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Identification of Fatty Acids, Amides and Cinnamic Derivatives in Supercritical-CO_2 Extracts of *Cinnamomum tamala* Leaves Using UPLC-Q-TOF-MS^E Combined with Chemometrics

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Abstract: Cinnamomum tamala leaf (CTL), also known as tejpat and Indian bay leaf, is used all over the world for seasoning, flavouring, and medicinal purposes. Numerous researchers are interested in exploring the nutritional and medicinal benefits of CTL due to their potential as nutraceuticals. These characteristics could be explained by the presence of several essential bioactive substances and lipid derivatives. There are no reports available on this species about its metabolites profile. In this work, rapid screen and identify chemical compounds in supercritical (SC)-CO_2 extracts of CTL by use of UPLC-Q-TOF-MS^E with multivariate statistical analysis approach was established in both negative and positive mode. As a results, a total of 166 compounds, including 66 monocarboxylic fatty acids, 52 dicarboxylic fatty acids, 27 fatty acid amides, and 21 others were tentatively identified based on accurate mass, and the mass spectrometric fragmentation pattern, out of which 142 compounds are common and found in all five CTL extracts. They displayed robust $[M+H]^+$ and/or $[M-H]^-$ ions in both low- and high-energy collision-induced dissociations (CIDs). Based on chemical profiling and chemometric analysis, CTL4 (300bar/55°C) extract was found significantly more potent in other CTL's extracts. A new mono- and di-carboxylic fatty acids, fatty acid amides and other essential bioactive compounds were separated within 20 min runtime and identified in CTL for the first time. The combination of UPLC-Q-TOF-MS^E and chemometric analysis is a powerful method to rapidly screen the metabolites profile for the quality control of C. tamala leaf.

Keywords: *Cinnamomum tamala*; chemometrics; fatty acids; fatty acid amides; SC-CO_2 extraction; UPLC-Q-TOF-MS^E

Introduction

Cinnamomum tamala (Buch.-Ham.) T.Nees & Eberm. is an evergreen tree that belongs to family Lauraceae and commonly known as Tejpat, Indian Cassia, and Indian bay leaf [1]. It is naturally distributed in North-East Himalaya, North-Western Himalaya and Southern parts of the country from tropical to subtropical regions at the altitudes of 900-2500 m [2,3]. *Cinnamomum tamala* leaves (CTL) are widely used as a food additive in numerous culinary preparations across the globe and in India used as spices in food, many applications in perfumery, flavoring and pharmaceutical industries [4]. CTL from the ancient time have been traditionally utilized as Ayurvedic and Unani medicine for the treatment of disease associated with scabies, anal, rectal, liver and spleen. These protective roles are due to the presence of high number of bioactive components such as terpenoids, lipids, flavonoids, glycosides, coumarins, and more are responsible for the biological, pharmaceutical and nutraceutical activities [5].

Previously, major constituents like cinnamaldehyde, cinnamic acids, coumarin, methyl eugenol, β-caryophyllene, caryophyllene oxide, linalool and cinnamyl acetate in CTL oil [6] and phenols,

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flavonoids, cinnamates, saponins, coumarins, alkaloids, terpenoids and fatty acids were detected in CTL extracts [7]. Documented studies have been reported the protective role of CTL against heart, gastrointestinal, renal/nephrotic diseases and central nervous system disorders such as anxiety and depression [8,9]. CTL extracts are further reported to cure complication associated with fever, anaemia, bad taste, cancer, coryza (inflammation in mucous membrane), anorexia, bleeding, cardiovascular diseases and blood circulation [8]. Apart from the leaf extracts, CTL oil commonly called as tejpat oil (a rich source of volatile flavour compounds) serve as a most common and important ingredient of spice that possess the ability to supress the progression of flatulent, diuretic, and in cardiac disorders [10–14]. Owing to its high medicinal value and being an important ingredient of the spices, the demand of CTL is increasing day by day [15,16]. Therefore, it is crucial to investigate the metabolic fingerprinting for a better understanding of the quality of CTL's oil and extracts.

Plant metabolites have a wide range of nutritional and pharmacological value in which fatty acids have a significant role, mainly linoleic acid and linolenic acid which cannot be produced by human body *in vivo* [17]. These fatty acids are responsible for regulation of lipid metabolism, anti-oxidation, anti-inflammation, lowering blood cholesterol and enhance the detoxification function of the liver [18–22]. Oleic acid, palmitic acid, linoleic acid, and hydroxy-linoleic acid were previously identified in *C. tamala* bark [7].

When a fatty acid and amine combine, fatty acid amides (FAAs) are produced in the form of an acyl tail with varying carbon length and unsaturations and an amide head-group. They are bioactive intracellular signalling molecules which is controlled by fatty acid amide hydrolases that convert the amide to the parent fatty acid [23,24]. FAAs are reported for many biological activities, for example analgesic, neuroprotection, sleep induction, anti-epilepsy, anti-convulsion, sedative and lipid metabolism. Oleamide, palmitamide and linoleamide have been reported for hypnotic effects, analgesic, inhibits the migration of cancer cells, preventing Alzheimer's disease, cardiovascular disease, and inflammation etc. [25–27]. There has been a lot of interest in FA and FAAs because of their diverse spectrum of biological functions, especially in the fields of pharmacy and nutrition. Several FAAs including palmitamide, oleamide, stearamide, and linoleamide have been previously detected in sesame oil, peanut oil, soybean, egg white and in different vegetable oils [28–31]. Due to their nutritional value, it is very important to screen FAAs in other source like herbal/medicinal plants, spices and oils.

The aim of present study is to establish an efficient, selective, and eco-friendliness method for identification of metabolites in CTL. On this basis, supercritical fluid extraction has gained the position for extraction of herbal materials which utilize smaller amount of organic solvent or no solvent, commonly observed with conventional extraction methods [32–36]. Supercritical with carbon dioxide (SC-CO_2) is an excellent technique for herbal extraction due to lack of toxicity, highly selective, no solvent residue, dynamic and low operating temperature, that means product are extracted at ambient temperature and high pressure to avoid degradation of active metabolites. The extraction parameters of SC-CO_2 had significant effect on the composition of bioactive compounds of the extracted oil.

Fatty acid (FA) in fatty oils and food products are generally analyzed by gas chromatography technique coupled to mass spectrometry (GC-MS) and/or flame ionization detection (GC-FID) detector [37], which is time consuming and require derivatization of fatty acids to their respective fatty acid methyl esters and utilise information of the analytical standards [38]. Contrary to the GC-MS or GC-FID, qualitative and quantitative estimation of fatty acid based on LC-MS methods possess advantage over GC methods as they process the sample without the derivatization with reduced analysis time [39]. Further, mass spectrometry coupled with liquid chromatography emerged as a powerful technique to screen chemical constituents in herbal extracts even in presence of sub ppm level [40–42]. The Xevo G2-XS Q-TOF-MS gives high sensitivity, superior robustness, and high selectivity with high accuracy qualitative information. The Q-TOF combined with UPLC brings not only conventional MS and MS/MS data but also gives MS^E for comprehensive accurate mass precursor and fragment ion information within a single analysis [43]. The MS^E is one of the data independent acquisition techniques in which pre-selection of analytes in the sample are not required

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but gives the mass information of all the compounds separated by the chromatographic column directly. This method can be used to consecutively scan, by "low collision energy" and "high collision energy" in two channels, which provide the high accurate information of parent ions and fragment ions in a single run.

In present study, an UPLC-Q-TOF-MS^E technique combined with chemometric approach was established for the rapid screening and identification of fatty acids, fatty acid amides and other essential metabolites in different SC-CO_2 extracts of CTL for the first time.

Result and Discussion

2.1. Optimization of Extraction Yield

Exhaustive drying experiments (110°C, continued until no weight decrease was registered) showed that the average moisture content was 6.3±0.28% of the shade dried *C. tamala* leaves powder. For efficient and appropriate SC-CO_2 extraction, the optimized parameters i.e. temperatures (55°C), desired pressure (100, 150, 250, 300 and 500 bar), particle diameter (<1.0 mm) and tested extraction time (3h) were applied with triplicate for each set of experiments. The extraction yields (%) of CTL extracts were 0.48±0.04% at 100 bar/55°C, 3.41±0.56% at 150 bar/55°C, 3.93±0.01% at 250 bar/55°C, 4.87±0.54% at 300 bar/55°C, 7.94±0.02% at 500 bar/55°C respectively.

2.1. UPLC-Q-TOF-MS^E Analysis and Identification of Bioactive Compounds

Optimized chromatographic and mass spectral analysis were performed to characterise the bioactive compounds in the SC-CO_2 extracts of CTL. Each extracts (1.0 mg/mL, *ca.* 1000 ppm) solution was prepared using HPLC analytical-grade solvent MeOH, filtered with a membrane disc filter, and then subjected to UPLC-Q-TOF-MS analysis. Isocratic and gradient UPLC methods was tested to optimize the conditions for maximum resolution of peaks. Different mobile phases (water/acetonitrile, 0.1% formic acid in water/acetonitrile, water/methanol, 0.1% formic acid in water/methanol) at variable flow rates (0.25, 0.3, 0.4, and 0.5 mL/min) were examined and compared for better chromatographic separation and appropriate ionization. A mobile phase consisting of 0.1% aqueous formic acid and acetonitrile at a flow rate of 0.3 mL/min resulted in satisfactory separation in a short analysis time. CTL extracts were analysed in the negative ionization modes using a Xevo G2-XS mass spectrometer, and the base peak chromatograms (BPCs) are shown in Figure 1. Due to the complexity of chemical composition in herbal extracts, we established a post-targeted screening strategy for the identification of lipids in different SC-CO_2 extracts of CTL. The accurate masses of targeted $[M+H]^+$ and/or $[M-H]^-$ ions of all possible fatty acids, fatty acid amides were extracted at the Waters Connect UNIFI workstation using a mass tolerance window of ±7 ppm, and the respective peak retention times (RT) are reported in Table 1. The mass spectra derived from these extracted ion chromatograms (EICs) show intense $[M+H]^+$ and/or $[M-H]^-$ ions with a mass error ≤6.5 ppm. Expected compound showed distinguishable MS/MS characteristic fragment ions with high mass accuracy. Compounds were tentatively identified by determining the elemental compositions of the precursor and product ions. The molecular formula and rational fragmentation patterns and pathways of these compounds were then identified based on a comparison of these data with chemical compound databases. In this way, we used the UPLC-Q-TOF-MS^E method in combination with databases to screen 166 compounds from CTL extracts.

