CHAPTER 3

Results and discussion

3.1 General

Fresh, steamed and fermented leaves of Assam tea were collected during spring to autumn (May to November, 2015) in Chiang Rai, Thailand. The physical appearance of each sample was leaf colour, collected temperature and pH value are shown in Table 3.1. During the submerged fermentation, the leaf color varied from slight yellow-green to dark brown.

The pH value has a positive correlation with acidity and is usually chosen as the main quality attribute and fermentation index of pickles.[80] Table 3.1, the pH value of fermented leaves occurred at four distinct stages during 150 days fermentation. First, the pH rapidly decreased from 5.53 to 4.21 very quickly with a significance ($P<0.01$) from fresh leaves to 15 days fermented leaves. Second, the pH gradually increased from 4.21 to 4.38 with a significance difference ($P<0.01$) from 15 to 60 days fermented leaves. Third, the pH significantly decreased ($P<0.01$) from 4.38 to 4.31 from 60 to 90 days fermented leaves. Finally, the pH gradually increased from 4.31 to 4.51 with a significance difference ($P<0.01$) from 90 to 150 days fermented leaves. The pH value of fermented water occurred at four distinct stages during 150 days fermentation. First, the pH rapidly decreased from 5.45 to 4.35 very quickly with a significance ($P<0.01$) from steamed water to 45 days fermented water. Second, the pH gradually increased from 4.35 to 4.57 with a significance difference ($P<0.01$) from 45 to 60 days fermented water. Third, the pH significantly decreased ($P<0.01$) from 4.57 to 4.48 from 60 to 90 days fermented water. Finally, the pH gradually increased from 4.48 to 4.59 with a significance difference ($P<0.01$) from 90 to 150 days fermented water. The fluctuation of pH values in fermented tea should be the result of the accumulated acid secreted by microbes and the transformation of tea components during the fermentation process.[81]

3.2 Volatile oils

3.2.1 Extract of volatile oils

Volatile oils from fresh, steamed, and fermented leaves (15, 30, 45, 60, 90,120, and 150 days) were extracted by SDE technique. As the results shown in Table 3.2, 150 days fermented leaves possessed the highest of percentage yield, followed by 120, 60, 90 and 45 days fermented leaves, respectively. The 30 days fermented leaves had the lowest yield. The majority of oils have been shown yellow colour.

Table 3.1 Physical properties of fresh, steamed, fermented leaves, steamed and fermented water from Assam tea

Samples	Collection		Colour	pH value	
	Days	Temperature (°C)		Leaves	Water
Fresh	19/05/15	32.5	green	5.53^{f}	-
Steamed	19/05/15	32.0	slight yellow-green	5.36^{e}	5.45^{f}
Fermented					
15 days	05/06/15	35.0	slight yellow-green	4.21^{a}	4.47^{b}
30 days	30/05/15	36.5	yellow-green	4.30^{b}	4.37^{a}
45 days	05/07/15	37.0	dark yellow-green	4.32^{b}	4.35^{a}
60 days	20/07/15	32.5	yellow-brown	4.38^{c}	4.57^{d}
90 days	19/08/15	32.0	dark yellow-brown	4.31^{b}	4.48^{b}
120 days	18/09/15	33.0	brown	4.34^{b}	4.49^{c}
150 days	18/10/15	33.0	dark brown	4.51^{d}	4.59^{e}

Values with different letters (a-f) within column of each solvents are significantly different at $P<0.01$

Table 3.2 Percentage yield (%yield) of volatile oils from Assam tea

Samples	Colour	% yield (w/w)
Fresh leaves	pale yellow	0.08
Steamed leaves	light yellow	0.04
Fermented leaves		
15 days	pale yellow	0.11
30 days	light yellow	0.07
45 days	pale yellow	0.12
60 days	pale yellow	0.15
90 days	pale yellow	0.14
120 days	yellow	0.22
150 days	yellow	0.24

3.2.2 Determination of chemical components of the volatile oils

Volatile constituents in fresh, steamed and fermented leaves (15, 30, 45, 60, 90, 120 and 150) of *C. sinensis* var. *assamica* were analyzed using GC-MS technique. The peak identification of essential constituents was mainly based on mass spectral data comparison with database libraries including, NIST 05 (National Institute of Standards and Technology, Gaithersburg, MD, USA), and Wiley 7n (Wiley, New York, USA). The retention indices data were calculated from retention times and compared to their retention indices reported by Adams 2007 in the literature reference. Moreover, the percentage area of compounds were calculated from total ion chromatogram (TIC) by a computerized integrator.

The volatile compounds of fresh, steamed, and fermented leaves could be classified into the following eight types, included alcohols, hydrocarbons, esters, acids, aldehydes, ketones, phenols and others. The volatile compounds of nine samples were denoted by the numbers shown in Table 3.3.

A total of 85 compounds representing 94.59% of fresh leaves were identified. These were including acids (5), hydrocarbons (17), alcohols (20), aldehydes (7), ketones (11), esters (14), phenols (1), and others (8) compounds. These compounds mainly

including methyl palmitate (16.16%), phytol (10.88%), methyl linolenate (7.08%) and *(E)*-linalool oxide (furanoid) (4.85%). The major class of compounds were ester (31.33%), alcohol (30.27%) and ketones (13.33%). For steamed leaves, the volatile compounds identified were 70 constituting a total content of 95.76%. These were including 1 acids, 14 hydrocarbons, 17 alcohols, 5 aldehyde, 10 ketones, 13 esters, 2 phenols, and 8 others compounds. The most abundance compounds were phytol (33.33%), linalool (18.26%), α-terpineol (6.08%) and geraniol (5.57%). Alcohol was a major class of compounds (73.10%). Eighty-nine compounds constituting 95.66% of all the volatile compositions were characterized from 15 days fermented leaves oil. These were including acids (7), hydrocarbons (16), alcohols (20), aldehydes (5), ketones (13), esters (17), phenols (3), and others (8) compounds. The dominant compounds were methyl linolenate (25.07%), methyl palmitate (23.07%), and methyl linoleate (15.01%). The major class of compounds were ester (69.32%) and alcohol (11.50%). For 30 days fermented leaves, a total of 87 components consisting of acids (9), hydrocarbons (15), alcohols (21), aldehydes (4), ketones (11), esters (15), phenols (4), and others (8) compounds were identified, representing 91.18% of oil obtained. *p*-Ethylphenol, linalool, ethyl hexadecanoate, methyl palmitate, linoleic acid ethyl ester and *(Z)*-linalool oxide (furanoid) were major compounds, accounting for 13.72%, 9.40%, 7.81%, 6.08%, 5.30%, and 4.83%, respectively. Alcohol and ester were dominant class of compounds. Methyl palmitate, methyl linolenate, methyl linoleate and *n*-hexadecanoic acid were major compound of 45, 60, 90, 120 and 150 days fermented oils, ranged between 20.76-22.69%, 18.62-21.06%, 9.01-23.37% and 7.49-4.27%, respectively, and phenylethyl alcohol was dominate of 120 days (5.65%) and 150 days fermented oils (5.12%). Ester and alcohol were the most abundant class of compounds of 45 days (61.95 % and 14.00%, respectively), 60 days (68.93% and 14.10%, respectively), 90 days (63.60% and 9.40, respectively), 120 day (55.55% and 19.96%, respectively) and 150 days (70.42% and 10.57%, respectively).

Comparing the volatile compounds of the analyzed fresh, steamed, and fermented leaves oil (15, 30, 45, 60, 90, 120, and 150 days), a total of 130 volatile compounds including acids (12), hydrocarbons (23), alcohols (30), aldehydes (12), ketones (19), esters (18), phenols (5), and others (12) compounds were identified. Alcohol and ester were the major class of compounds, ranged between 9.40-73.10% and 4.22-

70.42%, respectively. The major alcohol identified in nine samples were phytol, *p*-ethylphenol, phenylethyl alcohol, *(E)*-linalool oxide (pyranoid), linalool, *α*-terpineol, geraniol, *(Z)*-linalool oxide, and *(E)*-linalool oxide (furanoid). The content of phytol was higher in fresh and steamed leaves than in fermented leaves, while the content of *p*-ethylphenol and phenylethyl alcohol were found at highest content in fermented leaves. Among these, linalool, *α*-terpineol, and geraniol showed the highest content in steamed leaves, whilst (*E)*-linalool oxide (pyranoid) was the highest content in fresh leaves. Concerning the esters, the content of methyl palmitate and methyl linolenate were found at high content in 15 days fermented leaves, whereas ethyl palmitate, methyl stearate, and methyl salicylate were found in 30 days fermented leaves and the methyl linoleate was high content in 150 days fermented leaves. Figure 3.1 showed the structure of major compounds of Assam tea.

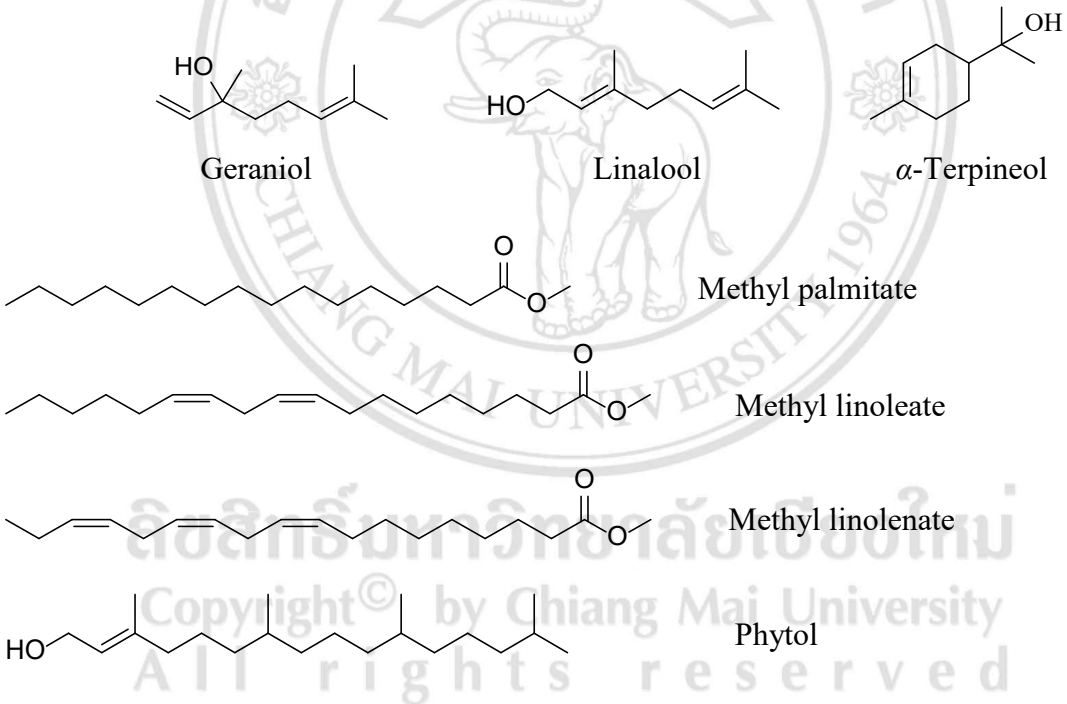

Figure 3.1 Structure of major compounds identified in volatile oil from Assam tea

Table 3.3 Chemical composition of volatile oils from Assam tea (n=2)

No.	Structure assignment	Class	KI[a]	KI[b]	FL	SL	Average amount (%)[c]						
							Fermented leaves (days)						
							15	30	45	60	90	120	150
1	Cyclohexen-1-one	KE	937	927	-	-	-	0.27	-	-	-	-	-
2	Benzaldehyde	ALD	960	960	0.87	-	-	0.26	0.05	tr	0.05	-	0.05
3	Phenol	Phe	989	984	-	-	-	0.49	-	-	-	-	-
4	Hexanoic acid	AC	992	997	-	-	-	-	0.38	-	-	-	-
5	*(E,E)*- 2,4 Heptadienal	ALD	1011	1007	-	-	-	-	-	0.14	0.14	-	0.15
6	2-Ethylhexanol	ALC	1027	1027	-	-	-	-	-	tr	-	-	tr
7	Benzyl alcohol	ALC	1032	1031	1.07	1.07	0.38	2.56	0.60	0.60	0.24	0.45	0.35
8	Benzene acetaldehyde	ALD	1041	1042	-	-	-	0.10	tr	0.13	0.76	0.10	0.15
9	Acetophenone	KE	1064	1065	0.21	tr	tr	0.05	tr	0.05	0.15	tr	tr
10	*(E)*-Linalool oxide	ALC	1071	1072	0.46	0.99	0.38	3.60	0.31	0.50	tr	0.74	0.44
11	*(Z)*-Linalool oxide (furanoid)	ALC	1089	1090	1.59	1.15	0.63	4.83	0.51	0.70	0.72	1.06	0.54
12	Linalool	ALC	1101	1097	1.63	**18.26**	0.70	9.40	0.57	0.44	0.87	0.40	0.24
13	Phenylethyl alcohol	ALC	1118	1114	1.86	0.22	1.14	4.57	2.53	3.46	0.54	5.65	5.12
14	Oxoisophorone	KE	1143	1145	-	-	0.07	0.17	0.07	0.05	5.07	0.06	-
15	*(E,Z)*-2,6-Nonadienal	ALD	1152	1154	-	-	0.05	-	-	-	0.06	0.12	0.06
16	*(E)*-2-Nonenal	ALD	1158	1161	-	0.05	tr	-	-	-	0.10	0.06	tr
17	*p*-Ethylphenol	ALC	1170	1168	1.98	0.27	3.97	**13.72**	**4.96**	3.40	0.06	6.97	1.94
18	*(E)*-Linalool oxide (pyranoid)	ALC	1175	1174	4.85	0.74	0.70	2.15	0.65	0.55	4.20	1.10	0.30
19	Octanoic acid	AC	1182	1180	-	-		-	-	0.15	0.63	0.07	0.11
20	*p*-Methyl acetophenone	KE	1185	1183	-	-	0.06	-	-	-	-	-	-
21	*α*-Terpineol	ALC	1191	1188	0.63	**6.08**	0.26	1.18	0.24	0.18	0.18	0.92	0.16
22	Methyl salicylate	ES	1193	1991	0.27	0.45	0.40	4.51	0.37	0.35	0.27	0.40	0.30
23	Dodecane	HD	1201	1200	0.33	0.25	0.23	-	0.28	0.33	0.38	0.64	0.35
24	Decanal	ALD	1206	1203	0.15	0.13	-	tr	-	0.05	0.49	-	tr
25	*β*-Cyclocitral	ALD	1219	1219	0.37	0.15	0.22	-	0.26	0.21	0.06	0.35	0.21
26	2,3-Dihydro benzofurane	O	1222	1221	-	0.28	-	-	-	-	-	-	-
27	Nerol	ALC	1227	1229	0.55	1.87	0.19	1.22	0.18	0.11	0.27	0.23	0.07
28	Carvotanaceton	KE	1242	1242	0.16	0.15	-	-	0.09	0.06	0.13	tr	tr
29	Caprolactam	O	1249	1244	0.04	-	-	-	-	-	0.09	0.11	tr
30	Anisaldehyde	ALC	1252	1250	tr	-	-	-	-	-	-	tr	tr
31	Geraniol	ALC	1254	1252	1.03	**5.57**	0.45	1.89	0.41	0.28	tr	0.66	0.15

