CHAPTER 3

RESULTS AND DISCUSSION

3.1 Screening of biological activity of crude extracts

The water and ethanolic extracts of all plants were prepared as described in section 2.3. Table 3-1 showed the percentage yields of both water and ethanolic extracts from each part of the plants investigated as well as Triphala formula.

Table 3-1 Percentage yields of the water and ethanolic extracts from the investigated each species and Triphala formula

Plant species	Part	Extracts	% Yield (w/w)
Phyllanthus emblica Linn.	Pulp	Water	44.53
		EtOH	38.39
	Seed	Water	17.75
		EtOH	2.90
Terminalia chebula Retz.	Pulp	Water	40.52
		EtOH	48.01
	Seed	Water	35.31
		EtOH	4.25
Terminalia bellerica Gaertn.	Pulp	Water	28.27
		EtOH	31.23
	Seed	Water	27.13
		EtOH	2.78
Triphala		Water	45.67
		EtOH	34.00

From Table 3-1, the highest yield for water extract was found in Triphala, however for ethanolic extract, it was found in *T. chebula* Pulp. These results indicated that the development of Triphala to manufacturing for health food should get the high yield for production.

3.1.1 Free radical scavenging activity

The antioxidant activities of the water and ethanolic extracts of the plants and their formula were evaluated by DPPH radical scavenging assay as described in section 2.4.1. The results are shown in Table 3-2.

Table 3-2 EC_{50} (µg/ml) of plant extracts and its formula on DPPH assay (n=3)

Plant Species	Part	Water extracts (Mean±SEM)	Ethanolic extracts (Mean±SEM)
Phyllanthus emblica Linn.	Pulp	4.942 ± 0.16	2.846 ± 0.83
	Seed	6.779 ± 0.70	6.275 ± 0.86
Terminalia chebula Retz.	Pulp	5.420 ± 0.42	3.807 ± 0.80
	Seed	4.622 ± 0.34	2.925 ± 0.67
Terminalia bellerica Gaertn.	Pulp	4.074 ± 0.27	3.413 ± 1.06
	Seed	5.629 ± 0.75	3.355 ± 0.52
Triphala		4.335 ± 0.35	3.913 ± 0.19
BHT (positive control)			12.647 ± 0.52

n = number of independent experiment which was performed in 3 replicates.

The results showed that the ethanolic extract of pulp of *P. emblica* had the highest antioxidant activity in this test with the EC_{50} value of 2.846 ± 0.83 µg/ml, followed by the ethanolic extract of seed of *T. chebula* with the EC_{50} value of 2.925 ± 0.67 µg/ml. The water and ethanolic extracts of all plants and its formula showed high antioxidant activity with the EC_{50} value in the range of 4.074 – 6.779 µg/ml and 2.846 – 6.275 µg/ml, respectively. Interestingly, all extracts possessed high antioxidant activities with their value of EC_{50} less than 7 µg/ml which were lower than that BHT (EC_{50} = 12.647 ± 0.52 µg/ml), the standard antioxidant. These results exhibited that all components of Triphala showed high antioxidant activity, resulting the higher antioxidant of the formula. The pulp and seed also showed high antioxidant activity so the whole

fruits should be used in preparation of Triphala. It is not nescessory for remove pulp from seed. These results are in accordance with the previous reports. For example, the fruit of *P. emblica* showed antioxidant activity by DPPH assay the IC_{50} values of 0.65 ± 0.05, 0.85 ± 0.04 µg/ml for free and bound phenolics of extracts, respectively (Kumar *et al*., 2006). Ascorbic acid content in the extract of the fruit of *P. emblica* showed antioxidant activity by DPPH, IC_{50} < 0.25 mg/ml (Scartezzini *et al*., 2006). The methanol, chloroform, ethylacetate, *n*-butanol, organic aqueous and water extracts of the fruit of *T. chebula* and four pure compounds including, casuarinin, chebulanin, chebulinic acid and 1,6-di-*O*-galloyl-ß-D-glucose showed free radical scavenging activities, especially chebulinic acid had the strongest activity the IC_{50} value of 2 µg/ml, casuarinin and all extracts, except organic aqueous extract possessed free radical scavenging activity with IC_{50} values in the range of 4 - 9 µg/ml (Cheng *et al*., 2003). The 75% methanolic extract of the fruit of *T. chebula*, *T. bellerica*, *E. officinalis* and their combination named "Triphala" were found to be scavenge hydroxyl and superoxide radicals in vitro. The concentration of plant extracts needed for the inhibition of hydroxyl radicals scavenging were 165.5 ± 8.5, 71.0 ± 2.7, 155.0 ± 8.1 and 151.0 ± 7.2 µg/ml, respectively and that for superoxide scavenging activity were found to be 20.5 ± 3.2, 40.5 ± 6.5, 6.5 ± 1.5 and 12.5 ± 2.2 µg/ml, respectively (Sabu and Kuttan, 2002). The 75% methanolic extract of *T. chebula*, *T. bellerica*, *E. officinalis* and Triphala were found that to scavenge oxygen radicals depended upon the types of radical encountered. The concentration needed for 50% inhibition of superoxide by *E. officinalis* was six times less than that of *T. bellerica*, but the hydroxyl radicals were more effectively scavenged by *T. bellerica* than the other two extracts (Chase, 2002). The fruits extracts of *T. bellerica, T. chebula*, *P. emblica* and Triphala were found to have high antioxidant activity (69.6 – 90.6%) (Bajpai *et al*., 2005). The aqueous extract of the fruits of *E. officinalis*, *T. chebula*, *T. bellerica* and Triphala were found to possess the ability to scavenge free radicals such as DPPH and superoxide. As the phenolic compounds present in these extracts are mostly responsible for their radical scavenging activity. These reports revealed that all three constituents of Triphala are active and they exhibited slightly different activities under difference conditions. *T. chebula* has greater radical scavenging activity. Thus, Triphala is expected to be more efficient due to the combined activity of the individual components (Naik *et al*., 2005(a)).