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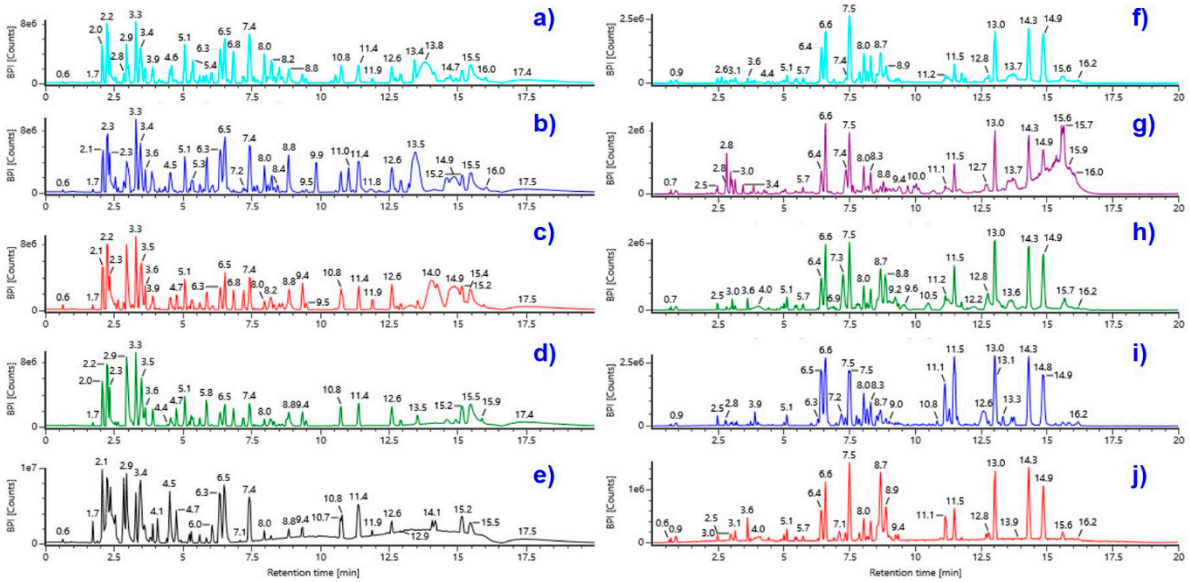

Figure 1. Base peak chromatograms (BPCs) of *C. tamala* leaf SC-CO_2 extracts a) CTL1, b) CTL2, c) CTL3, d) CTL4, e) CTL5 in positive ESI; f) CTL1, g) CTL2, h) CTL3, i) CTL4, j) CTL5 in negative ESI modes.

Table 1. Tentative identification of chemical constituents in supercritical-CO_2 extracts of *C. tamala* leaf using UPLC-Q-TOF-MS^E in both positive and negative polarity.

No.	RT (min)	Compound	Chemical Class	Molecular Ion	Observed Mass (*m/z*)	Error (ppm)	MS/MS Fragments	SC-CO_2 Extracts				
								CTL1	CTL2	CTL3	CTL4	CTL5
1	**1.62**	**Protocatechuic acid**	**PC**	**[M-H]⁻**	**153.0204**	**-0.7**	109.0297	+	–	–	–	–
2	1.67	3-(4-Hydroxyphenyl)lactic acid	PC	[M-H]⁻	181.0502	2.4	119.0502	+	+	+	+	+
3	1.86	Oxodecanedioic acid	DFA	[M-H]⁻	215.0928	-1.4	197.0786, 171.1076, 155.0751, 153.0952	–	+	+	–	+
4	1.88	Heptanedioic acid (Pimelic acid I)	DFA	[M-H]⁻	159.0667	-2.5	141.0542, 115.0772, 97.0673	+	+	+	+	–
5	1.90	Salicylic acid	OC	[M-H]⁻	137.0244	0.0	93.0348	+	+	+	+	+
6	2.16	Heptanedioic acid (Pimelic acid II)	DFA	[M-H]⁻	159.0665	-1.3	141.0542, 115.0772, 97.0673	+	+	+	+	–
7	2.18	Octanedioic acid (Suberic acid)	DFA	[M-H]⁻	173.082	-0.8	155.0687, 129.0986, 111.0816	+	+	+	+	+
8	2.21	2-Hydroxyhydrocinnamic acid	PC	[M-H]⁻	165.0559	-1.3	147.0906, 119.0502	+	+	+	+	+
9	2.22	Hydroxysebacic acid	DFA	[M-H]⁻	217.1095	-6.5	199.0984, 173.1111, 171.1049, 155.1108	+	+	+	+	+

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10	2.32	3-Hydroxy-4-methoxy-cinnamic acid	PC	$[M-H]^-$	193.0517	-5.7	193.0517	+	+	−	+	+
11	2.33	Hydroxyundecanedioic acid	DFA	$[M-H]^-$	231.1241	-1.3	213.1229, 195.0973, 187.1238, 169.1233	+	+	+	+	+
12	2.40	Syringaldehyde	PC	$[M+H]^+$	183.0653	-0.1	155.073, 140.050, 123.047, 105.0452, 95.053, 77.041	+	+	+	+	+
13	2.41	Oxododecanedioic acid I	DFA	$[M-H]^-$	243.1215	5.4	225.1170, 207.1074, 199.1328, 181.1243	+	+	+	+	+
14	2.45	Decenedioic acid I	DFA	$[M-H]^-$	199.0983	-3.5	181.0865, 155.1055, 137.0939	+	+	+	+	+
15	2.47	Nonanedioic acid (Azelaic acid)	DFA	[M-H]	187.0982	-3.2	169.0861, 143.1065, 125.0966	+	+	+	+	+
16	2.50	Oxododecanedioic acid II	DFA	$[M-H]^-$	243.1215	5.4	225.1170, 207.1074, 199.1328, 181.1243	+	+	+	+	+
17	2.60	Oxododecanedioic acid III	DFA	$[M-H]^-$	243.1214	5.8	225.1170, 207.1074, 199.1328, 181.1243	+	+	+	+	+
18	2.65	Dodecenedioic acid I	DFA	$[M-H]^-$	227.1301	-5.3	209.1197, 183.1368, 165.1287	+	+	+	+	+
19	2.65	Decenedioic acid II	DFA	$[M-H]^-$	199.0983	-3.5	181.0865, 155.1055, 137.0939	+	+	+	+	+
20	2.65	Hydroxydodecanedioic acid	DFA	$[M-H]^-$	245.1406	-4.9	227.1334, 209.1108, 201.1317	+	+	+	+	+
21	2.75	Sebacic acid	DFA	$[M-H]^-$	201.113	1.2	183.1021, 157.1214, 139.1119	+	+	+	+	+
22	2.77	4-Hydroxycinnamic acid	PC	$[M-H]^-$	163.0409	-5.0	119.0495	−	−	+	−	−
23	2.78	4-Methoxycinnamic acid	OC	$[M-H]^-$	177.0556	0.6	133.0653, 117.0340, 103.0577, 92.0285	+	+	+	+	+
24	2.79	Nonendioic acid	DFA	$[M-H]^-$	185.0815	2.2	167.0762, 141.0953, 123.0865	−	−	−	+	−
25	2.79	Salicylic acid	OC	$[M-H]^-$	137.0243	0.7	119.0515, 93.0348	+	+	+	+	+
26	2.82	Abscisic acid	OC	$[M-H]^-$	263.1296	-2.7	219.1398, 204.1162,	+	+	+	+	+

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							203.1083, 153.0899					
27	2.82	*p*-Hydroxybenzoic acid	PC	$[M-H]^-$	137.0249	-3.1	93.0348	+	+	+	+	+
28	2.86	4-Hydroxycinnamaldehyde	PC	$[M-H]^-$	147.0457	-3.9	119.0481, 117.0331	+	+	+	+	+
29	2.92	Undecanedioic acid	DFA	$[M-H]^-$	213.1128	1.9	195.1116, 169.1233, 151.1254	+	+	+	+	+
30	2.93	Decenoic acid	MFA	$[M-H]^-$	169.1233	0.6	169.1234, 151.1153, 125.1298	+	+	+	+	+
31	2.94	Coumarin	OC	$[M+H]^+$	147.0446	0.9	118.0454, 103.0603, 91.0597, 77.04313	+	+	+	+	+
32	2.95	Oxodecenoic acid	MFA	$[M-H]^-$	183.1028	-1.5	183.1027, 147.0874, 139.1129	+	+	+	+	+
33	3.04	Decenedioic acid	DFA	$[M-H]^-$	215.1292	-1.4	197.1188, 171.1410, 153.1279	+	+	+	+	+
34	3.06	Cinnamic acid	OC	[M-H]-	147.0457	0.5	103.0542, 77.0392	+	+	+	+	+
35	3.07	Dodecanedioic acid II	DFA	$[M-H]^-$	227.1301	-5.3	209.1197, 183.1368, 165.1287	+	+	+	+	+
36	3.15	9,10,13-Trihydroxy-11-octadenoic acid	MFA	$[M-H]^-$	329.2325	2.4	311.2251, 293.2155, 229.1466, 211.1319, 209.1196, 193.1255, 171.1019, 139.1128	+	+	+	+	+
37	3.20	2-Methoxycinnamic acid	OC	$[M-H]^-$	177.056	-1.5	133.0653, 117.0340, 103.0577, 92.0285	+	+	+	+	+
38	3.21	Cinnamyl alcohol	OC	$[M+H-H_2O]^+$	117.0695	0.5	115.0555, 91.0559, 77.0384	–	–	+	+	–
39	3.33	Dihydroxyhexadecanoic acid	MFA	$[M-H]^-$	287.2232	-1.4	287.2232, 269.2183, 241.2277	+	+	+	+	+
40	3.39	Dodecanedioic acid	DFA	$[M-H]^-$	229.1439	2.9	211.1342, 167.1434	+	+	+	+	+
41	3.48	Cinnamaldehyde I	OC	$[M+H]^+$	133.0648	0.9	115.0601, 105.0752, 103.0603, 91.0597, 89.0436, 79.0593	+	+	+	+	+
42	3.60	9,10,11-Trihydroxy-12-octadenoic acid	MFA	$[M-H]^-$	329.2325	2.4	311.2269, 293.2155,	+	+	+	+	+