Table 3.3 Chemical composition of volatile oils from Assam tea (n=2) (continued)

No.	Structure assignment	Class	KI[a]	KI[b]	Average amount (%)[c]								
					FL	SL	Fermented leaves (days)						
							15	30	45	60	90	120	150
32	*(E)*-2-Decenal,	ALD	1261	1263	0.15	-	-	-	-	-	-	-	-
33	*α*-Citral	ALC	1270	1267	0.29	-	-	-	-	-	-	-	-
34	*p*-Ethylguaiacol	ALC	1278	1280	-	-	0.06	1.61	-	0.04	0.09	0.07	-
35	Nonanoic acid	AC	1285	1281	-	-	-	0.04	-	0.03	-	-	-
36	Dihydroedulan II	O	1286	1285	-	-	0.04	0.32	-	-	0.05	-	-
37	Indole	O	1291	1291	0.18	0.12	0.07	-	-	0.05	0.07	0.18	0.08
38	Dihydroedulan IA	O	1292	1293	-	-	-	0.47	-	-	-	-	-
39	Tridecane	HD	1300	1300	-	-	-	-	-	-	-	-	tr
40	*p*-Vinylguaiacol	Phe	1311	1309	-	0.68	0.18	-	0.19	0.03	0.14	0.21	0.12
41	Edulan I	O	1312	1313	0.20	-	-	-	-	-	-	-	-
42	*(E,E)*-2,4-Decadienal	ALD	1315	1316	0.42	-	0.07	-	0.16	0.14	0.13	0.13	0.06
43	1,1,6-Trimethyl-1,2 dihydrona phthalene	O	1348	1349	0.09	tr	tr	0.35	0.06	0.08	0.05	tr	tr
44	Phenethyl propanoate	ES	1353	1354	-	0.06	tr	0.20	tr	0.05	0.07	0.06	tr
45	Eugenol	ALC	1356	1359	-	-	tr	0.18	tr	tr	tr	tr	tr
46	*(E)*-2-Undecenal	ALD	1363	1360	0.19	-	-	-	-	-	-	-	-
47	Cerulignol	Phe	1366	1369	-	-	-	0.07	-	-	-	-	tr
48	*n*-Decanoic acid	AC	1379	1377	-	-	0.07	0.07	0.05	tr	0.07	0.06	0.13
49	*(E)*-*β*-Damascenone	KE	1386	1384	0.13	0.43	-	0.17	-	tr	-	tr	0.05
50	1-Tetradecene	HD	1392	1389	-	-	-	-	-	-	tr	tr	tr
51	Tetradecane	HD	1401	1400	1.96	tr	0.66	0.14	0.98	0.06	0.97	2.59	0.65
52	*(E)*-*α*-Ionone	KE	1427	1430	0.98	0.29	0.56	0.06	0.66	0.86	0.36	0.83	0.24
53	Methyl 9-oxononanoate	ES	1435	1436	-	-	0.06	-	0.09	0.36	0.10	0.17	0.10
54	Isoeugenol	ALC	1447	1451	-	-	-	-	-	-	-	-	0.04
55	*(E)*-Geranyl acetone	KE	1455	1455	1.26	0.41	0.43	0.27	0.40	0.18	0.29	0.51	0.15
56	Caryophyllene	HD	1459	1459	-	-	-	-	-	tr	-	-	-
57	2,6-*di*-tert-Butylquinone	KE	1463	1464	-	0.07	-	-	-	-	tr	-	tr
58	Undecanoic acid	AC	1472	1466	-	0.05	-	0.28	-	0.24	tr	-	tr
59	1-Dodecanol	ALC	1475	1476	-	-	-	-	0.06	tr	0.05	0.0	tr
60	3,4-didehydro-*β*-Ionone	KE	1482	1485	-	-	0.59	-	0.05	tr	tr	tr	tr

Table 3.3 Chemical composition of volatile oils from Assam tea (n=2) (continued)

No.	Structure assignment	Class	KI[a]	KI[b]	Average amount (%)[c]								
					FL	SL	Fermented leaves (days)						
							15	30	45	60	90	120	150
61	*β*- Ionone	KE	1483	1485	2.25	1.48	0.56	0.23	1.13	tr	0.81	1.60	tr
62	1-Pentadecene	HD	1485	1489	-	-	-	0.37	-	-	-	-	-
63	Pentadecane	HD	1500	1500	0.07	0.11	0.38	-	0.03	0.68	tr	0.08	0.49
64	*(E,E)*-Farnesene	HD	1508	1505	-	1.40	-	0.45	-	-	-	-	-
65	2,4-bis(1,1'-dimethylethyl)phenol	Phe	1515	1513	0.19	0.66	0.12	0.16	0.15	tr	0.13	0.26	0.07
66	Dihydroactinidiolide	KE	1525	1525	2.00	0.45	2.09	0.15	2.49	0.12	1.56	1.79	1.26
67	Methyl dodecanoate	ES	1526	1526	0.10	-	tr	-	-	-	tr	0.13	-
68	*(Z)*-Nerolidol	ALC	1532	1532	-	0.13	-	-	-	-	-	-	-
69	*(E)*-Nerolidol	ALC	1563	1563	0.51	0.89	0.29	0.58	0.29	1.55	0.20	0.35	0.15
70	Dodecanoic acid	AC	1568	1566	0.22	-	tr	0.53	0.11	0.21	0.16	0.07	0.28
71	3-Methyl pentadecane	HD	1570	1570	0.03	-	-	-	-	-	-	-	-
72	Boronia butenal	ALD	1582	1584	0.04	0.17	0.02	0.11	0.03	0.12	tr	tr	tr
73	1-Hexadecene	HD	1593	1590	0.20	0.44	0.07	0.04	0.06	0.02	0.05	0.10	0.05
74	Hexadecane	HD	1601	1600	2.38	0.07	0.62	0.06	1.12	0.06	1.06	3.19	0.69
75	Dill apiole	O	1622	1625	-	0.56	tr	0.25	tr	0.98	tr	tr	tr
76	Benzophenone	KE	1623	1627	0.06	-	-	-	tr	tr	tr	tr	-
77	*τ*-Cadinol	ALC	1636	1639	-	0.12	0.05	tr	-	tr	tr	-	-
78	*α*-Muurolol	ALC	1642	1646	0.12	0.18	tr	tr	-	tr	-	tr	-
79	*α*-Cadinol	ALC	1651	1654	0.13	-	0.18	0.28	0.16	0.01	0.15	0.25	0.11
80	*(E)*-4-oxo-*β*-Ionone	KE	1666	1661	-	-	tr	-	-	-	-	-	-
81	2-Pentadecanone	KE	1699	1697	0.18	-	0.06	-	0.14	0.15	-	-	-
82	*δ*-Dodecalactone	KE	1705	1704	-	-	0.12	0.38	0.13	-	-	-	0.08
83	*(Z,Z)*-Farnesol	ALC	1714	1715	-	-	-	0.23	0.07	-	-	-	-
84	*(E,E)*-Farnesol	ALC	1721	1723	-	-	tr	0.17	-	-	tr	-	tr
85	Methyl tetradecanoate	ES	1727	1724	0.45	-	0.42	0.16	0.45	tr	0.40	0.41	0.45
86	*(E,E)*-Farnesal	ALC	1742	1741	0.13	-	-	-	tr	-	-	-	-
87	*α*-Hexylcinnamic aldehyde	ALD	1744	1746	-	tr	-	-	-	0.51	tr	0.09	tr
88	Benzyl benzoate	ES	1759	1760	0.28	0.38	0.11	0.12	0.11	0.19	0.09	0.12	0.07
89	Tetradecanoic acid	AC	1768	1769	0.12	-	0.05	0.15	0.16	0.08	0.16	-	-
90	3-Methyl heptadecane	HD	1772	1771	0.07	-	tr	0.25	tr	-	tr	0.09	0.32
91	1-Octadecene	HD	1787	1790	tr	-	tr	0.07	tr	0.29	tr	0.05	tr

Table 3.3 Chemical composition of volatile oils from Assam tea (n=2) (continued)

No.	Structure assignment	Class	KI[a]	KI[b]	Average amount (%)[c]								
					FL	SL	Fermented leaves (days)						
							15	30	45	60	90	120	150
92	*(E)*-3-Octadecene	HD	1794	1795	-	-	-	-	0.03	-	-	-	-
93	Octadecane	HD	1801	1800	1.71	0.23	0.53	tr	0.84	tr	0.77	1.97	0.53
94	Methyl pentadecanoate	ES	1828	1827	0.21	-	0.45	0.08	0.41	0.70	0.33	0.35	0.35
95	Caffeine	O	1837	1836	1.17	0.11	0.11	0.05	0.34	0.41	0.19	0.16	0.08
96	Hexahydrofarnesyl acetone	KE	1845	1847	4.44	1.35	0.30	tr	0.69	0.25	0.25	0.29	0.18
97	Benzyl salicylate	ES	1861	1865	0.26	0.30	0.16	0.71	0.27	0.31	0.16	0.19	0.15
98	Isobutyl phthalate	O	1865	1868	0.99	0.90	0.21	0.80	0.15	0.19	0.12	0.19	0.09
99	1-Hexadecanol	ALC	1873	1875	-	-	-	-	-	-	-	-	0.05
100	Cyclohexadecane	HD	1881	1883	0.77	0.78	0.18	0.08	0.16	0.12	0.11	0.13	-
101	Propyl tetradecanoate	ES	1892	1896	-	-	-	-	tr	0.09	-	-	-
102	*(Z)*-Methyl hexadec-7-enoate	AC	1900	1899	0.23	-	0.10	0.29	0.11	0.07	0.10	0.11	0.06
103	Methyl palmitoleate	AC	1906	1910	0.14	-	0.33	0.04	0.39	0.68	0.69	0.55	0.94
104	Farnesyl acetone	KE	1918	1915	1.68	0.14	0.33	0.04	0.52	0.28	0.33	0.13	0.15
105	Methyl palmitate	ES	1927	1922	**16.16**	0.57	**23.07**	**6.08**	**21.42**	**22.69**	**20.76**	**21.12**	**22.13**
106	Isophytol	ALC	1947	1947	1.20	1.95	0.16	1.32	0.31	0.22	0.24	0.14	0.22
107	Dibutyl phthalate	O	1959	1959	0.84	4.43	0.18	0.17	0.16	0.30	0.14	0.75	0.26
108	*n*-Hexadecanoic acid	AC	1968	1971	1.49	-	2.73	0.11	5.27	4.27	4.97	0.91	7.49
109	Ethyl palmitate	ES	1997	1993	0.54	0.15	0.24	7.81	0.27	0.21	0.19	0.20	-
110	Eicosane	HD	2001	2000	0.96	-	0.35	-	0.51	0.43	0.45	1.01	-
111	Methyl heptadecanoate	ES	2027	2024	0.72	0.14	0.51	0.35	0.68	0.63	0.49	0.36	0.56
112	Oleyl alcohol	ALC	2064	2060	-	-	tr	-	-	tr	-	-	-
113	1- Octadecanol	ALC	2083	2084	0.19	0.28	-	0.49	tr	-	-	-	-
114	Methyl linoleate	ES	2093	2095	2.67	0.28	**15.01**	0.12	**14.05**	**19.83**	**19.44**	**9.01**	**23.37**
115	Methyl linolenate	ES	2099	2100	**7.08**	0.73	**25.07**	1.28	**20.47**	**20.10**	**18.62**	**21.06**	**19.91**
116	Phytol	ALC	2111	2116	**10.88**	**33.33**	1.54	3.60	1.84	1.83	1.50	0.87	0.61
117	Methyl stearate	ES	2128	2125	1.61	0.65	2.86	4.18	2.41	2.54	2.21	1.59	2.55
118	Linoleic acid	AC	2136	2133	-	-	0.34	0.06	0.07	0.84	1.23	0.13	0.31
119	Oleic acid	ALC	2141	2142	1.17	-	0.37	1.19	0.25	0.10	-	-	-
120	Linoleic acid ethyl ester	ES	2157	2162	0.84	0.13	0.24	5.30	0.15	0.20	0.12	0.12	0.19

Table 3.3 Chemical composition of volatile oils from Assam tea (n=2) (continued)

No.	Structure assignment	Class	KI[a]	KI[b]	Average amount (%)[c]								
					FL	SL	Fermented leaves (days)						
							15	30	45	60	90	120	150
121	Linolenic acid ethyl ester	ES	2169	2169	0.14	0.11	0.53	0.29	0.51	0.35	0.33	0.28	0.12
122	Octadecanoic acid	AC	2171	2178	-	-	-	-	-	-	-	-	0.39
123	Ethyl stearate	ES	2193	2196	-	0.16	0.13	1.28	0.22	0.29	-	-	0.15
124	Docosane	HD	2200	2200	0.49	0.11	0.17	0.09	0.21	0.13	0.17	0.48	0.01
125	Tricosane	HD	2300	2300	0.18	0.22	0.11	0.65	0.09	-	-	-	0.26
126	Tetracosane	HD	2400	2400	0.47	-	0.15	0.33	0.19	0.10	0.18	0.30	0.12
127	Pentacosane	HD	2500	2500	0.36	0.34	0.12	0.02	0.15	0.08	0.20	0.18	0.02
128	Bis(2-ethylhexyl) phthalate	O	2548	2550	0.63	0.43	0.60	0.09	0.79	0.09	0.08	0.48	0.05
129	Hexacosane	HD	2600	2600	0.46	0.19	0.16	0.04	0.21	0.08	0.24	0.33	0.20
130	Heptacosane	HD	2700	2700	0.47	0.42	0.19	0.14	0.18	0.11	0.29	0.39	0.24
	Total identified				**94.68**	**95.76**	**95.66**	**97.14**	**96.35**	**97.69**	**97.95**	**97.61**	**98.46**
	Acids (AC)				2.20	0.05	3.65	1.57	6.54	6.58	8.03	1.90	9.73
	Hydrocarbons (HD)				10.92	4.70	3.95	2.78	5.03	2.52	4.98	11.58	3.95
	Alcohols (ALC)				30.27	73.10	11.50	54.62	14.00	14.10	9.40	19.96	10.57
	Aldehydes (ALD)				2.19	0.52	0.39	0.51	0.53	1.33	1.84	0.87	0.76
	Ketones (KE)				13.35	4.80	5.24	1.83	6.44	2.08	9.02	5.31	2.21
	Esters (ES)				31.33	4.22	69.32	32.48	61.95	68.93	63.60	55.55	70.42
	Phenols (Phe)				0.19	1.34	0.32	0.91	0.36	0.06	0.28	0.30	0.21
	Others (O)				4.14	7.03	1.29	2.49	1.52	2.11	0.81	2.14	0.61

a : Retention indices relative to C_7-C_{30} alkanes on HP-5MS column
b : Retention indices from Kovát and Van den Dool and literatures
c : Based on peak area
FL : Fresh leaves
SL : Steamed leaves
tr: trace (<0.05%)

3.2.3 Total phenolic contents of volatile oil

The total phenolic contents were performed according to Kahkonen *et.al.*[82] Phenolic compounds in the extract reacted with Folin-Ciocalteu reagent and resulted in green complex solution. After sodium carbonate were added, the green complex solution turned into blue complex solution which had maximal absorbance at wavelength 765 nm. Gallic acid was used as a standard to prepare a calibration curve as shown in Figure 3.2. The total phenolic content was expressed as milligrams of gallic acid equivalent per grams of dried weight of plant (mgGAE/g DW).