3.1.2 Lipid peroxidation of liposome assay

The antioxidant activity of the water and ethanolic extracts of the plants and their formula were evaluated by liposome assay as described in section 2.4.2. The results are shown in Table 3-3.

Table 3-3 EC_{50} (µg/ml) of plant extracts and its formula on liposome assay (n=3)

Plant Species	Part	Water extracts (Mean±SEM)	Ethanolic extracts (Mean±SEM)
Phyllanthus emblica Linn.	Pulp	125.410 ± 13.26	54.687 ± 10.74
	Seed	186.017 ± 7.49	110.541 ± 6.21
Terminalia chebula Retz.	Pulp	136.619 ± 8.72	6.040 ± 1.73
	Seed	102.910 ± 13.41	5.008 ± 1.30
Terminalia bellerica Gaertn.	Pulp	124.583 ± 17.17	9.800 ± 3.07
	Seed	128.348 ± 13.45	65.014 ± 8.34
Triphala		105.194 ± 4.94	25.667 ± 1.66
BHT (positive control)			7.803 ± 0.40

n = number of independent experiment which was performed in 3 replicates.

The results showed that the ethanolic extract of seed of *T. chebula* Retz. had the highest antioxidant activity in this test (EC_{50} = 5.008 ± 1.30 µg/ml), followed by the ethanolic extract of pulp of *T. chebula* (EC_{50} = 6.040 ± 1.73 µg/ml). The ethanolic extracts of pulp and seed of *T. chebula* and pulp of *T. bellerica* showed high antioxidant activity with the EC_{50} values of 6.040, 5.008 and 9.800 µg/ml, respectively. All ethanolic extracts showed high antioxidant activity with the EC_{50} value in the range of 5.008 – 65.014 µg/ml, except for the seed of *P. emblica* (EC_{50} value 110.541 µg/ml). In contrast, all water extracts exhibited less antioxidant activities with the EC_{50} values in the range of 102.910 – 186.017 µg/ml. Interestingly, the ethanolic extracts of the pulp and seed of *T. chebula* and pulp of *T. bellerica* showed higher

antioxidative effect than BHT (EC_{50} = 7.803 ± 0.40 µg/ml) which is the standard antioxidant substance.

There are some studies indicated that the butanol extract of water fraction of *P. emblica* fruits, orally administered to rat at the dose of 100 mg/kg body-weight showed cytoprotective effect on the stomach wall by increasing the secretion of hexosamine and gastric mucus against the indomethacin induced ulceration and decreasing the level of malonaldehyde (MDA) (Bandyopadhyay *et al*., 2000). Aqueous extract of *P. emblica* fruits showed antioxidant activity to inhibit γ-radiation-induced lipid peroxidaion (LPO) in rat liver microsomes damage in rat liver mitochondria. The antioxidant activity of *P. emblica* fruits extract was found to be both dose- and concentration-dependent (Khopde *et al*., 2001). An emblicanin-A (37%) and emblicanin-B (33%) enriched fraction of fresh fruit juice of *E. officinalis* (50 and 100 mg/kg body wt.) showed antioxidant effect that prevented ischemia-reperfusion (IRI)-induced oxidative stress in rat heart and may have a cardioprotective effect (Bhattacharya *et al*., 2002). The oral administration of flavonoid from methanol extract of *E. officinalis* at a dose of 10 mg/kg body weight/day showed significant antioxidant action in cholesterol-fed experiment rats. The antioxidant activity of free radical-scavenging enzymes were significantly elevated and lipid peroxide content was significantly decreased in flavonoid-treated hypercholesterolemic rats (Anila and Vijayalakshmi, 2003). The dehydrated powder of 5% and 10% levels of *E. officinalis* showed the ability to detoxify the dimethyl hydrazine (DMH) partly by enhancing the multicomponent antioxidant system in the rat (Anilakumar *et al*., 2004). Dried fruit rind of *P. emblica* was extracted with methanol and then separated into hexane, ethyl acetate and water fractions. Among these only the ethyl acetate phase showed strong NO scavenging activity in vitro, when compared with water and hexane phases. The ethyl acetate fraction was then to separation and purification. Aqueous extract of *T. chebula* inhibited γ-radiation-induced lipid peroxidation in rat liver microsomes and damage to superoxide dismutase enzyme in rat liver mitochondria. The extract inhibited xanthine/xanthine oxidase activity and also scavenger of DPPH radicals by using stopped-flow kinetic spectrometer. It can be concluded that the extract acts as a potent antioxidant and therefor, able to protect cellular organelles from the radiation-induced damage (Naik *et al*., 2004). This experiment showed that the ethanolic extract possessed higher antioxidant activity than the water extract. From the previous reports, aqueous extract of *T.*