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							201.1200, 171.1046					
43	3.72	Octadecanedioic acid I	DFA	$[M-H]^-$	313.2375	3.2	295.2280, 269.2425, 251.2289	+	+	+	+	+
44	3.87	Tridecanedioic acid	DFA	$[M-H]^-$	243.1601	0.4	225.1506, 199.1763, 181.1609	+	+	+	+	+
45	4.00	Nonanamide	FAA	$[M+H]^+$	158.1559	1.3	116.1119, 102.0963, 77.0431, 69.0753	+	+	+	+	+
46	4.00	Methylcinnamic acid	OC	$[M+H]^+$	163.0757	-1.1	105.0356, 103.0569, 91.0519, 77.0379	+	+	+	+	+
47	4.23	Cinnamyl acetate	OC	$[M+H]^+$	177.0913	-1.2	105.0356, 103.0569, 91.0519, 77.0379	+	+	+	+	+
48	4.41	Decanamide	FAA	$[M+H]^+$	172.1706	-5.8	128.0678, 116.1181, 115.0579, 105.0731, 91.0597, 69.0751	+	+	+	+	+
49	4.49	Tetradecanedioic acid I	DFA	$[M-H]^-$	257.1758	0.1	239.1580, 213.1841, 195.1700	+	+	+	+	+
50	4.75	Cinnamyl alcohol II	OC	$[M+H-H_2O]^+$	117.0695	0.6	115.0555, 91.0559, 77.0384	+	+	+	+	+
51	4.84	Hexadecanedioic acid	DFA	$[M-H]^-$	283.1912	1.1	265.1766, 221.1924	+	+	+	+	+
52	4.94	Octadecanedioic acid II	DFA	$[M-H]^-$	313.2375	3.2	295.2280, 269.2425, 251.2289	+	+	+	+	+
53	5.08	Cinnamaldehyde II	OC	$[M+H]^+$	133.0649	0.7	115.0579, 105.0752, 103.0582, 77.0431	+	+	+	+	+
54	5.26	Pentadecanedioic acid	DFA	$[M-H]^-$	271.1915	0.0	253.1779, 227.2038, 209.1932	+	+	+	+	+
55	5.40	Octadecanedioic acid I	DFA	$[M-H]^-$	311.2224	1.3	293.2123, 267.2316, 249.2220	+	+	+	+	+
56	5.46	Octadecanedioic acid III	DFA	$[M-H]^-$	313.2375	3.2	295.2280, 269.2425, 251.2289	+	+	+	+	+
57	5.50	Octadecanedioic acid II	DFA	$[M-H]^-$	311.2224	1.3	293.2123, 267.2316, 249.2220	+	+	+	+	+
58	5.53	Heptadecanedioic acid	DFA	$[M-H]^-$	297.2067	1.4	279.1973, 253.2210, 235.2145	+	+	+	+	+

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59	5.70	Octadecanedioic acid III	DFA	[M-H]-	311.2224	1.3	293.2123, 267.2316, 249.2220	+	+	+	+	+
60	5.98	Dihydroxystearic acid	MFA	$[M-H]^-$	315.2544	-1.0	315.2544, 297.2490	+	+	+	+	+
61	6.03	Hydroxystearidonic acid I	MFA	$[M-H]^-$	291.1964	0.7	273.1883, 255.2316, 245.1916	+	+	+	+	+
62	6.18	Hexadecanedioic acid	DFA	$[M-H]^-$	285.2072	-0.35	267.1978, 241.2069, 223.2130	+	+	+	+	+
63	6.32	Decanoic acid (Capric acid)	MFA	$[M-H]^-$	171.1392	-1.1	171.1396	+	+	+	+	+
64	6.40	Stearidonic acid I	MFA	$[M-H]^-$	275.2027	-3.6	275.2027, 257.1952, 231.2127, 229.1872	+	+	+	+	+
65	6.40	Lauramide	FAA	$[M+H]^+$	200.2015	-3.0	116.1121, 115.0578, 105.0731, 102.0851, 91.0577, 77.0431	+	+	+	+	+
66	6.42	9-Hydroxy-12,14,16-octadecatrienoic acid	MFA	$[M-H]^-$	293.2125	-1.0	275.2022, 235.1708, 183.1399, 171.1017	+	+	+	+	+
67	6.57	Hydroxyoctadecatrienoic acid I	MFA	$[M-H]^-$	293.2125	-1.0	275.2076, 257.1911, 185.1206, 171.1047	+	+	+	+	+
68	6.57	Stearidonic acid II	MFA	$[M-H]^-$	275.2027	-3.6	275.2027, 257.1952, 231.2127, 229.1872	+	+	+	+	+
69	6.80	Hydroxystearidonic acid II	MFA	$[M-H]^-$	291.1964	0.7	273.1883, 255.2316, 245.1916	+	+	+	+	+
70	6.98	Hydroxystearidonic acid III	MFA	$[M-H]^-$	291.1964	0.7	273.1883, 255.2316, 245.1916	+	+	+	+	+
71	7.16	Hydroxystearidonic acid IV	MFA	$[M-H]^-$	291.1964	0.7	273.1883, 255.2316, 245.1916	+	+	+	+	+
72	7.17	Tridecanamide	FAA	$[M+H]^+$	214.2194	0.5	128.0678, 116.1123, 115.05788, 105.0761, 91.0597, 81.0739, 77.0431, 69.0781					
73	7.22	Heptadecanedioic acid I	DFA	$[M-H]^-$	299.2242	-4.7	281.2143, 255.2352, 237.2166	+	+	+	+	-

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74	7.49	13-Hydroxy-9,11-octadecadienoic acid	MFA	$[M-H]^-$	295.2278	0.3	295.2278, 277.2161, 249.2215, 195.1418, 171.1046, 113.0973	+	+	+	+	+
75	7.85	Ricinoleic acid I	MFA	$[M-H]^-$	297.2438	-1.0	297.2438, 279.2322, 253.2534, 183.1396, 111.0840, 93.0349	+	+	+	+	+
76	8.30	Hydroxyoctadecatrienoic acid II	MFA	$[M-H]^-$	293.2125	-1.0	275.2076, 257.1911, 185.1206, 171.1047	+	+	+	+	+
77	8.33	Octadecanedioic acid IV	DFA	$[M-H]^-$	313.2375	3.2	295.2280, 269.2425, 251.2289	+	+	+	+	+
78	8.50	Hydroxyoctadecatrienoic acid III	MFA	$[M-H]^-$	293.2125	-1.0	275.2076, 257.1911, 185.1206, 171.1047	+	+	+	+	+
79	8.52	Ricinoleic acid II	MFA	$[M-H]^-$	297.2438	-1.0	297.2438, 279.2322, 253.2534, 183.1396, 111.0840, 93.0349	+	+	+	+	+
80	8.62	Ricinoleic acid III	MFA	$[M-H]^-$	297.2438	-1.0	297.2438, 279.2322, 253.2534, 183.1396, 111.0840, 93.0349	+	+	+	+	+
81	8.84	Dodecanoic acid (Lauric acid)	MFA	$[M-H]^-$	199.1704	-0.3	199.1704, 181.1572	+	+	+	+	+
82	9.01	Hydroxyhexadecenoic acid I	MFA	$[M-H]^-$	269.213	-3.0	269.2130, 251.2080, 225.2243, 223.2160	+	+	+	+	+
83	9.11	Palmitoleamide I	FAA	$[M+H]^+$	254.2483	-1.8	237.2203, 219.2092, 165.0745, 146.6038, 135.1205, 121.1049, 116.0634, 111.0859, 109.1058, 107.0884, 105.0752, 95.0898, 93.0747, 91.0577, 83.0915, 81.0758,	+	+	+	+	+

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							79.0593, 77.0431, 69.0753, 67.0591					
84	9.14	Linolenamide	FAA	$[M+H]^+$	278.2471	2.7	219.2102, 189.1640, 175.1480, 147.1168, 135.1170, 133.1010, 131.0860, 123.1170, 121.1010, 119.0860, 91.0578, 77.0449	+	+	+	+	+
85	9.17	Tetradecanedioic acid II	DFA	$[M-H]^-$	257.1758	0.1	239.1580, 213.1841, 195.1700	-	-	+	-	+
86	9.26	9-Hydroxy-10,12-octadecadienoic acid	MFA	$[M-H]^-$	295.2278	0.3	295.2280, 277.2229, 249.2215, 233.2223, 171.1064, 113.0973	+	+	+	+	+
87	9.29	Myristamide	FAA	$[M+H]^+$	228.2345	-1.3	116.1097, 115.0578, 105.0731, 102.0963, 91.0597, 88.0805, 77.0431, 69.0753	+	+	+	+	+
88	9.36	9-Hydroxy-10,12-octadecadienoic acid	MFA	$[M-H]^-$	295.2278	0.3	295.2280, 277.2229, 249.2215, 233.2223, 171.1064, 113.0973	+	+	+	+	+
89	9.51	Nonadecanedioic acid	DFA	$[M-H]^-$	327.2549	-2.4	309.2492, 283.2639, 265.2502	+	+	+	+	+
90	9.81	Hydroxyhexadecenoic acid II	MFA	$[M-H]^-$	269.213	-3.0	269.2130, 251.2080, 225.2243, 223.2160	+	+	+	+	+
91	9.96	Heptadecanedioic acid II	DFA	$[M-H]^-$	299.2242	-4.7	281.2143, 255.2352, 237.2166	+	+	+	+	+
92	10.14	Dihydroxyoctadecenoic acid	MFA	$[M-H]^-$	313.2378	1.9	295.2249, 277.2240, 269.2500, 171.1046, 155.1080, 125.0960	+	+	+	+	+
93	10.16	Octadecanedioic acid V	DFA	$[M-H]^-$	313.2375	3.2	295.2280, 269.2425,	+	+	+	+	+