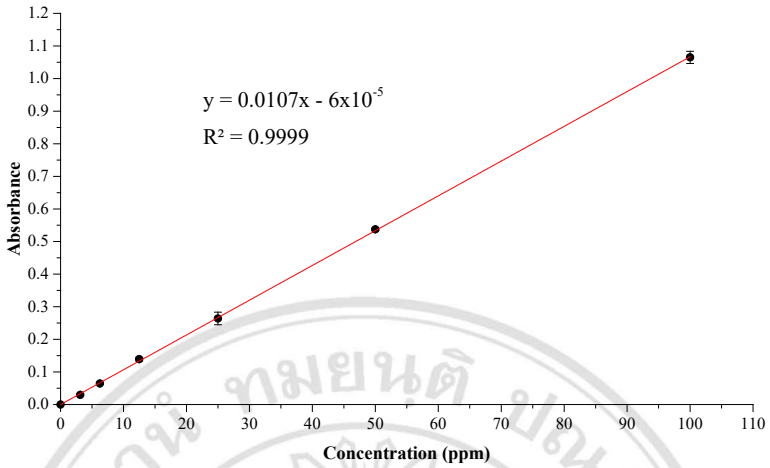

Figure 3.2 Calibration curve of gallic acid used for calculating the total phenolic content.

The total phenolic content (TPC) of the fresh leaves, steamed leaves, and fermented leaves volatile oils are presented in Table 3.4. In the volatile oil of 45 days fermented leaves, a high content of total phenols (40.60 ± 0.19 mgGAE/g extract) was demonstrated. The 120 days and 45 days fermented leaves essential oils were seem to be a less rich source of total phenols (34.09 ± 0.18 and 23.89 ± 0.12 mg GAE/g extract, respectively), while steamed leaves showed the lowest amount of total phenols. The phenolic content could be used as an important indicator of the antioxidant capacity, which may be used as a preliminary screen for essential oils when intended as natural sources of antioxidants in functional foods.[83]

3.2.4 Antioxidant activity of the volatile oils

The antioxidant activities of the volatile oils in fresh, steamed, and fermented leaves (15, 30, 45, 60, 90, 120, and 150 days) from Assam tea were evaluated using DPPH assay and FRAP assay. These extracts were compared with ascorbic acid, which is a synthetic antioxidant. The results of DPPH assay was expressed in % DPPH radical scavenging. Lower scavenging value indicate higher antioxidant activity. The FRAP assay was expressed as milligrams of gallic acid equivalent per grams of dried weight of plant (mgGAE/g DW), which gallic acid was used as a standard to prepare a calibration curve as shown in Figure 3.3.

1) DPPH assay of volatile oils

The DPPH free radical does not require any special preparation and is considered a simple and very fast method for determining antioxidant activity. In contrast, DPPH can only be dissolved in organic media, especially in methanol, which is an important limitation when interpreting the role of hydrophilic antioxidants. The radical scavenging capacity of the volatile oil was tested using the 'stable' free radical, DPPH. The strongest antioxidant activities were demonstrated the 45 days and 15 days fermented leaves essential oils (43.61 and 38.63 %, respectively). The weakest antioxidant activities were found in the fresh leaves and 30 days fermented leaves essential oils. All oils have lower antioxidant activity than that of synthetic antioxidant ascorbic acid (95.52%).

2) FRAP assay of volatile oils

The reducing power of the extract, which may serve as a reflection of its antioxidant activity, was determined using a modified Fe^{3+} to Fe^{2+} reduction assay, whereby the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of the sample. The presence of antioxidants in the sample causes the reduction of Fe^{3+}/ferricyanide complex to the Fe^{2+} form, which is monitored by measuring the formation of Perl's Prussian blue at 700 nm.[8] The FRAP was expressed as milligrams of gallic acid equivalent per grams of dried weight of plant (mgGAE/g DW). Gallic acid was used as a standard to prepare a calibration curve as shown in Figure 3.3.

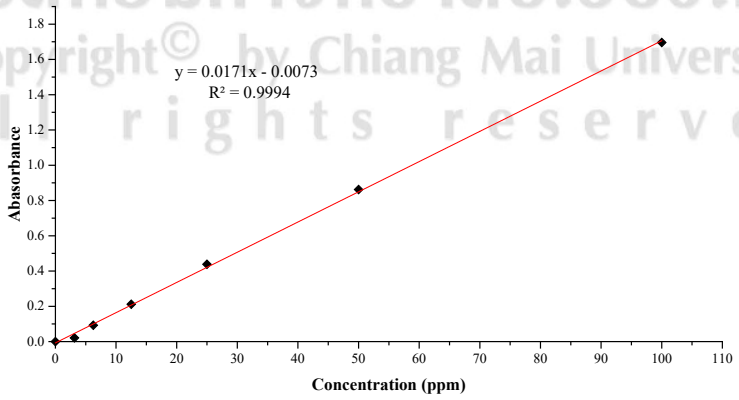

Figure 3.3 Calibration curve of gallic acid used for calculating FRAP value

The reducing capacity of the oil is presented in Table 3.4. The highest antioxidant activity was obtained for the 15 days and 45 days fermented leaves oils (3.39 and 2.96 mg GAE/g extract, respectively), followed by 120 days fermented leaves oil and fresh leaves (2.63 and 1.80 mg GAE/g extract, respectively).

Table 3.4 Total phenolic content and antioxidant activities of essential oils of Assam tea

Volatile oils	Total phenolic content (mgGAE/g DW)	DPPH assay (% DPPH radical scavenging)	FRAP assay (mgGAE/g DW)
Fresh leaves	8.75 ± 0.04^{b}	16.27 ± 0.19^{a}	1.80 ± 0.01^{e}
Steamed leaves	6.08 ± 0.03^{a}	13.30 ± 0.20^{c}	1.65 ± 0.00^{c}
Fermented leaves			
15 days	22.99 ± 0.11^{f}	38.63 ± 0.03^{g}	3.39 ± 0.01^{h}
30 days	16.01 ± 0.09^{e}	17.65 ± 0.18^{b}	1.19 ± 0.01^{b}
45 days	40.60 ± 0.19^{i}	43.61 ± 0.05^{h}	2.96 ± 0.00^{g}
60 days	23.89 ± 0.12^{g}	31.53 ± 0.54^{e}	1.75 ± 0.00^{d}
90 days	18.03 ± 0.09^{d}	31.80 ± 0.15^{e}	1.63 ± 0.00^{c}
120 days	34.09 ± 0.18^{h}	35.40 ± 0.15^{f}	2.63 ± 0.01^{f}
150 days	12.59 ± 0.06^{c}	26.07 ± 0.51^{d}	0.99 ± 0.01^{a}
Ascorbic acid		95.52 ± 0.00^{i}	

Data are given as means ± SD (n=3)

Values with different letters (a-i) within column of each solvents are significantly different at P<0.01

3.3 Optimization of solvent extraction

The selection of an ideal solvent for extraction is critical, as it determines the amount and type of phenolic compounds extracted because in each plant there are different phenolic compounds of varied chemical characteristics and polarities which may or may not be soluble in a particular solvent.[85]

Fresh leaves was used to optimize the condition of solvent extraction of catechins and caffeine. Results of solvent effects on extraction efficiency of chemical constituents from fresh leaves are shown in Table 3.5, total phenolic content and antioxidant activities of the extracts (Table 3.6–3.7). The fresh leaves extracts obtained from various solvents show variations in the contents of phenolic compounds extracted. Total phenolic contents

in these extracts was measured at 765 nm using a UV-VIS spectrophotometer according to the Folin-Ciocalteau method as shown in Section 2.7 and antioxidant activity was evaluated by DPPH and FRAP method as shown in Section 2.8 and 2.9, respectively.

The yield of extraction depends on the solvent with varying polarity, pH, temperature, extraction time, and composition of the sample. Under the same extraction time and temperature, solvent and composition of sample are known as the most important parameters.[86] In this work, the selection of solvent system for extraction of catechins and caffeine from the fresh leaves of Assam tea was performed using thirteen different solvents; acetone : water (25%, 50%, 80% and 100%), ethanol : water (25%, 50%, 80%, and 100%), methanol : water (25%, 50%, 80% and 100%) and water as described in Section 2.6.

Extraction yields ranged from 2.04% for 100% acetone extract to 18.76% for 80% aqueous acetone extract (Table 3.5). The yields of extraction by various solvents decreased in the following order: 80% aqueous ethanol > 50% aqueous acetone > 50% aqueous ethanol > 100% methanol > 100% ethanol > 50% aqueous methanol >25% aqueous acetone > 80% aqueous methanol > 25% aqueous methanol > 25% aqueous ethanol > RO water > 100% acetone. It can be seen that the extraction yield of pure methanol (11.95%) and pure ethanol (11.61%) are higher than that pure acetone (2.04%). This shows that the extraction yield increases with increasing polarity of the solvent used in extraction. It can also be found that the yield of the water extract (3.42%) is only slightly less than that of the pure methanol extract, whereas the yield of aqueous solvent extract (from 9.12% for 25% aqueous ethanol to 16.03 % for 80% aqueous ethanol) is higher than that of the pure solvent extracts. These results indicate that increasing in water concentration in the solvent enhances extraction yield. The present investigation showed similar result to the report of Drużyńska *et.al*, where the 80% acetone solvent the most effective in extracting phenolic compound of green tea from China.[87]

Table 3.5 Physical properties and percentages yields (%yield) of solvent extract of fresh leaves from Assam tea

Solvents	% v/v	Colour	% yield (w/w)
Acetone : water	25	orange-brown	11.28
	50	dark maroon	15.17
	80	olive-brown	18.76
	100	light olive	2.04
Ethanol : water	25	dark maroon	9.12
	50	dark brown	13.85
	80	dark brown	16.03
	100	olive	11.61
Methanol : water	25	light brown	9.76
	50	dark brown	11.57
	80	dark brown	11.08
	100	dark olive	11.95
Water	100	pale orange-brown	3.42

3.3.1 Total phenolic contents of solvents extract

The fresh leaves extracts using different solvents show variations in different total contents of phenolic compounds. It can be seen that the extract obtained by 80 % aqueous acetone yielded the highest amount of phenolic contents with the value of 786.65 ± 0.08 mgGAE/g DW, followed by 50% methanol, 100% methanol, 25% methanol and 25 % acetone : water, respectively as show in Table 3.6. The total phenolic content of 80% aqueous acetone extract is significantly higher than that of other solvents ($P<0.01$). Gallic acid was used as a standard to prepare a calibration curve as shown in Figure 3.2.

Table 3.6 Total phenolic contents of fresh leaves extract obtained from various type of solvent extraction

Solvents	% v/v	Total phenolic contents (mg GAE/g DW)
Acetone : water	25	230.99 ± 0.01^{e}
	50	197.73 ± 0.00^{de}
	80	786.65 ± 0.08^{i}
	100	147.72 ± 0.01^{bc}
Ethanol : water	25	174.24 ± 0.02^{cd}
	50	210.77 ± 0.09^{de}
	80	189.25 ± 0.02^{cde}
	100	172.91 ± 0.09^{cd}
Methanol : water	25	109.39 ± 0.01^{b}
	50	651.93 ± 0.08^{h}
	80	403.27 ± 0.02^{f}
	100	476.38 ± 0.03^{g}
Water	100	64.71 ± 0.01^{a}

Data are given as means ± SD (n=3)

Values with different letters (a-i) within column of each solvents are significantly different at $P<0.01$

3.3.2 DPPH assay of the extract by various solvents

DPPH radical is a stable organic free radical with an absorption band at 517 nm. It loses this absorption when accepting an electron or a free radical species, which results in a visually noticeable discoloration from purple to yellow. It can accommodate many samples in a short period and is sensitive enough to detect active ingredients at low concentrations.[88] Figure 3.4 shows the DPPH scavenging activities of the extracts in a concentration-dependent manner. The extract obtained by 80% aqueous acetone yielded the highest DPPH radical scavenging activity at concentrations ranging from 7.81 ppm to 2000 ppm. All extracts obtained by pure and aqueous organic solvent gave stronger radical scavenging capacity than that of the water extract. The IC_{50} of a compound is inversely related to its antioxidant capacity, as it expresses the amount of antioxidant

required to decrease the DPPH concentration by 50%, which is obtained by interpolation from a linear regression analysis.[89] A lower IC_{50} indicates a higher antioxidant activity of a compound. Figure 3.4 shows the IC_{50} values in the DPPH radical scavenging activity assay of the extracts. It was found that the 80% aqueous acetone extract possesses the strongest DPPH radical activity (IC_{50}= 0.24 mg/mL).

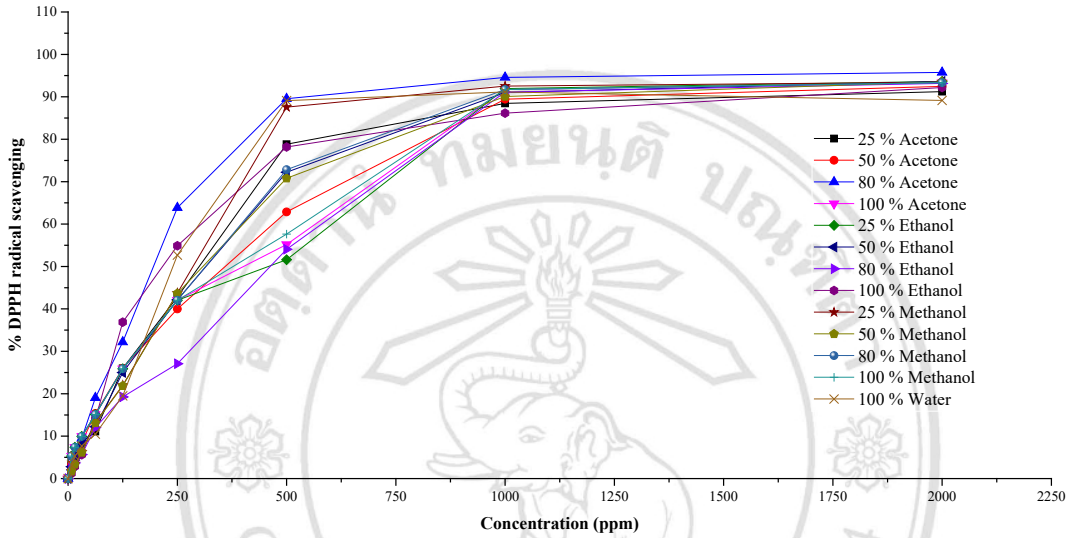

Figure 3.4 % DPPH radical scavenging of fresh leaves extract obtained by different solvent extractions.

