sv* leaf aqueous and methanolic extracts includes Fabianine, beta-Solamarine (Kupchan, 1965), Solasonine (Li, 2016), Sychotridine (Roth, 1986), Laurelliptine (Gutiérrez-Grijalva et al., 2020), beta-Solanine (Jabamalairaj et al., 2019), 5-alpha-Tomatidan-3-one (Mohmmad et al., 2023), Veratramine (Khanfar et al., 2013), Aconine, Ritterazine A, and Irinotecan (Fujita et al., 2015).

Additionally, flavonoids such as myricitrin, kaempferol (Kim & Choi, 2013), gambiriin (Desdiani et al., 2020), Mammeisin (Franchin et al., 2016), and Pedaliin are identified in the extracts. Notably, Fabianine, Aconine, Ritterazine A, myricitrin, and Pedaliin have not been previously reported in *Sv* plants nor studied as anti-proliferative bioactive molecules in breast cancer therapeutics.

Moreover, Koryogenoside R1 (triterpenoid), Sulfamethopyrazine (sulfonamide), Caffeoylquinic acid, and quinic acid are also identified, none of which have been previously evaluated for their potential against breast cancer.

These novel findings highlight the diverse array of secondary metabolites in *Sv* leaf extracts, with several compounds showing potential for further exploration as anti-proliferative agents in breast cancer therapeutics.

In conclusion, this chapter presents the screening of potential bioactive compounds from Sv leaf extracts through GC-MS and HR-LCMS/MS analysis. Among the more than 30 phyto-compounds identified, nine were selected based on previous literature on studies on breast cancer cell lines. These selected phyto-compounds underwent further in-silico analysis, as detailed in Chapter 4.