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							251.2289					
94	10.22	Tridecanoic acid	MFA	$[M-H]^-$	213.1856	1.9	213.1856, 195.1645	+	+	+	+	+
95	10.27	Hydroxyhexadecenoic acid III	MFA	$[M-H]^-$	269.213	-3.0	269.2130, 251.2080, 225.2243, 223.2160	+	+	+	+	+
96	10.29	Hydroxyhexadecanoic acid I	MFA	$[M-H]^-$	271.2293	-5.2	271.2293, 225.2244	+	+	+	+	+
97	10.35	Pentadecanamide	FAA	$[M+H]^+$	242.2466		116.0578, 115.0578, 102. 0954, 91.059	+	+	+	+	+
98	10.50	Dihydroxyoctadecadienoic acid I	MFA	$[M-H]^-$	311.2222	1.9	293.2160, 275.1958, 265.2173, 257.2183	+	+	+	+	+
99	10.60	Palmitadienoic acid	MFA	$[M-H]^-$	251.2016	0.4	251.2016	+	+	+	+	+
100	10.66	Linoleamide I	FAA	$[M+H]^+$	280.2631	1.4	263.2333, 245.2219, 161.1178, 133.0839, 119.0683, 109.0826, 95.0667, 91.0353, 81.0513, 79.0352	+	+	+	+	+
101	10.70	Dihydroxyoctadecadienoic acid II	MFA	$[M-H]^-$	311.2222	1.9	293.2160, 275.1958, 265.2173, 257.2183	+	+	+	+	+
102	10.74	Eicosanedioic acid	DFA	$[M-H]^-$	341.2695	0.6	323.2603, 297.2877, 279.2632	+	+	+	+	+
103	10.77	Nonadecanedioic acid	DFA	$[M-H]^-$	325.2368	4.9	307.2291, 281.2480, 263.2364, 237.2231	+	+	+	-	+
104	11.01	Dihydroxyoctadecadienoic acid III	MFA	$[M-H]^-$	311.2222	1.9	293.2160, 275.1958, 265.2173, 257.2183	+	+	+	+	+
105	11.10	Ceriporic acid I	DFA	$[M-H]^-$	351.2534	1.9	333.2467, 307.2613, 289.2500	+	+	+	+	+
106	11.14	Oleic acid I	MFA	$[M-H]^-$	281.248	2.1	281.248, 263.2364, 237.2231	-	+	+	+	+
107	11.17	Pentacosanedioic acid I	DFA	$[M-H]^-$	411.3474	1.5	393.3307, 367.3678, 349.3567	+	+	+	+	+
108	11.24	Stearic acid I	MFA	$[M-H]^-$	283.2642	0.2	283.2642, 265.2568	+	+	+	+	+

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109	11.35	Eicosenedioic acid	DFA	$[M-H]^-$	339.2542	-0.3	321.2497, 295.2707, 277.2547	+	+	+	+	+
110	11.37	Hydroxyhexadecanoic acid II	MFA	$[M-H]^-$	271.2293	1.5	271.2293, 225.2244	+	+	+	+	+
111	11.38	Pentadecenoic acid	MFA	$[M-H]^-$	239.2015	0.8	239.2115, 221.1918	+	+	+	+	+
112	11.48	Linolenic acid	MFA	$[M-H]^-$	277.2173	0.0	259.2143, 233.2348, 211.1382	+	+	+	+	+
113	11.61	Myristic acid	MFA	$[M-H]^-$	227.2015	0.7	227.2015, 209.1939	+	+	+	+	+
114	11.80	Oxotetracosanedioic acid	DFA	$[M-H]^-$	411.3118	-0.5	393.3081, 375.2944, 367.3244, 349.3106	+	+	+	+	+
115	11.80	Palmitamide	FAA	$[M+H]^+$	256.2636	-0.4	116.1119, 105.0730, 102.0963, 88.0805, 77.0431, 69.0752	+	+	+	+	+
116	11.83	Heptadecadienoic acid	MFA	$[M-H]^-$	265.2167	2.3	265.2167, 247.2089	+	+	+	+	+
117	11.88	Ceriporic acid II	DFA	$[M-H]^-$	351.2534	1.9	333.2467, 307.2613, 289.2500	-	+	+	+	-
118	11.88	Eicosadienoic acid I	MFA	$[M-H]^-$	307.2649	-2.0	289.2500, 263.2529, 261.2602	-	-	-	+	-
119	11.97	Heneicosanedioic acid	DFA	$[M-H]^-$	355.285	1.1	337.2845, 311.2908, 293.2897	+	+	+	+	+
120	12.10	Ceriporic acid III	DFA	$[M-H]^-$	351.2534	1.9	333.2467, 307.2613, 289.2500	-	-	-	+	-
121	12.25	Palmitoleic acid I	MFA	$[M-H]^-$	253.2177	-1.6	253.2177, 235.2183	+	+	+	+	+
122	12.49	Ricinoleic acid IV	MFA	$[M-H]^-$	297.2438	-1.0	297.2438, 279.2322, 253.2534, 183.1396, 111.0840, 93.0349	+	+	+	+	+
123	12.51	Oleamide I	FAA	$[M+H]^+$	282.2787	1.4	265.2504, 247.2419, 177.1642, 165.0929, 149.1350, 135.1205, 121.1049, 111.0859, 107.0905, 97.1062,	+	+	+	+	+

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							91.0597, 83.0896, 81.0758, 79.0593, 69.0753, 55.059					
124	12.52	Arachidamide	FAA	$[M+H]^+$	312.3257	1.3	207.0339, 165.0693, 159.1176, 145.1033, 116.0678, 115.0579, 105.0731, 102.0963, 91.0597, 77.0431, 69.0753, 67.05909	+	+	+	+	+
125	12.70	Pentadecanoic acid	MFA	$[M-H]^-$	241.2173	0.0	241.2173, 223.2073	+	+	+	+	+
126	12.70	Palmitic acid I	MFA	$[M-H]^-$	255.2328	0.6	255.2351, 237.2227	+	+	+	+	+
127	12.70	Eicosenoic acid	MFA	$[M-H]^-$	309.2783	5.2	309.2799, 291.2735	+	-	+	+	+
128	12.85	Heptadecanamide I	FAA	$[M+H]^+$	270.2778	4.8	107.0884, 115.0579, 105.0752, 91.0597, 77.04313, 69.0753	+	+	+	+	+
129	13.04	Linoleic acid	MFA	$[M-H]^-$	279.2329	0.4	279.2329, 261.2203, 243.2081	+	+	+	+	+
130	13.19	Docosanedioic acid	DFA	$[M-H]^-$	369.301	0.0	335.3020, 325.3030, 307.2972	+	+	+	+	+
131	13.22	Heptadecanamide II	FAA	$[M+H]^+$	270.2778	4.8	107.0884, 115.0579, 105.0752, 91.0597, 77.04313, 69.0753	+	+	+	+	+
132	13.33	Palmitoleic acid II	MFA	$[M-H]^-$	253.2177	-1.6	253.2177, 235.2183	+	+	+	+	+
133	13.40	Arachidinic acid I	MFA	$[M-H]^-$	311.295	1.9	311.2950, 293.2899, 267.2970	+	+	+	+	-
134	13.41	Heptadecenamide	FAA	$[M+H]^+$	268.2641	-2.3	175.1539, 165.0745, 133.1033, 115.0573, 111.0876, 105.0731, 97.1032, 91.0578, 79.0598, 77.0431, 69.0763, 67.0591	+	+	+	+	+
135	13.41	Behenamide I	FAA	$[M+H]^+$	340.3575	-0.3	144.0966, 130.0794,	+	+	+	+	+

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							116.1097, 117.0733, 102.0963, 88.0805					
136	13.48	Palmitoleamide II	FAA	$[M+H]^+$	254.2481	-1.0	237.2203, 219.2092, 165.0745, 146.6038, 135.1205, 121.1049, 116.0634, 111.0859, 109.1058, 107.0884, 105.0752, 95.0898, 93.0747, 91.0577, 83.0915, 81.0758, 79.0593, 77.0431, 69.0753, 67.0591	+	+	+	+	+
137	13.51	Erucamide I	FAA	$[M+H]^+$	338.3438	-6.1	321.2128, 303.3040, 177.1675, 163.1533, 149.1386, 135.1241, 121.1086, 111.1242, 97.1100, 83.0933, 81.0795, 69.0789, 55.0626	+	+	+	+	+
138	13.57	Heptadecenoic acid I	MFA	$[M-H]^-$	267.2331	-0.4	267.2331, 249.2276	+	+	+	+	+
139	13.66	Palmitoleic acid III	MFA	$[M-H]^-$	253.2177	-1.6	253.2177, 235.2183	+	+	+	+	+
140	13.70	Palmitic acid II	MFA	$[M-H]^-$	255.2328	0.6	255.2351, 237.2227	+	+	+	+	+
141	13.77	Heneicosanoic acid	MFA	$[M-H]^-$	325.3113	-0.3	325.3113, 307.3052, 281.3201	+	+	+	+	+
142	13.77	Heptadecanamide III	FAA	$[M+H]^+$	270.2778	4.8	107.0884, 115.0579, 105.0752, 91.0597, 88.0805, 77.0431, 69.0753	+	+	+	+	+
143	13.79	Oleamide II	FAA	$[M+H]^+$	282.2789	0.7	265.2504, 247.2419, 177.1642, 165.0929, 149.1350, 135.1205,	+	+	+	+	+