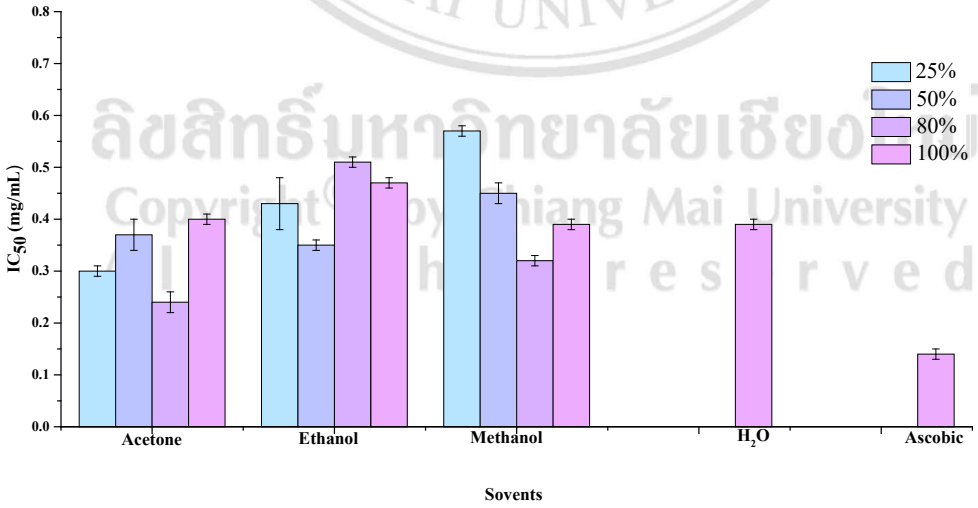

Figure 3.5 IC_{50} values of fresh leaves extract obtained by different solvents

3.3.3 FRAP assay of the extract by various solvents

In Figure 3.6, all extracts show some degrees of electron-donating capacity in a concentration-dependent manner. The 80% aqueous acetone extract once again gave the highest reducing power and is significantly higher ($P < 0.01$) than that of the other extracts at all concentrations studied, followed by that of the 50% aqueous methanol extract and the 100% methanol extract. The lowest reducing power was found in the 25% aqueous methanol extract. Its value is also significantly lower than that of the other extracts at all concentrations studied. Gallic acid was used as a standard to prepare a calibration curve as shown in Figure 3.3.

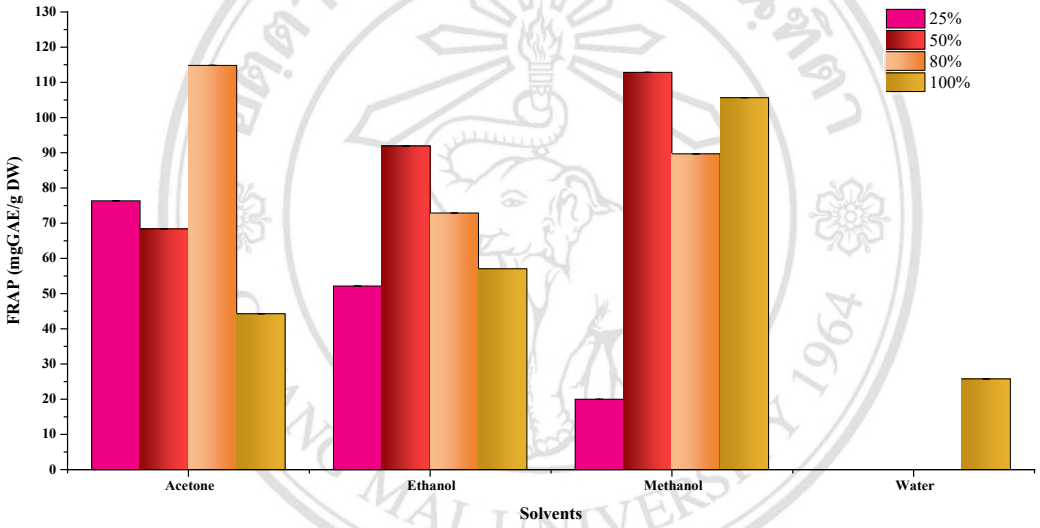

Figure 3.6 FRAP (mgGAE/g DW) of fresh leaves extract obtained by different solvent extractions.

Table 3.7 showed the antioxidant activity of different solvents, the antioxidant activity of 80% aqueous acetone extract exhibited the greatest antioxidant activities in all test assay. Almost all of their results showed higher total phenolic content and total flavonoid content as well as antioxidant activity when the pure organic solvent was used. In this study, it was also observed that the total phenolic content and antioxidant activity of the aqueous organic solvent system, 80% aqueous acetone and 50% aqueous methanol are higher than pure methanol, water, and the other solvents. The differences between the results of this study and those of other studies may be attributed to several factors: (1) the difference in plant matrix; (2) different solvents used in extraction resulted in differences

in compositions and antioxidant activities of the extracts; (3) an extract possessing a phenolic compound that contains a higher number of hydroxyl groups has a higher antioxidant activity; (4) the method and conditions of extraction (temperature and time) also affected to antioxidant activities.[90]

Table 3.7 Antioxidant activities of fresh leaves extract obtained by different solvent extractions.

Solvents	**% v/v**	**DPPH assay IC_{50} (mg/mL)**	**FRAP assay (mgGAE/g DW)**
Acetone : water	25	0.30 ± 0.01^{b}	76.36 ± 0.04^{h}
	50	0.37 ± 0.03^{e}	68.38 ± 0.02^{f}
	80	0.24 ± 0.02^{a}	114.83 ± 0.04^{m}
	100	0.40 ± 0.01^{g}	44.27 ± 0.05c
Ethanol : water	25	0.43 ± 0.05^{h}	52.16 ± 0.02^{d}
	50	0.35 ± 0.01^{d}	91.92 ± 0.02^{j}
	80	0.51 ± 0.01^{k}	72.89 ± 0.02^{g}
	100	0.47 ± 0.01^{j}	57.05 ± 0.02^{e}
Methanol : water	25	0.57 ± 0.01^{m}	20.00 ± 0.06^{a}
	50	0.45 ± 0.02^{i}	112.85 ± 0.02^{l}
	80	0.32 ± 0.01^{c}	89.68 ± 0.03^{i}
	100	0.39 ± 0.01^{f}	105.65 ± 0.04^{k}
Water	100	0.59 ± 0.01^{l}	25.76 ± 0.05^{b}

Ascorbic acid : standard (IC_{50} = 14.19 ± 0.00 ppm)

Data are given as means ± SD (n=3)

Values with different letters (a-m) within column of each solvents are significantly different at $P<0.01$

In this study, thirteen solvents were used to examine the effects of extraction solvent on total phenolic content and antioxidant activity evaluation. It was found that 80% aqueous acetone provide significantly better result, including extraction yield (18.76%), total phenolic content (786.65 mgGAE/g DW), and antioxidant activity (IC_{50} = 0.24 mg/mL and FRAP = 114.83 mgGAE/g DW), than those of the other solvent system.

3.4 Optimization of HPLC condition

The optimum HPLC conditions for separation of phenolic compounds containing GA, GC, caffeine, EGC, C, EC, EGCG, GCG and ECG and caffeine were achieved by adjusting detection wavelength, mobile phase composition, flow rate of the mobile phase and type of column.

3.4.1 Effect of type column on separation

Two columns, VertiSep UPS C18 (5 µm, 4.6 × 250 mm) and Wakosil-II 5C18 HG (5 µm, 0.3 x 250 mm) were tested under the same condition. Both column gave similar responses. However, Wakosil-II 5C18 HG give full separation for all analytes (Figure 3.8), whereas VertiSep UPS C18 HPLC could not separate the caffeine, GCG and EC as shown in Figure 3.7. As a result, the Wakosil-II 5C18 HG (5 µm, 0.3 x 250 mm) column was selected for this investigation.

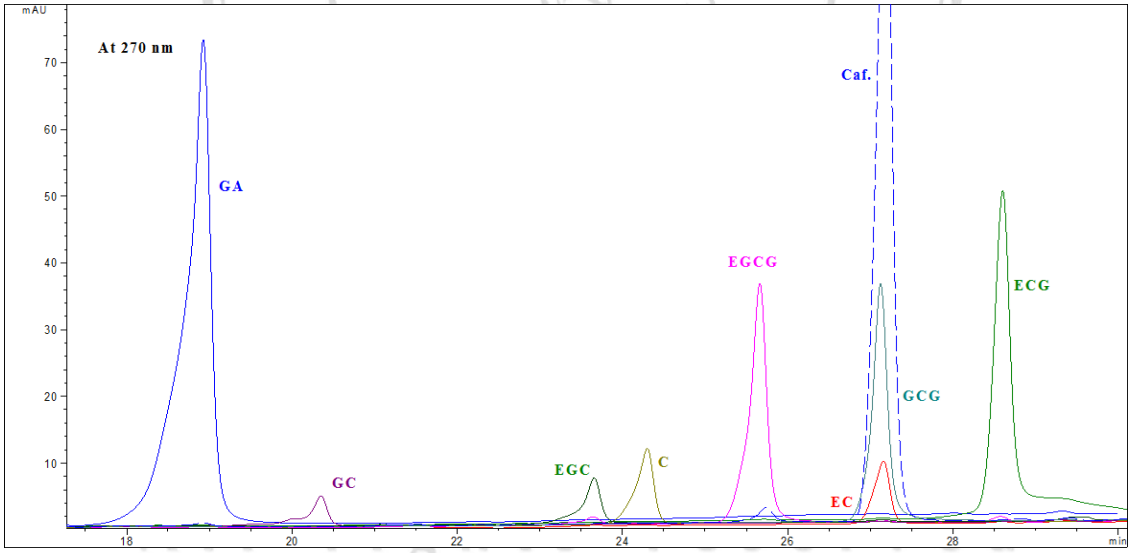

Figure 3.7 HPLC chromatograms of GA, GC, caffeine, EGC, C, EC, EGCG, GCG and ECG standard obtained using VertiSep UPS C18

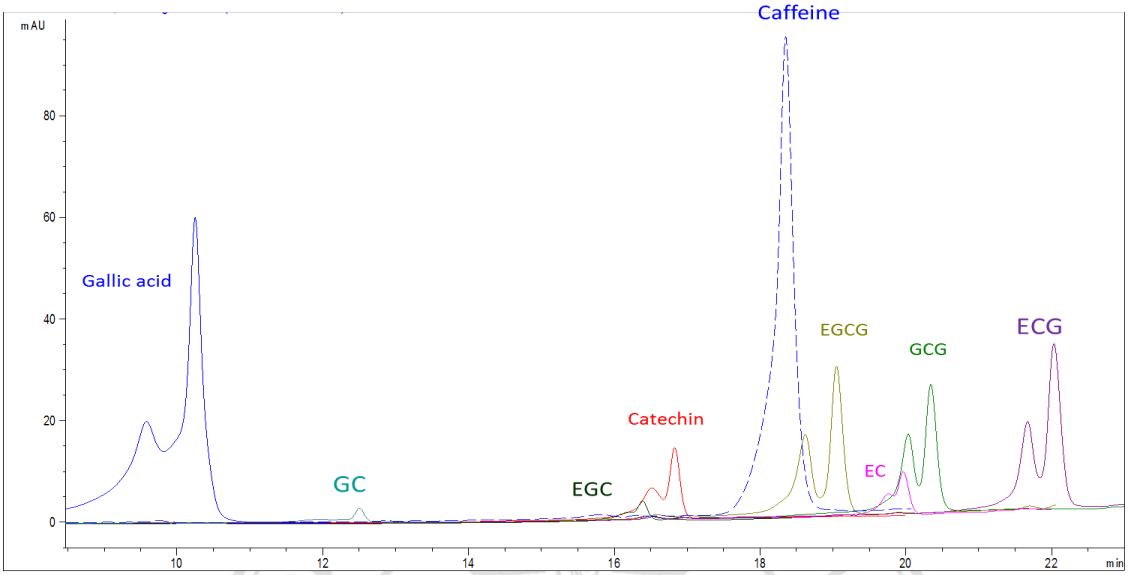

Figure 3.8 HPLC chromatograms of GA, GC, caffeine, EGC, C, EC, EGCG, GCG and ECG standard obtained using Wakosil-II 5C18 HG

3.4.2 Effect of mobile phase composition

Methanol, 1% ethyl acetate in methanol, 0.05% (v/v) acetic acid in water, and 0.1% (v/v) phosphoric acid in water, which are ordinary solvents used as mobile phase in HPLC system, were used in this work. Previous studies revealed that the addition of a low amount of acid, for example, phosphoric acid, to the mobile phase could reduce the retention time.[91]Three series of mobile phase gave similare response. The 1% ethyl acetate in metanol/ 0.1% (v/v) phosphoric acid in water system gave a complete separatton of the six catechins, caffeine and gallic acid, wherease the methanol/0.05% (v/v) acetic acid in water and methanol/0.1% (v/v) phosphoric acid in water gave a split peak of catechins (racemic compound) such as catechin, EGCG and GCG as shown in Figure 3.9. Therefore, a mobile phase consisting of 1% ethyl acetate in metanol/ 0.1% (v/v) phosphoric acid in water was tested in the present study.

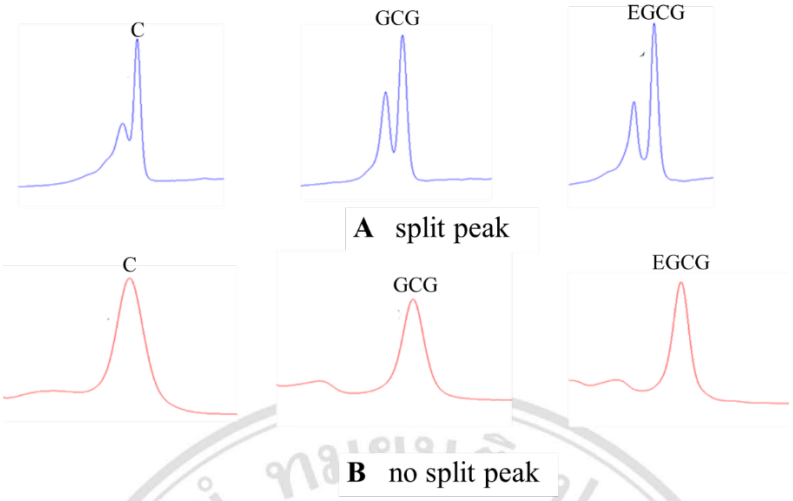

Figure 3.9 The characteristic peak of catechin, GCG and EGCG using two moblie phase system. A: methanol/0.1% (v/v) phosphoric acid in water; B: 1% ethyl acetate in metanol/ 0.1% (v/v) phosphoric acid in water.