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							121.1049, 111.0859, 107.0905, 97.1062, 91.0597, 83.0896, 81.0758, 79.0593, 69.0753, 55.059					
144	13.82	Heptadecenoic acid II	MFA	$[M-H]^-$	267.2331	-0.4	267.2331, 249.2276	+	-	+	+	+
145	14.12	Arachidinic acid II	MFA	$[M-H]^-$	311.295	1.9	311.2950, 293.2899, 267.2970	+	+	+	+	+
146	14.30	Palmitic acid III	MFA	$[M-H]^-$	255.2328	0.6	255.2351, 237.2227	+	+	+	+	+
147	14.37	Heptadecanoic acid I	MFA	$[M-H]^-$	269.2482	1.5	269.2482, 251.2439, 225.2305	+	+	+	+	+
148	14.39	Tricosanedioic acid	DFA	$[M-H]^-$	383.3176	-2.4	365.3100, 339.3257, 321.3157	+	+	+	+	+
149	14.41	Octadecanedioic acid VI	DFA	$[M-H]^-$	313.2375	3.19	295.2280, 269.2425, 251.2289	+	+	+	+	+
150	14.65	Stearamide	FAA	$[M+H]^+$	284.2957	-3.2	207.0338, 116.1119, 102.0851, 88.0805, 81.0739, 74.0649, 69.0753	+	+	+	+	+
151	14.67	Erucamide II	FAA	$[M+H]^+$	338.3401	4.9	321.2128, 303.3040, 177.1675, 163.1533, 149.1386, 135.1241, 121.1086, 111.1242, 97.1100, 81.0795, 69.0789, 55.0626	+	+	+	+	+
152	14.87	Stearic acid II	MFA	$[M-H]^-$	283.2642	0.2	283.2642, 265.2568	+	+	+	+	+
153	14.87	Ocatdecanoic acid II	MFA	$[M-H]^-$	281.2478	2.8	281.2478, 263.2364	+	+	+	+	+
154	14.95	Tetracosanoic acid	MFA	$[M-H]^-$	367.3573	2.4	367.3573	+	+	+	-	-
155	14.95	Behenamide II	FAA	$[M+H]^+$	340.3575	-3.0	144.0966, 130.0794, 116.1097, 117.0733, 102.0963, 88.0805	+	+	+	+	+
156	15.04	Nonadecanoic acid	MFA	$[M-H]^-$	297.2798	0.3	297.2798, 279.2667	+	+	+	+	-

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157	15.16	Eicosenamide	FAA	$[M+H]^+$	310.3092	3.8	283.2647, 256.2669, 211.1508, 177.1669, 165.0719, 149.1350, 135.1205, 121.1049, 111.0859, 107.0884, 105.0752, 102.0942, 97.1063, 93.074, 91.0597, 81.0739, 79.0593, 77.0431, 69.0753, 55.0592	+	+	+	+	+
158	15.50	Tricosanoic acid	MFA	$[M-H]^-$	353.3405	5.7	353.3405	+	+	-	+	-
159	15.56	Eicosadienoic acid II	MFA	$[M-H]^-$	307.2649	-2.0	289.2500, 263.2529, 261.2602	+	+	+	+	+
160	15.70	Docosanoic acid (Behenic acid)	MFA	$[M-H]^-$	339.3272	-0.9	339.3272, 295.3106, 139.0407, 119.0496	+	+	+	-	+
161	15.70	Nonadecanamide II	FAA	$[M+H]^+$	298.3085	6.4	145.1033, 133.1057, 119.0907, 105.0752, 116.0642, 91.0597, 88.0845, 77.0431, 69.0745	+	+	+	+	+
162	15.70	Linoleamide II	FAA	$[M+H]^+$	280.2628	2.4	263.2333, 245.2219, 161.1178, 133.0839, 119.0683, 109.0826, 95.0667, 91.0353, 81.0513, 79.0352	-	+	-	-	-
163	15.81	Tetracosanedioic acid	DFA	$[M-H]^-$	397.3307	4.03	379.3195, 353.3482, 335.3321	+	+	+	+	+
164	15.87	Heptadecanoic acid II (Margaric acid)	MFA	$[M-H]^-$	269.2482	1.5	269.2482, 251.2439, 225.2305	+	+	+	+	+
165	16.07	Henicosanamide	FAA	$[M+H]^+$	326.3426	-2.6	165.0772, 159.1202, 121.1027, 109.1058, 105.0731, 102.0579,	-	+	-	+	+

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							91.0597, 69.0753, 67.0624						
166	16.23	Pentacosanedioic acid II	DFA	[M-H]-	411.3474	1.16	393.3307, 367.3678, 349.3567	+	-	+	+	+	

2.1.1. CTL1) 100bar/55°C; CTL2) 150bar/55°C; CTL3) 250bar/55°C, CTL4) 300bar/55°C; CTL5) 500bar/55°C; MFA) Monocarboxylic fatty acid; DFA) Dicarboxylic fatty acid; FAA) Fatty acid amide; Phenolic Compound (PC); Organic Compound (OC); I-VI, Indicates presence of isomers; a) Identification based on mass spectrometry data and comparison with the online database with the reference standards; (+)/(-) sign indicates presence/absence of compound in corresponding extract.Identification of Fatty Acids

FAs are a group of chemical compounds that contains carboxylic acid functional group (-COOH) at one end of their hydrocarbon chain. In this study two types of FAs detected, one is monocarboxylic FAs containing one -COOH group while the second one is dicarboxylic FAs, containing two -COOH group. Total 66 peaks have been extracted from TICs and tentatively identified as monocarboxylic FAs. The 19 peaks out of 66 have been observed as saturated monocarboxylic FA, as they contain no double bonds in their carbon chain, based on their HRMS, empirical formula and double bond equivalents (DBE). Saturated FAs showed a positive relationship between retention time and the length of FA which indicates the elusion time increases as the carbon length of fatty acid increases. They showed strong $[M-H]^-$ ion in both channels i.e., low-energy CID and high-energy CID. The lack of detection of fragment ions of the linear hydrocarbon backbone is steady with the previous reports [44]. In high-energy CID channel, the $[M-H]^-$ ion did not lead to decrease when using the highest energy in MS^E experiment up to 85eV. They showed characterisation ions corresponding to $[M-H-18]^-$, $[M-H-46]^-$ and $[M-H-44]^-$ ions, resulting from a loss of one water molecule, loss of -HCOOH, and decarboxylation from quasimolecular ions, respectively (Figure 2). Eleven peaks at 66, 67, 74, 76, 106, 108, 112, 129, 146, 153 and 158 have been detected as most abundant monocarboxylic FAs in five different SC-CO_2 CTL extracts and tentatively identified as 9-hydroxy-12,14,16-octadecatrienoic acid (t_R = 6.42 min), hydroxyoctadecatrienoic acid (t_R = 6.57 min), 13-hydroxy-9,11-octadecadienoic acid (t_R = 7.49 min), hydroxyoctadecatrienoic acid II (t_R = 8.30 min), oleic acid (t_R = 11.14 min), stearic acid I (t_R = 11.24 min), linolenic acid (t_R = 11.48 min), linoleic acid (t_R = 13.04 min), palmitic acid III (t_R = 14.30 min), ocatdecanoic acid II (t_R = 14.87 min) and tetracosanoic acid (t_R = 15.50 min), respectively, based on exact mass and MS/MS data supporting with previous reports. Monocarboxylic FAs have been detected the most abundant in CTL4 (300bar/55°C) SC-CO_2 extract.

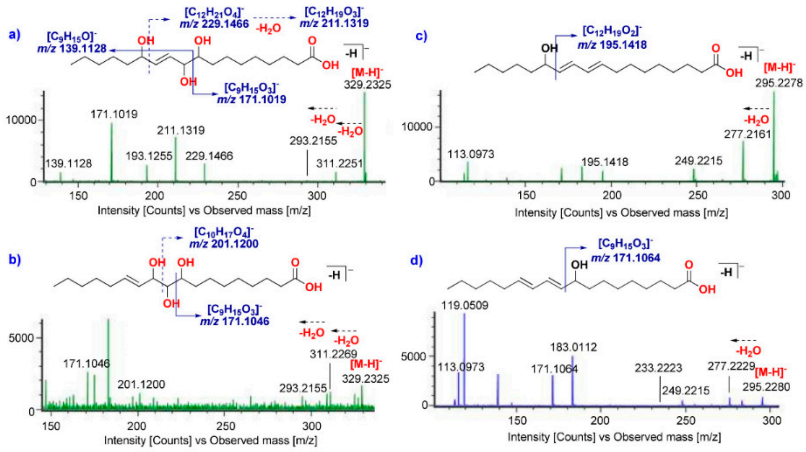

Figure 2. MS/MS spectra of hydroxyl derivatives of monocarboxylic fatty acids a) 9,10,13-trihydroxy-11-octadenoic acid, b) 9,10,11-trihydroxy-12-octadenoic acid, c) 13-hydroxy-9,11-octadecadienoic acid, d) 9-hydroxy-10,12-octadecadienoic acid.

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Similarly, total 52 dicarboxylic fatty acids have been tentatively identified in CTL extracts. Monitoring of high-energy CID channel, fragment spectra revealed no fragmentation for many fatty acids while the formation of $[M-H-18]^-$, $[M-H-44]^-$, and $[M-H-18-44]^-$, ions were observed in low intensity, resulting from a loss of water molecule, decarboxylation and simultaneous loss of water and CO_2 molecules respectively (Table 1 and Figure 2). 28 peaks out of 52 have been tentatively identified as saturated dicarboxylic fatty acids having carbon chain length 7 to 25. The $[M-H]^-$ ion of 13 peaks were tentatively identified as unsaturated dicarboxylic FA having one unsaturation while three peaks at 105 (t_R = 11.10 min), 117 (t_R = 11.88 min) and 120 (t_R = 12.10 min) having two unsaturation. Eight peaks have been identified as oxygenated dicarboxylic FA based on their exact mass, empirical formula, DBE, characteristic fragment ions and with the literature support. Peaks 15, 55, 56, 57, 59, 107, 119, 130, 148, 149 and 163 have been identified as the most abundant peaks corresponding to azelaic acid (*m/z* 187.0982), octadecenedioic acid I (*m/z* 311.2224), octadecanedioic acid (*m/z* 313.2375), octadecenedioic acid II (*m/z* 311.2224), octadecenedioic acid III (*m/z* 311.2224), pentacosanedioic acid (*m/z* 411.3474), heneicosanedioic acid (*m/z* 355.2850), docosanedioic acid (*m/z* 369.3010), tricosanedioic acid (*m/z* 383.3176), octadecanedioic acid VI (*m/z* 313.2375), and tetracosanedioic acid (*m/z* 397.3307), respectively. Dicarboxylic FAs have been also detected maximum intensity in CTL4 (300bar/55°C) SC-CO_2 extract.

2.1.1. Identification of Fatty Acid Amides

Twenty seven peaks were observed as the $[M+H]^+$ ion in positive ion mode (ESI+) and their empirical formula assigned C, H, O and single N atom that are present in the structure. Out of 27 peaks, 16 peaks were tentatively identified as saturated FAAs based on their exact mass, empirical formula and one double bond equivalent (DBE) and they were similar regardless of the acyl chain length ranging from C_9 to C_{22}. They were discovered to have similar fragment ion peaks containing carbon, hydrogen, oxygen, and nitrogen, which were fragments having the amide head group with varied in the acyl fragmentation site. The MS/MS spectra of the $[M+H]^+$ ion of these peaks showed the fragment ions at the *m/z* 116.1123 $[C_6H_{14}NO]^+$, *m/z* 102.0897 $[C_5H_{12}NO]^+$, *m/z* 88.0739 $[C_4H_{10}NO]^+$ and *m/z* 74.0631 $[C_3H_8NO]^+$ corresponding to the cleavage of acyl chain (Figure 3), accordingly these peaks were identified as lauramide (t_R = 6.40 min), palmitamide (t_R = 11.80 min), myristamide (t_R = 9.29 min), stearamide (t_R = 14.65 min), respectively [25,45]. The empirical formula of the $[M+H]^+$ ion of eight peaks (83, 123, 134, 136, 137, 143, 151 and 157) were showed two double bond equivalent (DBE), one corresponds to amide group and one correspond to unsaturation in acyl chain. The MS/MS spectra of these compounds showed fragments correspond to the cleavage of acyl fragmentation site. Palmitoleamide (C16:1, t_R = 9.11 min) (*m/z* 254.2483), heptadecenamide (C17:1, t_R = 13.41 min) (*m/z* 268.2641), oleamide (C18:1, t_R = 12.51 min) (*m/z* 282.2787), eicosenamide (C20:1, t_R = 15.16 min) (*m/z* 310.3092) and erucamide (C22:1, t_R = 13.51 min) (*m/z* 338.3438) were tentatively identified as monosaturated FAAs in CTL extracts based on their exact mass and literature support [46]. In addition to saturated and monosaturated FAAs, di- and trisaturated FAAs were also identified in CTL extracts based on their exact mass, empirical formula and DBE. Peaks 100 (t_R = 10.66 min) at *m/z* 280.2631 and 162 (t_R = 15.70 min) at *m/z* 280.2628 were observed as $[M+H]^+$ ion with empirical formula $[C_{18}H_{34}NO]^+$ and DBE three. The MS/MS spectra of these peaks showed similar fragment ions, showing presence of isomeric peaks. These peaks were tentatively assigned as linoleamide (C18:2) based on their fragment ion reported earlier [46]. Peak 87 (t_R = 9.14 min) at *m/z* 278.2471, empirical formula $[C_{18}H_{32}NO]^+$ showed four DBE (i.e., three double bonds in acyl chain) was tentatively assigned as linolenamide (C18:3) based on their fragment ions which were observed due to cleavages of acyl chain.

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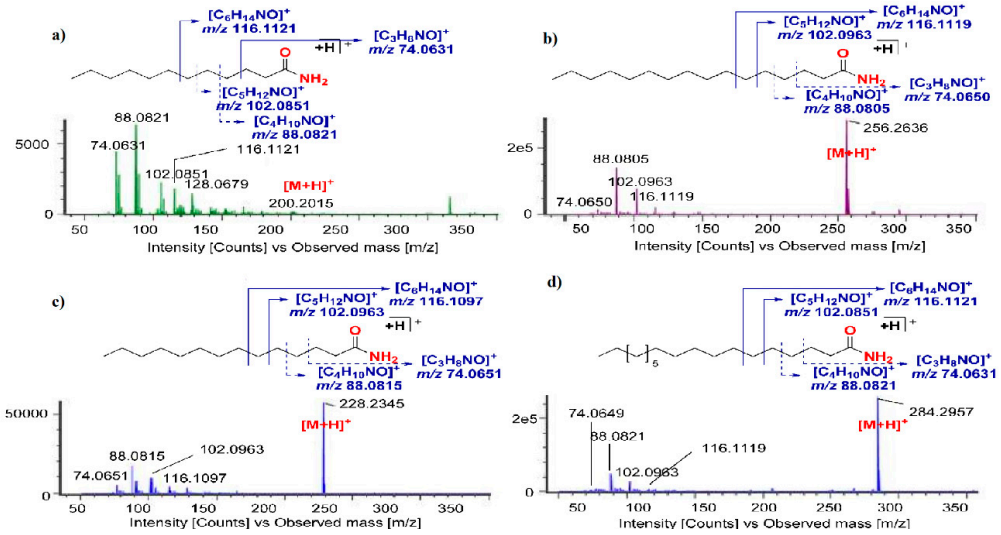

Figure 3. MS/MS spectra of fatty acid amides a) Lauramide, b) Palmitamide, c) Myristamide, d) Stearamide.

2.1.1. Identification of Cinnamic Acid Derivatives

Apart from FAs and FAAs, twelve compounds have been tentatively identified as cinnamic acid derivatives based on their HR-MS, MS/MS and literature support. Nine peaks out of twelve detected as $[M-H]^-$ ion in (-)-ESI while two peaks were detected as $[M+H]^+$ ion. Peak 50 (t_R = 3.21 min) at *m/z* 117.0695 was observed as $[M+H-H_2O]^+$ and confirmed as cinnamyl alcohol with the reference compound. Peak 34 (t_R = 3.06 min) at *m/z* 147.0457 was observed as $[M-H]^-$ ion with empirical formula $[C_9H_8O_2]^-$ confirmed as cinnamic acid, which was supported by its characteristic fragment ions of *m/z* 103.0553 $[M-H-CO_2]^-$ (Figure 4). Peak 31 (t_R = 2.94 min), 41 (t_R = 3.48 min) and 53 (t_R = 5.08 min) were confirmed as coumarin, *trans*-cinnamaldehyde and *cis*-cinnamaldehyde with the reference compounds as $[M+H]^+$ ion at *m/z* 147.0446 $[C_9H_7O_2]^+$, 133.0648 $[C_9H_9O]^+$ and 133.0649 $[C_9H_9O]^+$, respectively. These compounds were detected as the major component in CTL extracts. Peak 26 (t_R = 2.82 min) was detected as $[M-H]^-$ ion at *m/z* 263.1296 $[C_{15}H_{19}O_4]^-$ and tentatively identified as plant hormone abscisic acid with the assistance of library and database [7]. They were found most intense in CTL2 (150bar/55°C) SC-CO_2 extract.

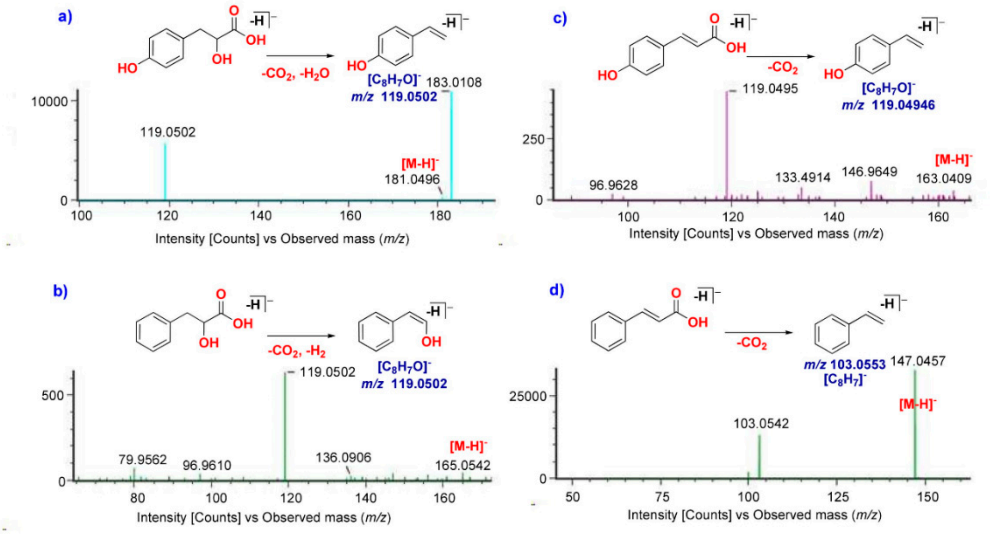

Figure 4. MS/MS spectra of a) 3-(4-hydroxyphenyl)lactic acid, b) 3-phenyllactic acid, c) 4-hydroxycinnamic acid and d) cinnamic acid.