3.4.3 Effect of gradient profile of mobile phase

Figure 3.10. showed the effect of gradient elution series for separation of phenolic compounds and caffeine. Both gradient elution gave similar respones. However, the batch 2 gave full separation for all anaytes, whereas batch 1 gave a basline drift in gradient elution. As a result, the batch 2 was selected for this investigation.

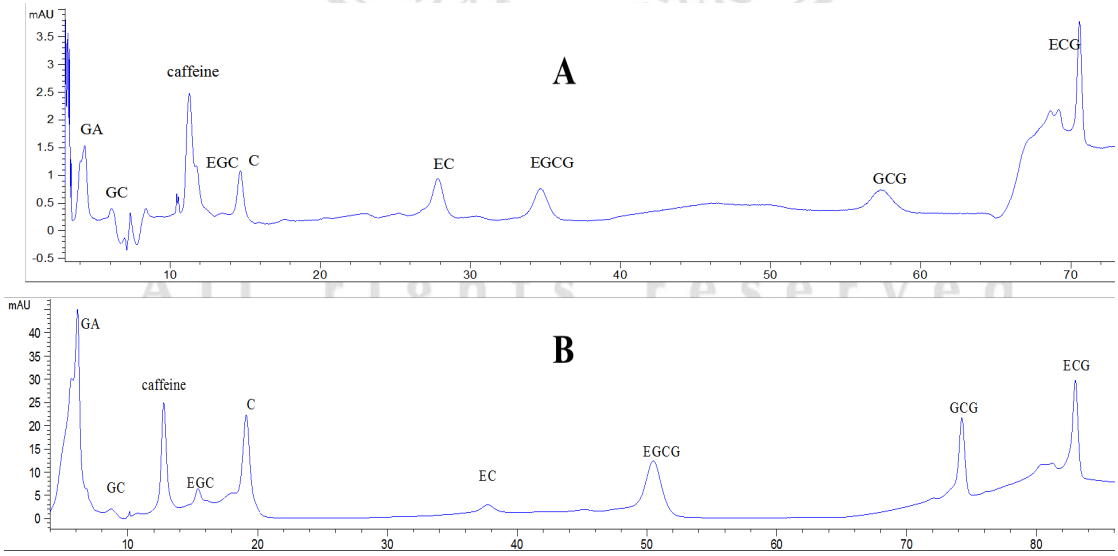

Figure 3.10 Chromatograms of phenolic compounds and caffeine in various gradient elutions. A: batch 1; B: batch 2.

3.4.4 Effect of wavelength

The detection wavelength was set 270 nm and 280 nm for measurement of standard GA, GC, EGC, C, EC, EGCG, GCG, and ECG, and caffeine. As the result shown in Table 3.8, the detection wavelenght at 270 nm gave the highest senstivity for phenolic compounds and caffeine, therefor it was employed for further study.

Table 3.8 Peak areas of phenolic compounds at various wavelenghs

Compounds	Concentration (ppm)	Peak area (mAU/s)	
		270 nm	280 nm
GA	10	358.378	352.340
GC	30	27.445	15.979
Caffeine	10	1232.800	1087.800
EGC	30	91.232	52.306
C	20	374.200	345.100
EC	30	253.563	250.300
EGCG	20	569.747	504.900
GCG	20	492.600	485.900
ECG	20	436.646	437.812

3.4.5 Summary of the optimum HPLC condition

The optimum HPLC conditions obtained including column detection wavelength and composition mobile phase of was with a 20 µL injection volume are listed below. These optimal condition resulted used in subsequent studies in this work. Under the optimum condition, all analytes were separated ($R_s > 0.1$). Chromatogram of phenolic compound and caffeine standard is shown in Figure 3.11.

Column	Wakosil-II 5C18 HG (5 µm, 0.3 x 250 mm)	
Mobile phase	A: 1 % ethyl acetate in methanol	
	B: 0.1 % phosphoric acid in water	
Gradient profile:	Time (minute)	% A
	0	15
	60	15
	70	30
	75	30
	80	15
Flow rate	0.45 ml/min	
Injection volume	20 µL	
Detection wavelength	270 nm	

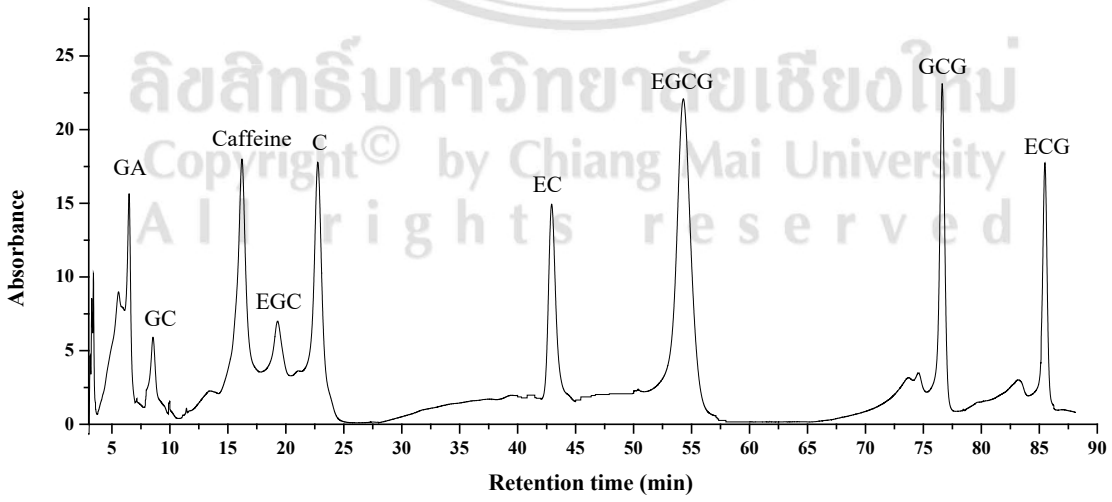

Figure 3.11 Chromatogram of phenolic compound standards and caffeine

3.5 Method validation

After establishing the optimized condition of HPLC, detection limit, precision and percent recovery were investigated.

3.5.1 Detection limit

The detection limit values were calculated from the linear regression line of the calibration curves based on a method described by Miller and Miller.[92] Peak areas of phenolic compounds and caffeine at various concentrations are shown in Table 3.9. Limits of detection (LOD) and limit of quantification (LOQ) of eight phenolic compounds and caffeine are summarized in Table 3.10.

Table 3.9 Peak areas of phenolic compounds and caffeine at various concentrations for determination of LOD and LOQ (n=3)

Compounds		1	2	3	4
GA	Conc. (ppm)	0.60	0.90	1.20	1.50
	Peak area (mAU*s)	14.80	24.24	37.13	46.22
GC	Conc. (ppm)	5.00	10.00	15.00	20.00
	Peak area (mAU*s)	38.36	63.97	97.96	139.15
Caffeine	Conc. (ppm)	0.10	0.15	0.20	0.25
	Peak area (mAU*s)	8.93	15.06	21.66	30.65
EGC	Conc. (ppm)	1.60	2.40	3.20	4.00
	Peak area (mAU*s)	10.42	14.68	19.41	40.19
C	Conc. (ppm)	1.00	1.50	2.00	2.50
	Peak area (mAU*s)	4.50	7.16	11.26	13.20
EC	Conc. (ppm)	1.40	2.10	2.80	3.50
	Peak area (mAU*s)	5.12	8.18	14.17	20.37
EGCG	Conc. (ppm)	1.20	1.80	2.40	3.00
	Peak area (mAU*s)	21.30	41.40	59.30	68.20
GCG	Conc. (ppm)	1.30	1.95	2.60	3.25
	Peak area (mAU*s)	18.40	27.66	39.98	62.69
ECG	Conc. (ppm)	0.90	1.35	1.80	2.25
	Peak area (mAU*s)	23.41	33.17	50.51	68.50

Table 3.10 Summary of LOD and LOQ of phenolic compounds and caffeine

Compounds	Regression line equation	Correlation coefficient (R^2)	LOD (ppm)	LOQ (ppm)
GA	y = 32.246x - 7.0869	R^2 = 0.9831	0.10	0.32
GC	y = 0.5567x - 1.3751	R^2 = 0.9585	2.45	8.19
Caffeine	y = 66.683x + 2.9298	R^2 = 0.9729	0.05	0.13
EGC	y = 13.007x - 12.745	R^2 = 0.9627	0.75	2.49
C	y = 6.7681x - 1.2213	R^2 = 0.9670	0.35	1.03
EC	y = 7.4551x - 3.7981	R^2 = 0.9590	0.55	1.65
EGCG	y = 24.19x - 3.8306	R^2 = 0.9972	0.48	1.56
GCG	y = 23.352x - 9.9102	R^2 = 0.9965	0.75	2.16
ECG	y = 31.754x - 8.5108	R^2 = 0.9898	0.23	0.70

3.5.2 Precision

The precision, repeatability and reproducibility give a measure of error in the development methodology and these are usually reported as a percentage of relative standard deviation (%R.S.D.). In this study, the repeatability was determined by five injections of standard mixture of phenolic compounds and caffeine onto the C18 column under the optimum condition in the same day. The reproducibility was determined by five injections in different days. Results are shown in Tables 3.11 – 3.12. The repeatability and reproducibility of peak area of each compound expressed as R.S.D. were in the range of 0.05 – 1.03%, and 0.16 – 3.35%, respectively. These values are considered acceptable for analytical purposes as it indicates < 5% (high precision).

Table 3.11 Repeatability of the peak area of each phenolic compound and caffeine

No. of injection	Peak area (mAU*s)								
	GA	GC	Caffeine	EGC	C	EC	EGCG	GCG	ECG
1	142.61	41.88	367.14	294.00	106.76	127.39	284.49	515.68	149.20
2	141.48	42.15	366.60	294.03	106.67	126.37	248.73	515.45	149.07
3	142.80	41.75	368.28	295.03	106.89	127.99	283.48	515.66	149.95
4	142.75	42.99	367.01	293.70	106.44	125.36	284.78	514.97	149.31
5	142.11	42.45	365.77	292.44	105.93	126.14	284.64	515.54	149.16
Average	142.35	42.25	366.96	293.84	106.54	126.65	284.22	515.46	149.34
SD	0.50	0.44	0.82	0.83	0.34	0.93	0.55	0.26	0.31
%RSD	0.35	1.03	0.22	0.28	0.32	0.74	0.19	0.05	0.21

Table 3.12 Reproducibility of the peak area of each phenolic compound and caffeine

No. of injection		Peak area (mAU*s)								
		GA	GC	Caffeine	EGC	C	EC	EGCG	GCG	ECG
Day 1	1	142.61	41.88	367.14	294.00	106.76	127.39	284.49	515.68	149.20
	2	141.48	42.15	366.60	294.03	106.67	126.37	248.73	515.45	149.07
	3	142.80	41.75	368.28	295.03	106.89	127.99	283.48	515.66	149.95
	4	142.75	42.99	367.01	293.70	106.44	125.36	284.78	514.97	149.31
	5	142.11	42.45	365.77	292.44	105.93	126.14	284.64	515.54	149.16
Day 3	1	141.23	41.20	364.43	283.76	100.13	124.41	266.03	515.42	147.25
	2	141.15	40.58	363.48	283.67	100.29	124.40	267.58	515.62	147.15
	3	141.84	42.23	364.68	283.84	100.08	125.73	267.21	515.80	147.38
	4	140.19	41.27	364.79	283.20	100.51	124.79	267.78	513.76	147.75
	5	141.23	40.40	365.77	283.54	100.22	124.71	267.07	513.47	147.70
Day 5	1	134.61	38.89	362.11	287.63	95.15	117.55	266.89	514.09	142.78
	2	133.90	38.95	360.56	286.66	96.90	118.80	266.56	514.15	142.63
	3	134.37	38.83	360.06	286.48	95.08	116.40	266.72	515.53	142.75
	4	135.12	39.43	360.84	287.21	94.70	118.80	265.86	514.74	142.10
	5	133.86	39.71	360.45	287.28	96.49	118.60	265.13	516.13	142.19
	Average	139.28	40.85	364.13	288.16	100.82	123.16	270.20	515.07	146.42
	SD	3.54	1.37	2.65	4.29	4.49	3.79	9.63	0.80	2.90
	%RSD	2.54	3.35	0.73	1.49	4.45	3.08	3.57	0.16	1.98

3.5.3 Recovery assay

The extraction of fresh leaves and 45 days fermented leaves from Assam tea samples was carried out by spiking each sample with mixed phenolic compounds and caffeine at 5LOQ and 10LOQ concentrations. The results shown as percentage recovery of nine compounds are summarized in Table 3.13.

Table 3.13 The percentage recovery of extraction for fresh and 45 days fermented leaves from Assam tea at 5LOQ and 10LOQ concentrations (n=3)

Compounds	%Recovery			
	5LOQ		10LOQ	
	Fresh leaves	45 days fermented leaves	Fresh leaves	45 days fermented leaves
GA	96	81	90	93
GC	95	92	84	85
Caffeine	92	89	92	98
EGC	93	90	97	96
C	97	94	95	92
EC	97	86	97	96
EGCG	92	92	83	87
GCG	92	92	85	91
ECG	94	95	92	92

The accuracy expressed in terms of percentage recovery was obtained by spiking the mixture of phenolic compounds and caffeine into fresh leaves and 45 days fermented leaves from Assam tea. The percentage recoveries of this proposed method for GA, GC, caffeine, EGC, C, EC, EGCG, GCG and ECG were 90-96, 84-95, 92, 89-98, 93-97, 95-97, 97, 83-92, 85-92, and 92-94, respectively of fresh leaves and 81-93, 85-92, 89-98, 90-96, 92-94, 86-96, 87-92, 91-92 and 92-95, respectively, of 45 days fermented leaves.

3.6 Quantitative HPLC analysis of phenolic compounds and caffeine in samples

3.6.1 Extraction yield

In the sample extract of fresh, steamed and fermented leaves (15, 30, 45, 60, 90, 120, and 150 days) from Assam tea were extracted by 80% aqueous acetone. The highest yield was obtained from 15 days fermented leaves extract, followed by 120, 90, 60, 30 days fermented leaves, steamed leaves, 45 days fermented leaves, 150 days fermented leaves, and fresh leaves extract, respectively (Table 3.14).

Table 3.14 Physical properties and percentages yields of various extracts from Assam tea

Samples	Appearance	Colour	% yield (w/w)
Fresh leaves	liquid	olive-brown	17.49
Steamed leaves	liquid	dark olive-brown	22.52
Fermented leaves			
15 days	liquid	dark-brown	25.75
30 days	liquid	dark-brown	23.27
45 days	liquid	dark-brown	22.48
60 days	liquid	dark-brown	24.05
90 days	liquid	dark-brown	24.86
120 days	liquid	dark-brown	24.99
150 days	liquid	dark-brown	19.58

3.6.2 HPLC analysis of phenolic compounds and caffeine in samples

In order to demonstrate the suitability of the proposed method in this study for quantification of phenolic compounds and caffeine in real samples, fresh, steamed, fermented leaves (15, 30, 45, 60, 90, 120, and 150 days), steamed and fermented waters (15, 30, 45, 60, 90, 120 and 150 days) were analyzed. Calibration curves based on peak area were linear (R^2=0.9917–0.9992) over a concentration range of 5.0-25.0 ppm for GA, 15.0-120.0 ppm for GC, 1.0-5.0 ppm for caffeine, 10.0-50.0 ppm for EGC, 10.0-50.0 ppm for C, 10.0–50.0 ppm for EC, 10.0-50.0 ppm for EGCG, 5.0-25.0 ppm for GCG and 5.0-25.0 ppm for ECG as illustrated in Figure. 3.12-3.20.

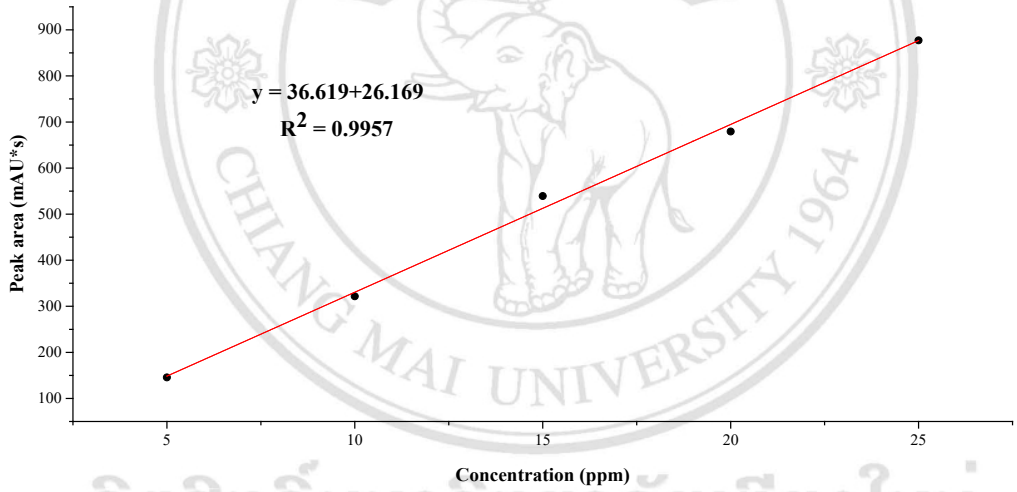

Figure 3.12 Calibration curve used for calculating concentration of GA in samples

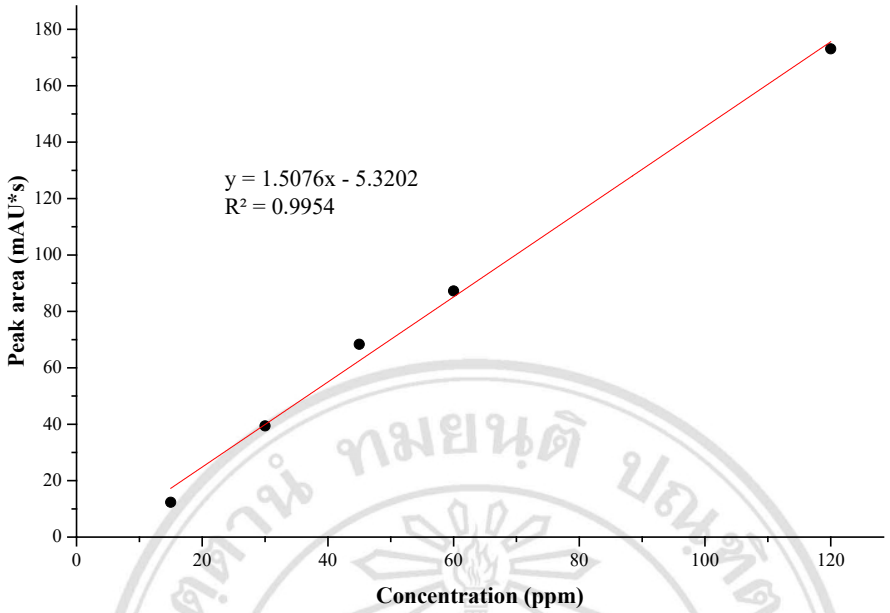

Figure 3.13 Calibration curve used for calculating concentration of GC in samples

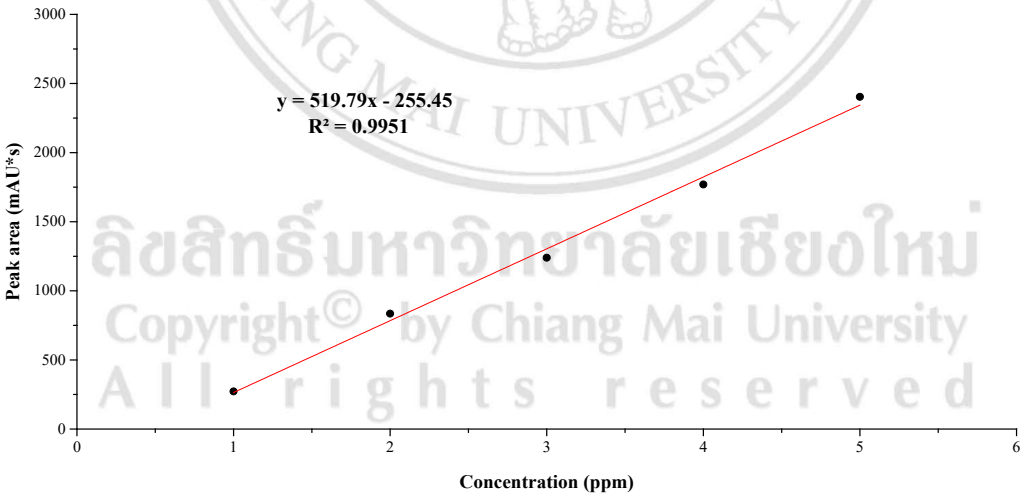

Figure 3.14 Calibration curve used for calculating concentration of caffeine in samples

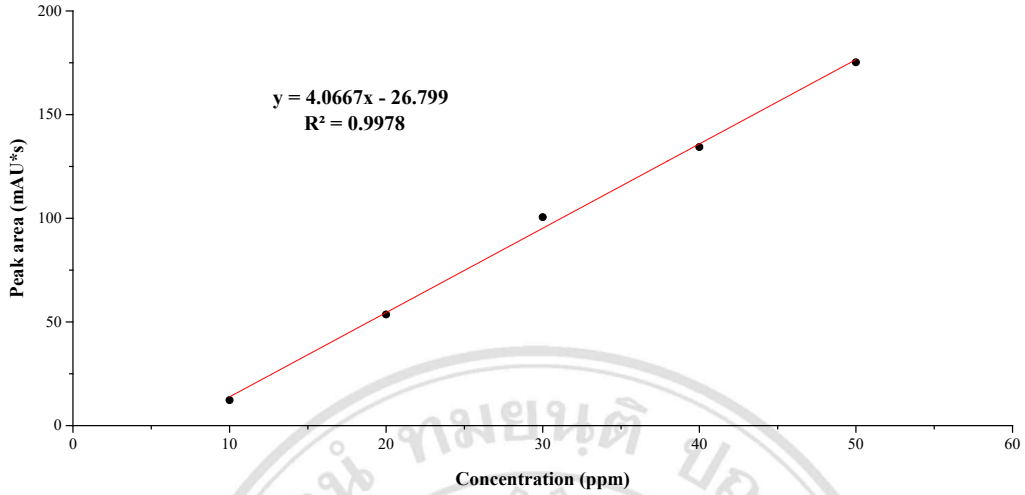

Figure 3.15 Calibration curve used for calculating concentration of EGC in samples

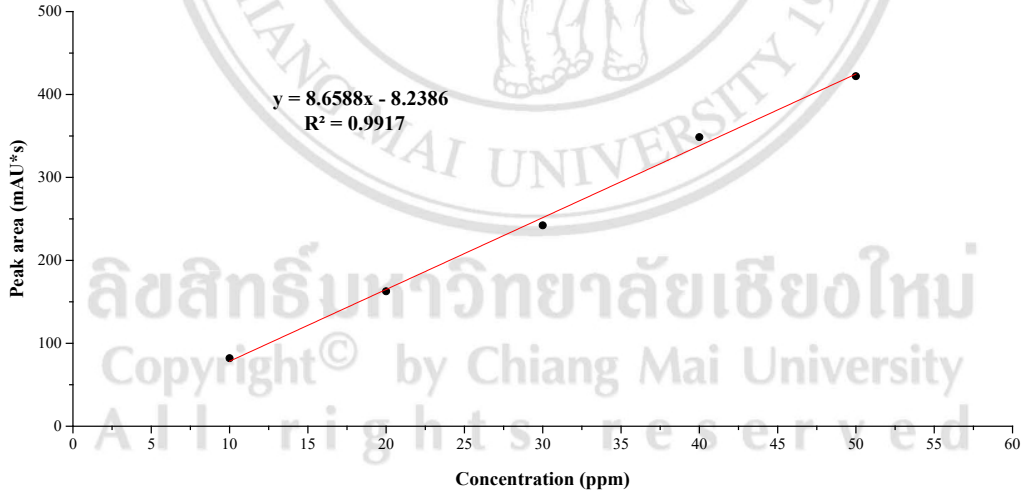

Figure 3.16 Calibration curve used for calculating concentration of C in samples

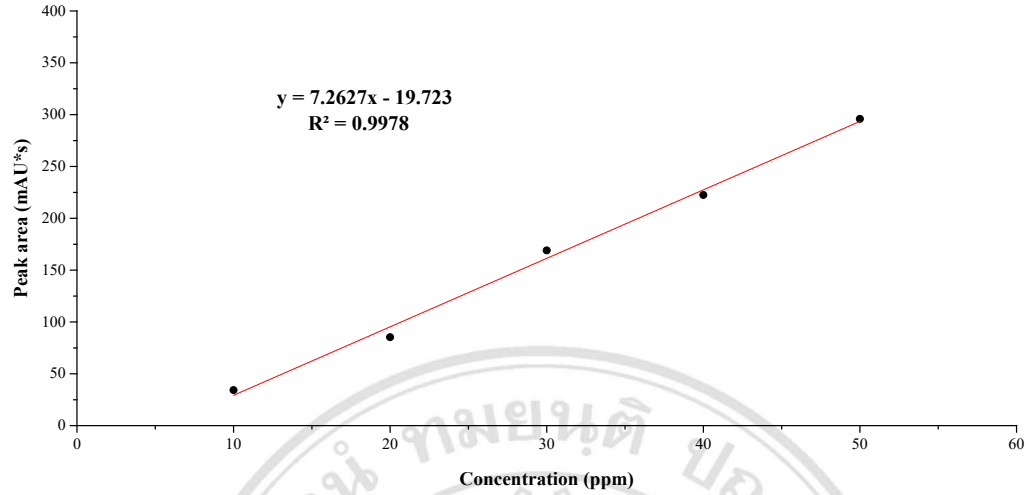

Figure 3.17 Calibration curve used for calculating concentration of EC in samples

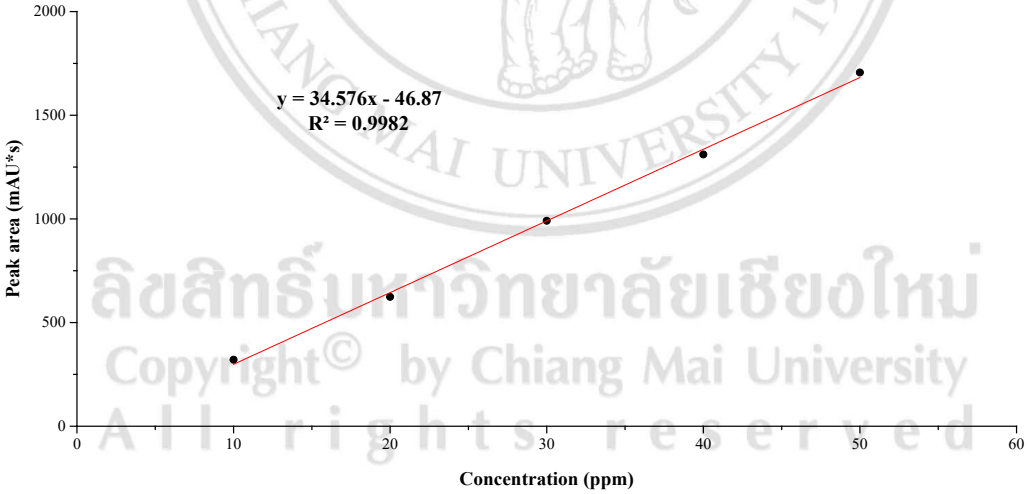

Figure 3.18 Calibration curve used for calculating concentration of EGCG in samples

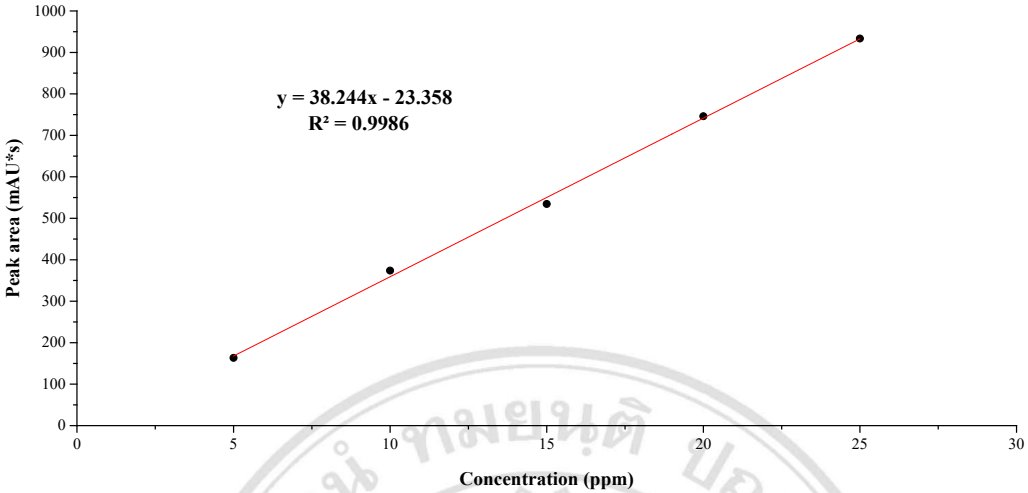

Figure 3.19 Calibration curve used for calculating concentration of GCG in samples

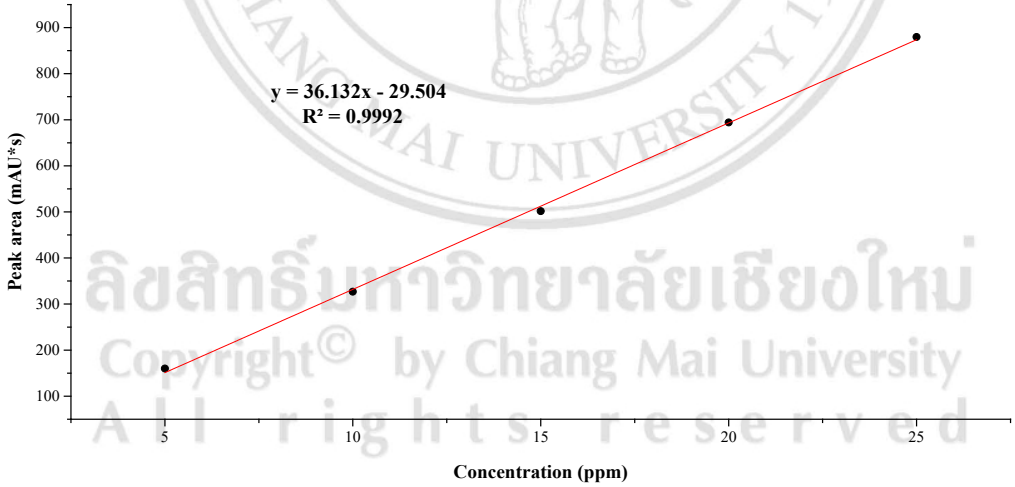

Figure 3.20 Calibration curve used for calculating concentration of ECG in samples

A HPLC profile of the phenolic compounds and caffeine of some sample extracts is presented in Figure 3.21. The caffeine content was decreased to 71.25% from the actual extract. Table 3.15–3.16 were showed the content of catechins and caffeine in the leave extracts and water samples after some caffeine extraction. The concentrations of GA, GC, caffeine, EGC, C, EC, EGCG, GCG and ECG in all sample extracts ranged from 0.123-0.368, 0.145-0.305, 0.071-0.625, 0.018-0.343, 0.230-0.494, 0.185-1.400, 0.034-16.976, 0.037-4.814 and 0.049-15.357 mg/g DW, respectively. The highest amount of GA was detected in steamed leaves extract, whilst the 45 days fermented leaves extract showed the lowest amount of GA as shown in Figure 3.22. GC and EGC were detected in high amount in fresh leaves extract, while the steamed leaves had the highest amount of C, EGCG, GCG, and ECG (0.494, 16.976, 4.814, and 15.357 mg/g DW, respectively). The highest amount of caffeine and EC was detected in 120 and 15 days fermented leaves extracts.