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2.1. Chemometric Analysis

Data representing the chemometric distribution of fatty acid and fatty acid amides obtained in positive and negative ionization mode in UPLC-Q-TOF-MS from the SC-CO_2 extracts at different pressure are graphically represented in Figure 5 and Figure 6, and the normalize data used to draw these diagrams are given in Table S1. From the Figure 5a and 6a, it can be observed that the SC-CO_2 extracts behaves differently in both modes. Two principal components (PC1 and PC2) contribute to 91.9% and 86.6% variation for both positive and negative mode ionization mode, respectively.

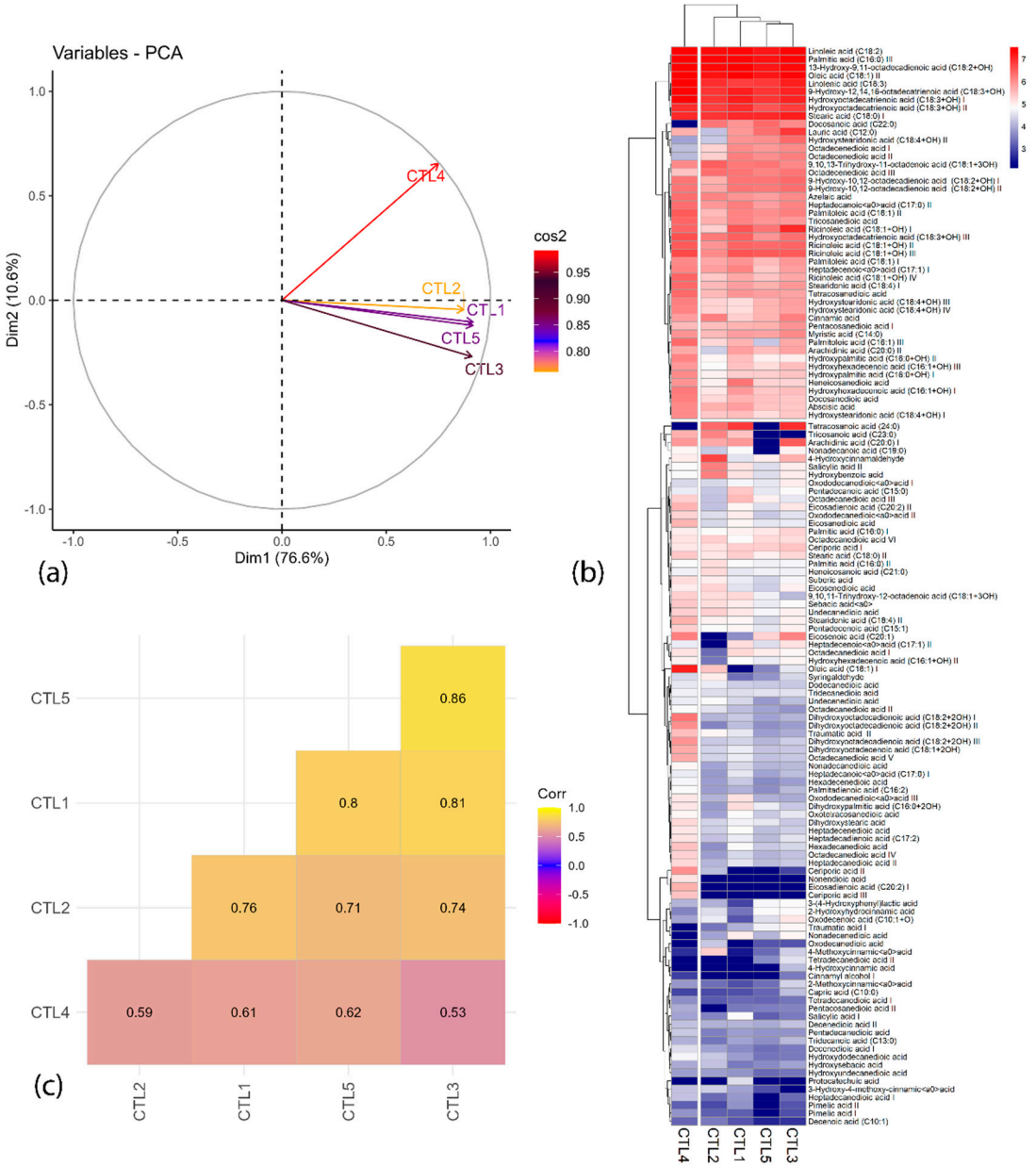

Figure 5. Data representing the (a) PCA biplot (b) heatmap representing the cluster hierarchical analysis and (c) correlation among different SC-CO_2 extracts of *C. tamala* leaf in (-)-ESI mode.

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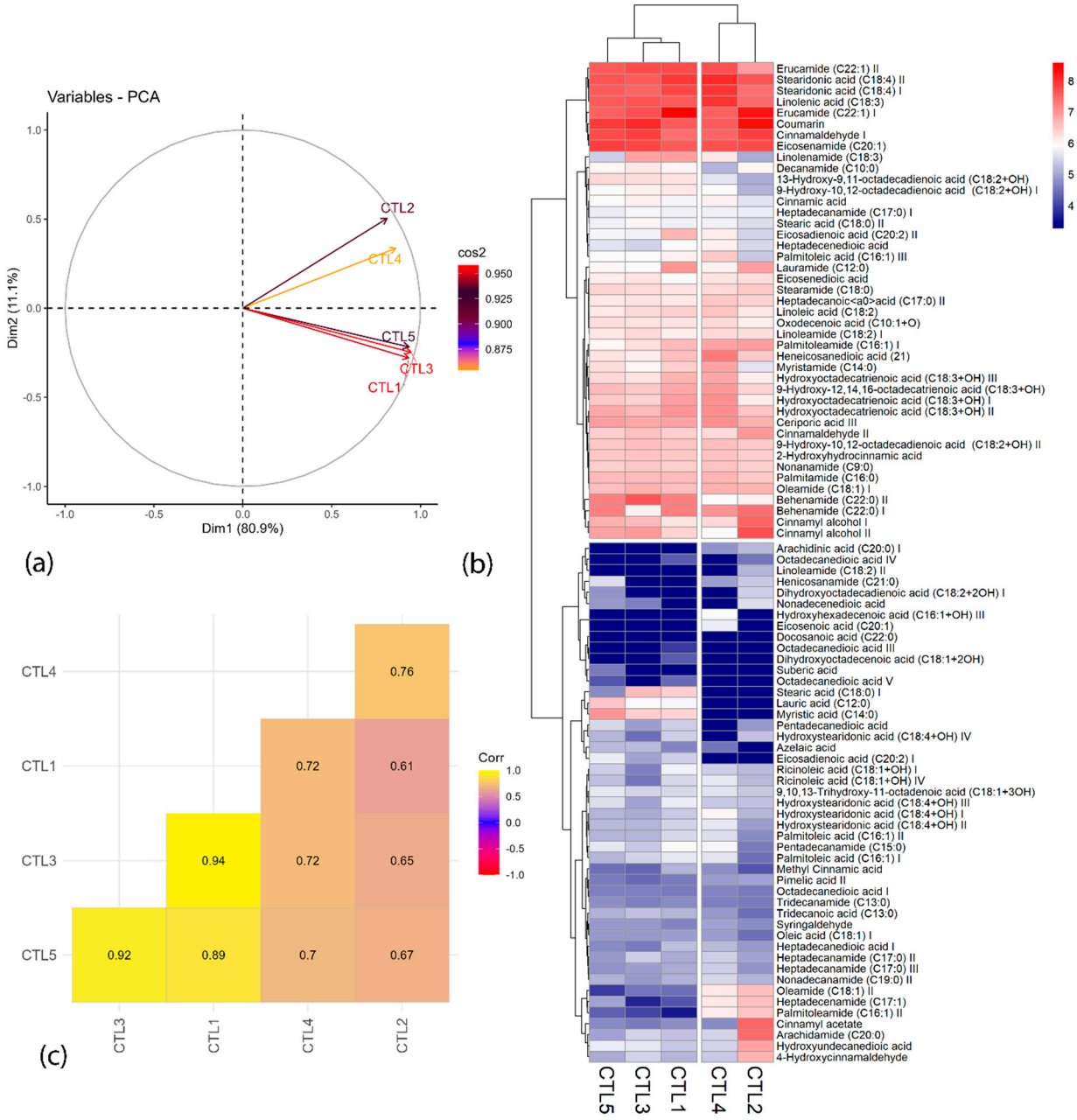

Figure 6. Data representing the (a) PCA biplot (b) heatmap representing the cluster hierarchical analysis and (c) correlation among different SC-CO_2 extract of *C. tamala* leaf in (+)-ESI mode.