In the water samples, the concentration of GA, GC, caffeine, EGC, C, EC, EGCG, GCG, and ECG in all sample waters ranged from 0.293-3.559, 0.228-0.396, 0.076-0.267, 0.027-0.081, 0.383-3.679, 0.388-3.424, 0.104-0.861, 0.059-0.240 and 0.008-0.210 mg/g DW, respectively. The 30 days fermented water contained the highest amount of C, and EGCG at 3.679 and 0.861 mg/g DW, respectively, while the highest amount of GC and EC was detected in the 150 days fermented water (Figure 3.23). The 60 days fermented water contained the highest amount of GCG, and ECG at 0.258 and 0.210 mg/g DW, respectively. The highest amount of GA, caffeine and EGC contents were detected in 120, 15 days fermented water and steamed water, respectively. The steamed leaves contained higher total phenolic compound (both GA, EGCG, ECG, and GCG) compared with non-steamed sample (fresh leaves). This might be due to the phenolic compound in fresh leaves being all in polymerized (undetectable) form and they may be decomposed by heat into single molecules.[93]

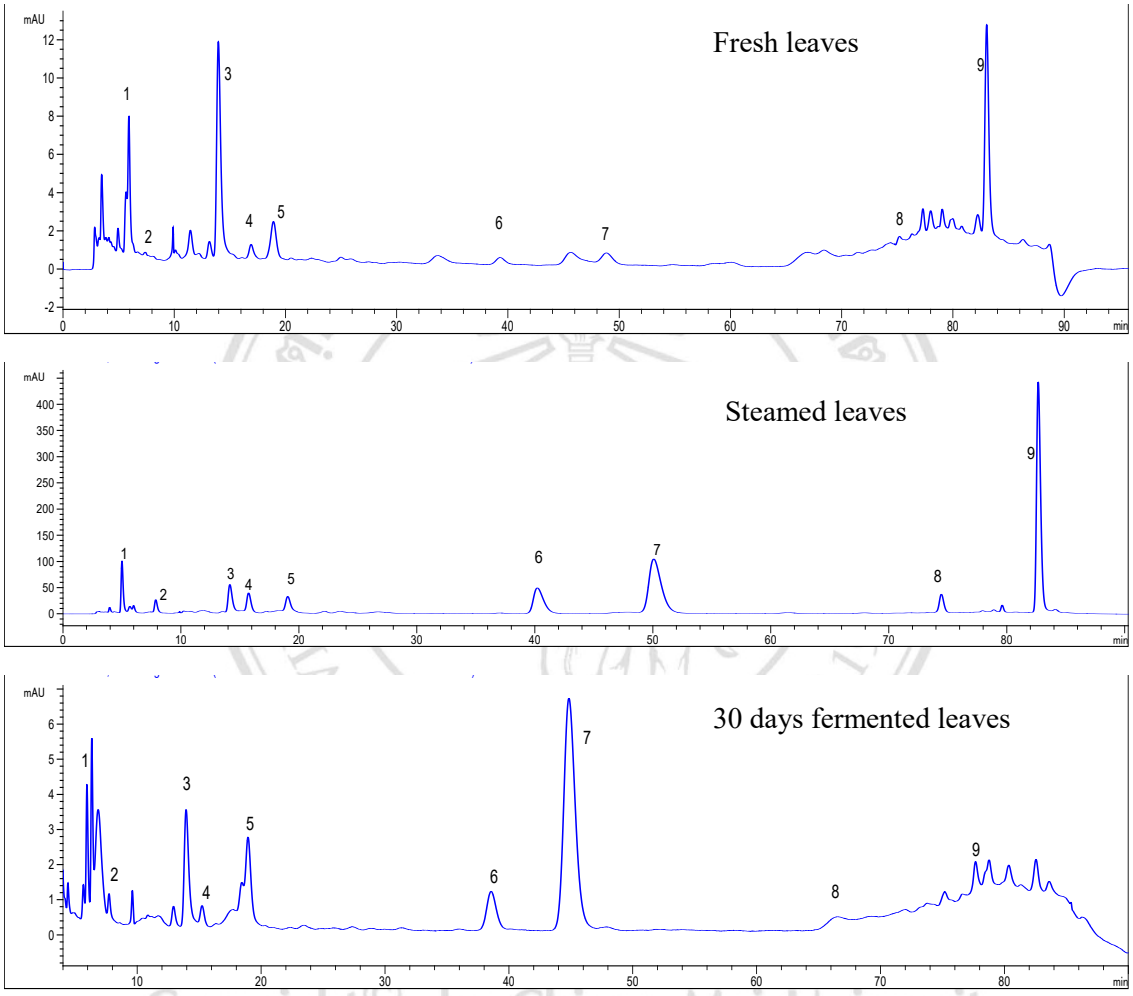

Figure 3.21 Chromatograms of phenolic compounds in sample extracts obtained using 1% ethyl acetate in methanol/ 0.1% (v/v) phosphoric acid in water

Peak identification: (1) GA, (2) GC, (3) caffeine, (4) EGC, (5) C, (6) EC, (7) EGCG, (8) GCG and (9) ECG

Table 3.15 Contents of phenolic compounds and caffeine of leaves extracts from Assam tea

Samples	Content (mg/g DW)								
	GA	**GC**	**Caffeine**	**EGC**	**C**	**EC**	**EGCG**	**GCG**	**ECG**
Fresh leaves	0.170	**0.305**	0.079	**0.343**	0.369	0.210	0.062	0.108	0.189
Steamed leaves	**0.368**	0.184	0.223	0.090	**0.494**	0.318	**16.976**	**4.814**	**15.357**
15 days fermented	0.217	0.285	0.071	0.054	0.230	**1.400**	0.034	0.167	0.189
30 days fermented l	0.208	0.170	0.131	0.018	0.257	0.210	1.082	0.042	0.107
45 days fermented	0.123	0.145	0.172	0.045	0.230	0.185	0.165	0.063	0.074
60 days fermented	0.132	0.188	0.193	0.054	0.230	0.204	0.153	0.056	0.070
90 days fermented	0.359	0.275	0.180	0.036	0.209	0.592	0.365	0.038	0.049
120 days fermented	0.207	0.194	**0.625**	0.038	0.267	1.068	0.305	0.037	0.146
150 days fermented	0.210	0.192	0.623	0.038	0.262	1.074	0.304	0.040	0.145

Table 3.16 Content of phenolic compounds and caffeine of water samples from Assam tea

Samples	Content (mg/g DW)								
	GA	**GC**	**Caffeine**	**EGC**	**C**	**EC**	**EGCG**	**GCG**	**ECG**
Steamed	0.463	0.351	0.234	**0.081**	0.383	0.388	0.104	nd	0.008
15 days fermented	2.539	0.315	**0.267**	0.045	0.480	2.081	0.590	0.219	0.049
30 days fermented	0.293	0.242	0.261	0.045	**3.679**	2.024	**0.861**	0.240	0.198
45 days fermented	0.434	0.267	0.182	0.027	1.050	0.522	0.588	0.035	0.066
60 days fermented	1.624	0.311	0.123	0.036	1.314	2.247	0.699	**0.258**	**0.210**
90 days fermented	0.481	0.228	0.090	0.036	0.862	0.681	0.191	0.059	0.041
120 days fermented	**3.559**	0.311	0.117	0.045	1.106	3.392	0.759	0.195	0.111
150 days fermented	3.172	**0.396**	0.076	0.063	3.102	**3.424**	0.769	0.237	0.107

nd, not detected (less than the detect limit value)

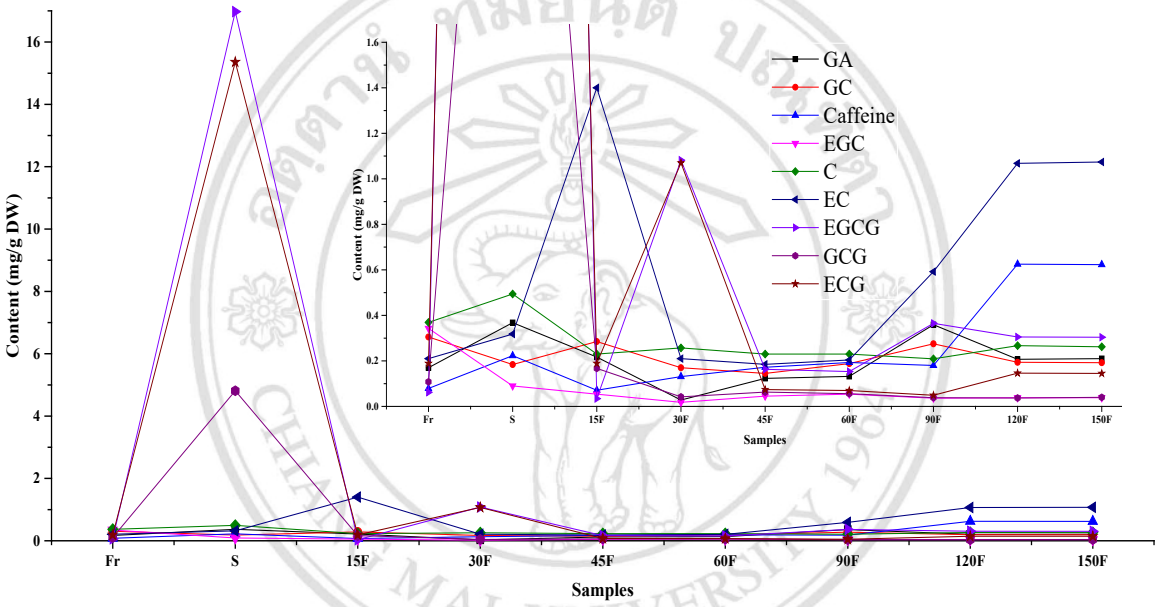

Figure 3.22 Content of GA, GC, caffeine, EGC, C, EC, EGCG, GCG, and ECG from extract samples

Note; Fr: Fresh leaves, S: Steamed leaves, 15F: 15 days fermented leaves, 30F: 30 days fermented leaves, 45F: 45 days fermented leaves, 60F: 60 days fermented leaves, 90F: 90 days fermented leaves, 120F: 120 days fermented leaves, and 150F: 150 days fermented leaves

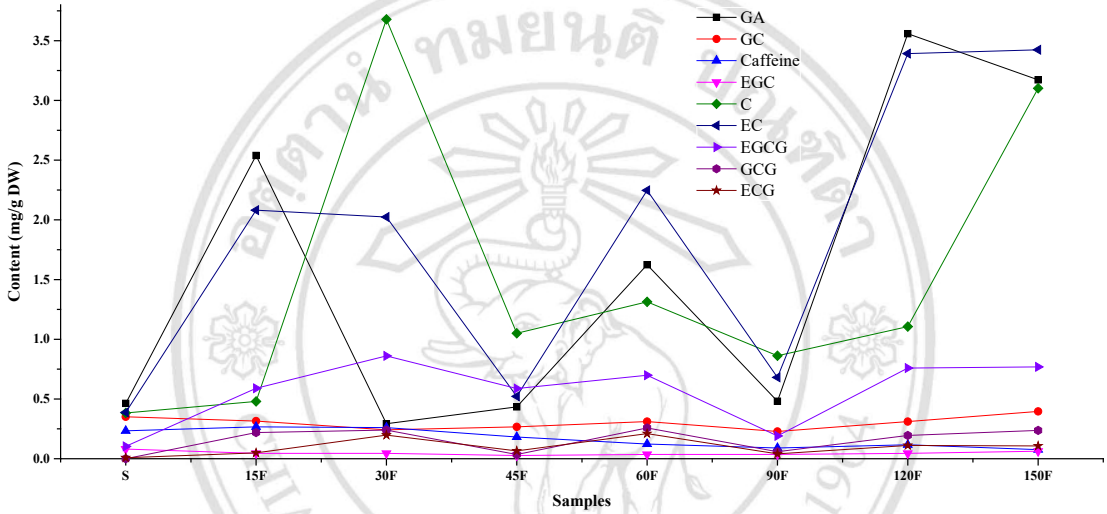

Figure 3.23 Content of GA, GC, caffeine, EGC, C, EC, EGCG, GCG, and ECG from water samples

Note; S: Steamed water, 15F: 15 days fermented water, 30F: 30 days fermented water, 45F: 45 days fermented water, 60F: 60 days fermented water, 90F: 90 days fermented water, 120F: 120 days fermented water, and 150F: 150 days fermented water

3.7 Total phenolic content and antioxidant activities of Assam tea

3.7.1 Total phenolic contents of extract

Table 3.17 shows the total phenolic content of the leaves extracts (fresh, steamed and fermented) and water (steamed and fermented water) were determined spectrometrically according to Folin-Ciocalteau method. The part of leaves extracts, the 150 days fermented leaves extract shown the highest phenolic content with the value of 457.26 mgGAE/g DW, whereas fresh leaves had the lowest amount of phenolic content. For the water sample, the 30 days fermented water had the highest concentration of phenolic substances, followed by 120 days fermented water, 60 days fermented water and 150 days fermented water. These results corresponded with previous research. During the microbial fermentation of fermented tea, the chemical constituents of fresh tea are qualitatively and quantitatively changed and some new metabolites were found.[94-96]

Table 3.17 Total phenolic contents of leaves extracts and water samples

Samples	Total phenolic content (mgGAE/g DW)	
	Leaves	Waters
Fresh	231.75 ± 0.18^{a}	–
Steamed	413.80 ± 0.01^{f}	68.28 ± 0.01^{b}
Fermented		
15 days	428.56 ± 0.01^{g}	38.34 ± 0.00^{a}
30 days	369.94 ± 0.00^{d}	644.04 ± 0.01^{h}
45 days	436.35 ± 0.01^{h}	311.61 ± 0.14^{c}
60 days	323.01 ± 0.01^{c}	594.50 ± 0.03^{f}
90 days	258.66 ± 0.02^{b}	548.71 ± 0.04^{e}
120 days	380.87 ± 0.02^{c}	622.54 ± 0.03^{h}
150 days	457.26 ± 0.02^{i}	534.69 ± 0.01^{d}

Data are given as means ± SD (n=3)

*; Leaves from extraction with 80% aqueous acetone

**; Water from steaming and fermented processing

Values with different letters (a-i) within column of each samples are significantly different at $P<0.01$

3.7.2 Antioxidant activity of the leaves extracts and waters

The fresh, steamed, fermented leaves (15, 30, 45, 60, 90, 120, and 150 days), steamed and fermented waters (15, 30, 45, 60, 90, 120, and 150 days) from Assam tea were evaluated antioxidant activities using DPPH and FRAP assay, as described in Section 2.8 and 2.9.

1) DPPH assay of leaves extracts and water

Figure 3.24 showed DPPH radical scavenging activity of the leaves extracts and water sample in a concentration-dependent manner. DPPH radical scavenging activity is determined by IC_{50} values. The IC_{50} value can be calculated from this graph at 50 % inhibitory concentration. The results showed that the steamed leaves extract had significantly the highest activity. The highest DPPH radical scavenging activity was observed in steamed leaves extract at IC_{50} value of 12.78 ppm followed by the 15 days, 45 days, 150 days, and 90 days fermented leaves as shown in Figure 3.25 and Table 3.18. In water samples, the strongest antioxidant activities were demonstrated the 150 days and 60 days fermented water (14.61 and 18.67 ppm, respectively).

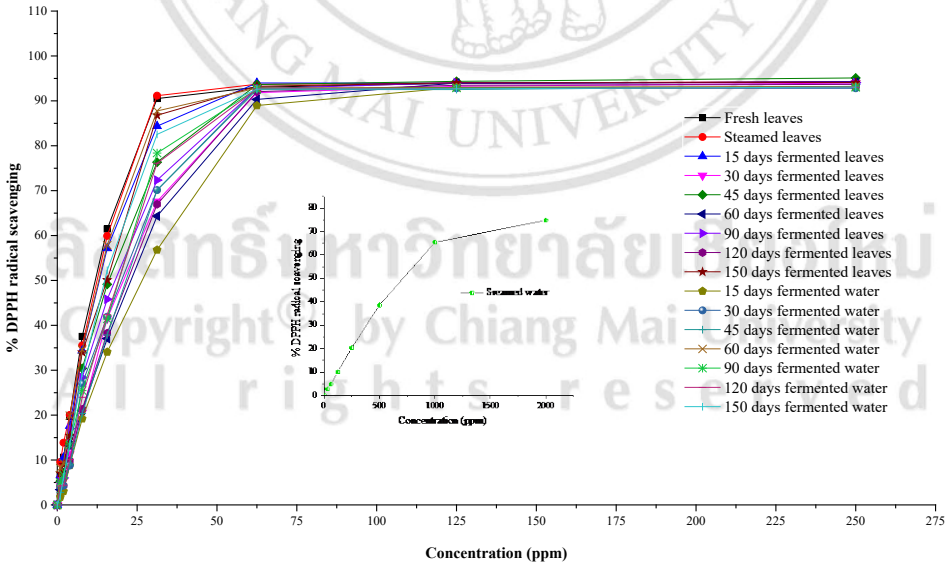

Figure 3.24 %DPPH radical scavenging activity of sample extracts and water samples

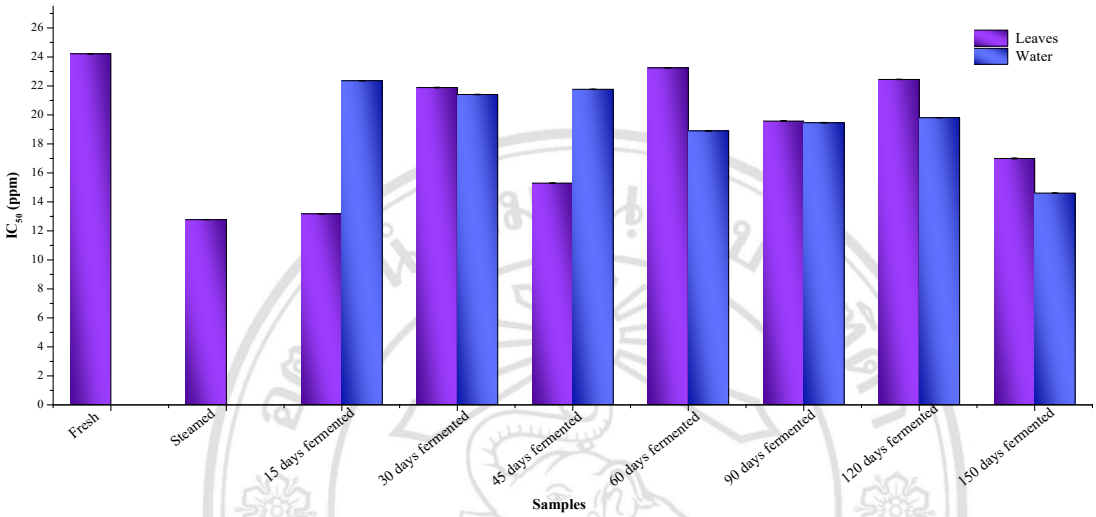

Figure 3.25 IC_{50} values of sample extracts and water samples

2) FRAP assay of leaves extracts and water

The reducing capacity of the samples is presented in Figure 3.26. The highest antioxidant activities were obtained for the 150 days and steamed leaves extracts (307.34 and 305.34 mg GAE/g DW, respectively), followed by the 45 days and 15 days fermented leaves extract (302.45 and 290.53 mg GAE/g DW, respectively). In part water samples, the 30 days fermented water showed the highest reducing ability (480.47 mgGAE/g DW), followed by 60 days and 120 days fermented water (471.71 and 464.68 mgGAE/g DW, respectively). The steamed water is significantly lower than all the samples (44.91 mgGAE/g DW)

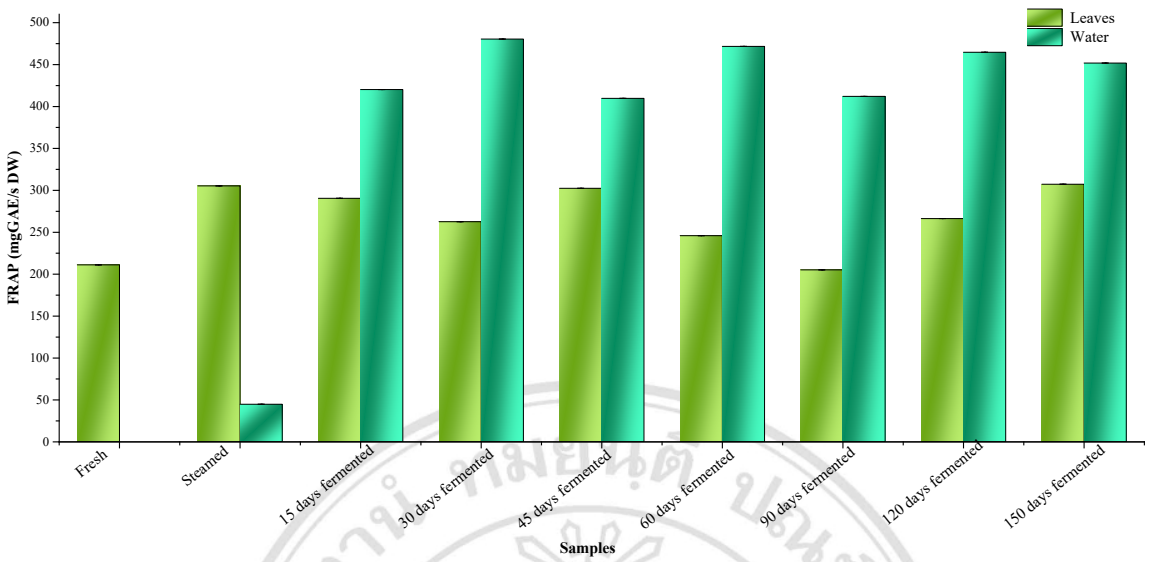

Figure 3.26 FRAP of sample extracts and water samples

Table 3.18 Antioxidant activities of fresh, steamed, fermented leaves, streamed, and fermented water of Assam tea

Samples	DPPH assay IC_{50} (ppm)		FRAP assay (mgGAE/g DW)	
	Leaves	Water	Leaves	Water
Fresh	24.22 ± 0.02^{i}	–	211.12 ± 0.04^{b}	–
Steamed	12.78 ± 0.01^{a}	643.09 ± 0.01^{h}	305.34 ± 0.02^{h}	44.91 ± 0.00^{a}
Fermented				
15 days	13.18 ± 0.01^{b}	22.36 ± 0.02^{g}	290.53 ± 0.00^{f}	420.23 ± 0.01^{d}
30 days	21.89 ± 0.02^{f}	21.41 ± 0.02^{e}	262.62 ± 0.02^{d}	480.47 ± 0.00^{h}
45 days	15.30 ± 0.01^{c}	21.77 ± 0.01^{f}	302.45 ± 0.03^{g}	409.71 ± 0.01^{b}
60 days	23.25 ± 0.01^{h}	18.67 ± 0.01^{b}	245.95 ± 0.07^{c}	471.70 ± 0.00^{g}
90 days	19.58 ± 0.02^{e}	19.46 ± 0.02^{c}	205.13 ± 0.01^{a}	412.05 ± 0.00^{c}
120 days	22.45 ± 0.01^{g}	19.81 ± 0.01^{d}	266.37 ± 0.03^{e}	464.68 ± 0.02^{f}
150 days	17.00 ± 0.03^{d}	14.61 ± 0.01^{a}	307.34 ± 0.05^{i}	451.81 ± 0.01^{e}

Ascorbic acid : standard (IC_{50} = 15.82 ± 0.00 ppm)

Data are given as means ± SD (n=3)

Values with different letters (a-i) within column of each solvent are significantly different at $P<0.01$

The result of all samples is shown in Table 3.18. The highest antioxidant activity were obtained for the steamed leaves, 45 days fermented leaves and 150 days fermented leaves in DPPH and FRAP assay. In water samples, the antioxidant activity of the 60 days fermented water in DPPH and FRAP assays were higher than other water samples. The chemical structures required for effective antioxidant activity of flavonoids and polyphenols have been identified recently.[98] The most important features that have been identified in these studies are an *ortho*-dihydroxy catechol (3', 4'–OH) arrangement on the B-ring (present in flavan-3-ols) that promotes formation of stable phenoxyl radical due to effective electron delocalization and a C2–C3 double bond in the C-ring in combination with a C4 carbonyl group and the hydroxypyranone structure (Figure 3.27). In addition to acting as efficient free radical scavengers based on these structural features, flavonoids can also chelate free metal ion via the catechol group on the B-ring, thus reducing metal-catalyzed ROS production. The iron-chelating ability of catechins has been ranked: EGC > ECG = EGCG > EC.[99-101]

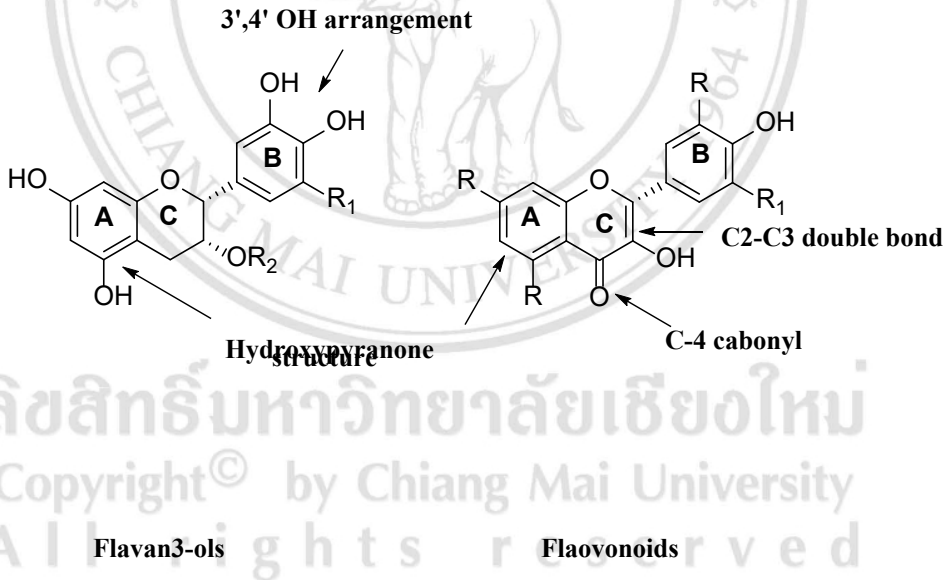

Figure 3.27 Structural features of flavonoids important to antioxidant chemistry

3.8 Correlation between total phenolic contents and antioxidant activities

The correlation was established between the phenolic contents and their antioxidant activities in the solvent extract, essential oils, sample extracts and water sample as shown in Table 3.19. The correlation coefficient between total phenolic contents and the DPPH (%radical scavenging) activity of solvent extracts, volatile oils, leave extracts and water samples was r^2 = 0.716, 0.782, 0.704 and 0.576, respectively and FRAP activity was r^2 = 0.854, 0.667, 0.977 and 0.668, respectively, which showed significant correlation ($P<0.01$). It suggested that the phenolic compounds may contribute to the antioxidant properties of these samples from Assam tea. The remaining of the FRAP and DPPH radical scavenging activity may be due to the presence of bioactive compounds such as carotenoids, vitamins and other glycosides. Moreover, correlation coefficient between DPPH (%radical scavenging) and FRAP assay showed significant correlation coefficient, r^2 = 0.646, 0.677, 0.735 and 0.982 in solvent extracts, volatile oils, leave extracts and water samples, respectively.

Table 3.19 Correlation coefficient between total phenolic contents and the DPPH activity of solvent extracts, essential oils sample extracts and sample waters

Correlation	Correlation coefficient (r^2)			
	Solvent extracts	**Essential oils**	**Leave extracts**	**Water sample**
TPC-DPPH	0.716**	0.782**	0.704**	0.576**
TPC-FRAP	0.854**	0.667**	0.977**	0.668**
DPPH-FRAP	0.646**	0.677**	0.735**	0.982**

** Correlation of significant at the 0.01 level (2-tailed)