In negative ionization mode among all extracts (CTL1-CTL5), CTL4 extract behaves differently and contribute to the maximum variation from the other SC-CO_2 extracts, whereas in positive ionization mode (Figure 6a) a least variation was observed between CTL2 and CTL4 as they are clustered together and other three extracts are clustered together. These results are supported by multivariate heatmap (Figure 5b and Figure 6b) clusters drawn based of ward clustering method where the rows and column are distanced apart based on the Euclidean distance. From the heat map it can be observed that CTL4 extract is grouped in a single separate cluster whereas the other three extracts behave similarly and are grouped in a separate cluster. Correlation plots (Figure 5c and 6c) on the other hand shows a correlation between the qualitative analysis of different extracts. From the Figure 6c a good correlation (R^2>0.7) can be observed between the CTL3, CTL4 and CTL5, whereas a low correlation of these with CTL2 and CTL4 extracts which are separating them from each other. Whereas, for negative ESI mode CTL4 extract behave differently from other extracts and exhibit a

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low correlation (R^2 <0.7) with other SC-CO_2 extracts (Figure 5c). Venn diagram was constructed to summarise the number of metabolites that differentially accumulated in different SC-CO_2 extracts of CTL leaves, which relatively overlap between each set of metabolites (Figure 7). Total 166 metabolites were identified in leaves extracts, out of these 142 metabolites were common to all five extracts, projected in the centre of diagram. In CTL1 extract (100bar/55°C) protocatechuic acid was found exclusively. Biologically, protocatechuic acid having anti-inflammatory, neuroprotective, antiviral, anticancer, antiaging activities; protection from metabolic syndrome; and preservation of liver, kidneys, and reproductive functions [47]. CTL2 (150bar/55°C), has linoleamide II as a fatty acid amide which has been reported to exert the sedative and hypnotic effects, and inhibits the migration of cancer cells in human [25,48]. An exclusive compound 4-hydroxycinnamic acid (HCA) of CTL3 (250bar/55°C), having health-beneficial effects and uses as cosmeceutical ingredients. HCA's mainly recognized as potent antioxidants and involved in the prevention of several diseases connected to oxidative stress, i.e., cardiovascular and neurodegenerative diseases, and cancer [49]. Nonanedioic acid is an alpha, omega-dicarboxylic acid having a role as an antibacterial agent, an antineoplastic agent, a dermatologic drug and a plant metabolite. Nonendioic acid, eicosadienoic acid I, ceriporic acid III were identified in CTL4 (300bar/55°C). CTL5 (500bar/55°C) extract did not have any exclusive compound, further it has least 151 compounds as compare to other extracts. The less number of compounds may be due to the SC-CO_2 extraction parameters (pressure/temperature), because high selectivity of lipophilic bioactive compounds can be easily achieved by lowering the pressure and/or temperature in the separator [50]. Based on the chemometric data we can observed that CTL4 extract have behaved differently from the other SC-CO_2 extracts of CTL in both ionization mode and make it the optimum extraction method for extraction of SC-CO_2 extract rich in fatty acids, fatty amides and cinnamic acid derivatives.

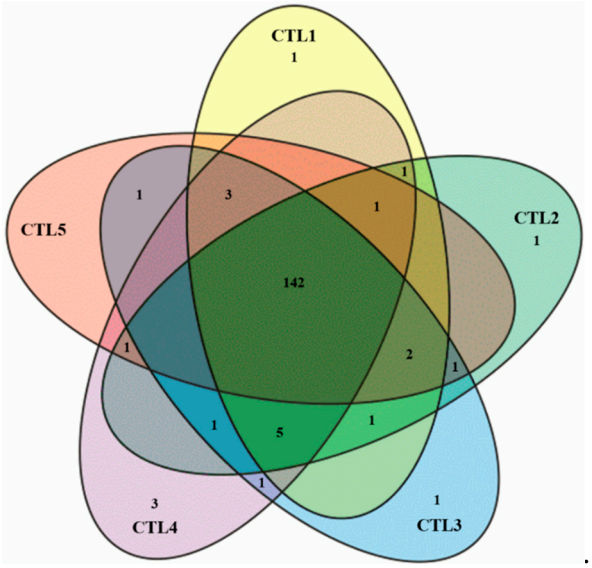

Figure 7. Venn diagram representing untargeted metabolites distribution in different SC-CO_2 extracts of CTL leaves.

3. Experimental

3.1. Chemicals and Materials

Cinnamaldehyde (93%), cinnamyl alcohol (98%), cinnamyl acetate (99%) and coumarin (99%) were purchased from (Sigma Aldrich, St Louis, MO, USA). Acetonitrile and methanol (LC-MS grade) were obtained from J.T. Baker (Deventer, Netherlands). Formic acid (LC-MS grade) was obtained from Sigma-Aldrich (St. Louis, MO, USA). The type 1 grade water, produced by Adrona Crystal, was used for all experimental procedures. High-purity gases (99.995%) for extraction were obtained from Linde (Dehradun, Uttarakhand, India).

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3.2. Plant Materials

C. tamala leaves were collected from the campus of Centre for Aromatic Plants (CAP) under Doon Valley climatic condition of Uttarakhand (30º36′22.13″ N, 77º84′95.38″ E) in month of October, 2021. The plant was authenticated by plant taxonomist Dr. Sunil Sah (Senior Scientist) and voucher specimen was deposited in the Herbarium of the centre. Leaves were washed thoroughly with normal tap water followed by Type-1 grade water and dried at room temperature (25-30°C). All dried leaves were crushed into coarsely ground powder (particle size <1.0 mm, 18 mesh) using pulveriser machine (Decibel, Lab Willey Grinder, and Model No. DB 5581-4, New Delhi, India) and stored in airtight container at room temperature until analysis. The moisture content of the powder was estimated to be 6.3±2.8% on a dry weight basis.

3.3. Supercritical Fluid (CO_2) Extraction and Sample Preparation

The coarsely ground leaves powder (2.5 kg) was charged into a 12 L extraction vessel (SS316) with maintained the constant flow rate of CO_2 (food grade) at 0.9-1.0 kg/min (Thar SFE 2000-2-FMC50, Thar Instruments, Pittsburgh, Pennsylvania, USA) for the first 15 min and the system was on static period. After completion of static period the system was run at continuous flow of CO_2 (1.0 kg/min, 120 min), which connected to a collection chambers (separators 1 and 2), where pressure was reduced to 8.0 MPa (80 bar). The optimized extraction parameters, temperatures (55°C) and desired pressure (100, 150, 250, 300, and 500 bar) were applied with triplicate for each set of experiments. The pressure in both the extraction and separation vessels was controlled by pressure regulator valve. The extract in form of oleoresin was collected from separator and average amount (%) of extracts were calculated. All extracts were stored in amber-coloured screw capped glass vials at 4°C until further analysis. 1.0 mg/mL solution of the dried SC-CO_2 CTL extract was prepared in methanol and filtered through a 0.22 µm nylon syringe filter (AXIVA Sichem Biotech, Delhi, India) prior to analysis.

3.4. UPLC-Q-TOF-MS^E Analysis

The UPLC analysis was performed on a Waters Acquity UPLC™ system (Waters, Milford, MA, USA) interfaced with a Waters Xevo G2-XS Quadrupole time-of-flight mass spectrometer (Waters Corporation, Milford, MI, USA) equipped with an electrospray ion source. The Waters Acquity UPLC™ system was equipped with a binary solvent manager, sample manager, column oven, and photodiode array detector. A Waters ACQUITY UPLC HSS T3 analytical column (100 mm × 2.1 mm, 1.8 µm) was used for chromatographic separation of compounds in SC-CO_2 extract of CTL. The chromatographic parameters were set as follows: column temperature, 40°C; flow rate, 0.3 mL/min; temperature of the autosampler, 4°C; mobile phase, solvent A (0.1% formic acid in water) and solvent B (acetonitrile). Linear gradient was applied for elution as follows: 0-1 min, 10%-30% B; 1-2 min, 30%-50% B; 2-8 min, 50%-70% B; 8-13 min, 70%-85% B; 13-15 min, 85% B; 15-19 min, 85%-10% B; 19-20 min, 10% B. The sample injection volume was 2 µL. The PDA spectra were obtained by scanning the samples in the range of 190-400 nm.

The mass spectrometric (MS) data was acquired in MS^E experiment under sensitivity mode in both positive and negative electrospray ionization (ESI+/-). The acquisition parameters for MS were set as follows: capillary voltage, 2.5 kV; sample cone voltage, 30.0 V; source temperature, 120°C; desolvation temperature, 450°C; cone gas flow rate, 50 L/h; desolvation gas flow rate, 900 L/h; source offset, 80V; acquisition time 20 min for both polarities. The low-energy collision-induced dissociation (CID) of the MS^E experiment was 6 eV, the high-energy CID was 30-85 eV, and the scanning range was *m/z* 50-1,200. Nitrogen was used as drying, nebulising and collision gas. Leucine Enkephalin (200 pg/mL, 5 µL/min) was used as a real-time correction fluid generating a reference ion for the positive ion mode [$(M+H)^+$ *m/z* 556.2726] and negative ion mode [$(M-H)^-$ *m/z* 554.2620]. The lock-spray scan time was set at 0.25 s with an interval of 30 s. The data was acquired and processed by Waters Connect UNIFI version 3.0.0.15.

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3.5. Chemometric Analysis

For the analysis of qualitative data, the PCA , correlation plots and hierarchical cluster analysis heatmap diagrams were made with the open-source R software by using ggplot2 (https://ggplot2.tidyverse.org/), factoextra (https://cran.r-project.org/web/packages/factoextra/index.html), and ggcorrplot (https://cran.r-project.org/web/packages/ggcorrplot/readme/README.html) packages from the CRAN (Comprehensive R Archive Network) database. Venn diagrams were generated using a web tool.

Conclusions

Chromatographic (UPLC-Q-TOF-MS^E) separation with chemometric analysis permitted to determine the metabolites composition and classify the SC-CO_2 extracts of *C. tamala* leaves collected from Doon Valley climatic condition of Uttarakhand. A total 166 metabolites, of which 118 fatty acids, 27 fatty amides, and 21 (phenolic and organic) essential metabolites identified in both positive and negative ion mode, out of which 142 compounds are common and found in all five extracts. The ability to employ the high-resolution MS provided the capability for tentatively identification of major compounds. PCA and cluster hierarchical analysis provide a statistical model that clearly discriminate the chemical profile of analysed extracts and allowed the selection of SC-CO_2 extract rich in fatty acids, fatty amides and other bioactive constituents for the use of food and nutraceutical industries. As per authors knowledge, this is the first study regarding the detection of different metabolites in SC-CO_2 extracts of *C. tamala* leaf by UPLC-Q-TOF-MS, and results open the new dimensions where the work can be further proceed.

Author Contributions: H.L. and N.K.C. conceptualization and methodology. A.K. and V.B. designed the experiments, acquired and analyzed data. A.K. and L.A. wrote the paper and interpreted the data. S.Z.H. helped in statistical experiment, editing manuscript. H.L. and N.C. provided guidance for the experiments. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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