chebula could inhibit the formation of thiobarbituric acid reactive substances (TBARS) (Naik *et al*., 2003) and the ethanol extract from the fruit of *T. chebula* exhibited significant inhibitory activity on oxidative stress. In the peroxidation model, it showed a notable cytoprotective effect on the human epidermal keratinocytes-Neonatal/Foreskin (HEK-N/F) cells at a concentration of 50 µg/ml (Na *et al*., 2004). The other research showed that the methanol, chloroform, ethylacetate, *n*-butanol, organic aqueous and water extracts of the fruit of *T. chebula* and 4 pure compounds, including, casuarinin, chebulanin, chebulinic acid and 1,6-di-*O*-galloyl-ß-D-glucose contained anti-lipid peroxidation activities with IC_{50} values less than 9.00 mg/ml, except casuarinin, IC_{50} value was 29.67 mg/ml (Cheng *et al*., 2003). An aqueous extract of fruit of *T. chebula* significantly reversed the tert-butyl hydroperoxide (t-BHP)-induced cell cytotoxicity and lactate dehydrogenase leakage. In addition, this extract exhibited in vitro ferric-reducing antioxidant activity and DPPH free radical-scavenging activities. The in vivo study showed that pretreatment with the extract (500 or 1000 mg/kg) significantly lowered the serum levels of the hepatic enzyme markers aspartate and alanine aminotransferases and reduced the indicators of oxidative stress in the liver, such as the glutathione disulfide content and lipid peroxidation, in a dose-dependent manner. It was concluded that *T. chebula* extract has the potential play a role in the hepatic prevention of oxidative damage in living systems (Lee *et al*., 2005). Prophylactic treatment of rats with methanolic extract of *T. chebula* (25 and 50 mg/g body weight) resulted in the diminution of $NiCl_2$ mediated damage as evident from the down regulation of glutathione content, glutathione-S-transferase (GST), glutathione reductase (GR), lipid peroxidation (LPO), H_2O_2 generation, blood urea nitrogen (BUN), serum creatinine, DNA synthesis ($p<0.001$) and ornithine decarboxylase (ODC) activity ($p<0.01$) with concomitant restoration of glutathione peroxidase (GPx) activity. It suggested that *T. chebula* extract can blocks or suppresses the events associated with chemical carcinogenesis (Prasad *et al*., 2006). Treatment with the ethanolic extract of *T. bellerica* (200, 400 and 800 mg/kg, p.o.) and gallic acid (50, 100 and 200 mg/kg, p.o.) showed dose-dependent recovery in aspartate aminotransferase, serum alanine aminotransferase, serum alkaline phosphatase, lipid peroxidation and glutathione level but the effect was more pronounced with gallic acid and at 200 mg/kg dose of gallic acid most effective against carbon tetrachloride induced liver and kidney damage (Jadon *et al*., 2006). The 75% methanolic extract of the fruit of *T. chebula*, *T. bellerica*, *E. officinalis* and Triphala were found

to inhibit in vitro lipid peroxide formation. The concentration of plant extracts that inhibited 50% of lipid peroxidation induced with Fe^{2+}/ascorbate were found to be 85.5 $\pm$ 6.5, 27.0 $\pm$ 3.2, 74.0 $\pm$ 5.8 and 69.0 $\pm$ 5.1 µg/ml, respectively (Sabu and Kuttan, 2002). The 75% methanolic extract of *T.chebula*, *T. bellerica*, *E. officinalis* and Triphala were found to inhibit in vitro lipid peroxide formation. *T. bellerica* was the most effective in inhibiting lipid peroxidation (Chase, 2002). The aqueous extract of the fruits of *E. officinalis*, *T. chebula*, *T. bellerica* and Triphala inhibited radiation induced lipid peroxidation in rat liver microsomes effectively with IC_{50} values less than 15 µg/ml. *E. officinalis* showed greater efficiency in lipid peroxidation (Naik *et al*., 2005(a)). Aqueous extracts of *T. bellerica*, *T. chebula*, *E. officinalis* and Triphala are good antioxidants. They also inhibited gamma-radiation-induced lipid peroxidation in rat liver microsomes (Naik *et al*., 2005). The aqueous extract of Triphala also exhibited dose-dependent NO scavenging activities (Jagetia *et al*., 2004).

Figure 3-1 shows the comparative histogram of antioxidant activity of three plants and Triphala formula on DPPH and lipid peroxidation assay. It was evaluated that the ethanolic extract of Triphala showed higher antioxidant activity in both methods, compared to the water extract. This indicated that the ethanolic extract is more suitable than the water extract for manufacturing Triphala health product.

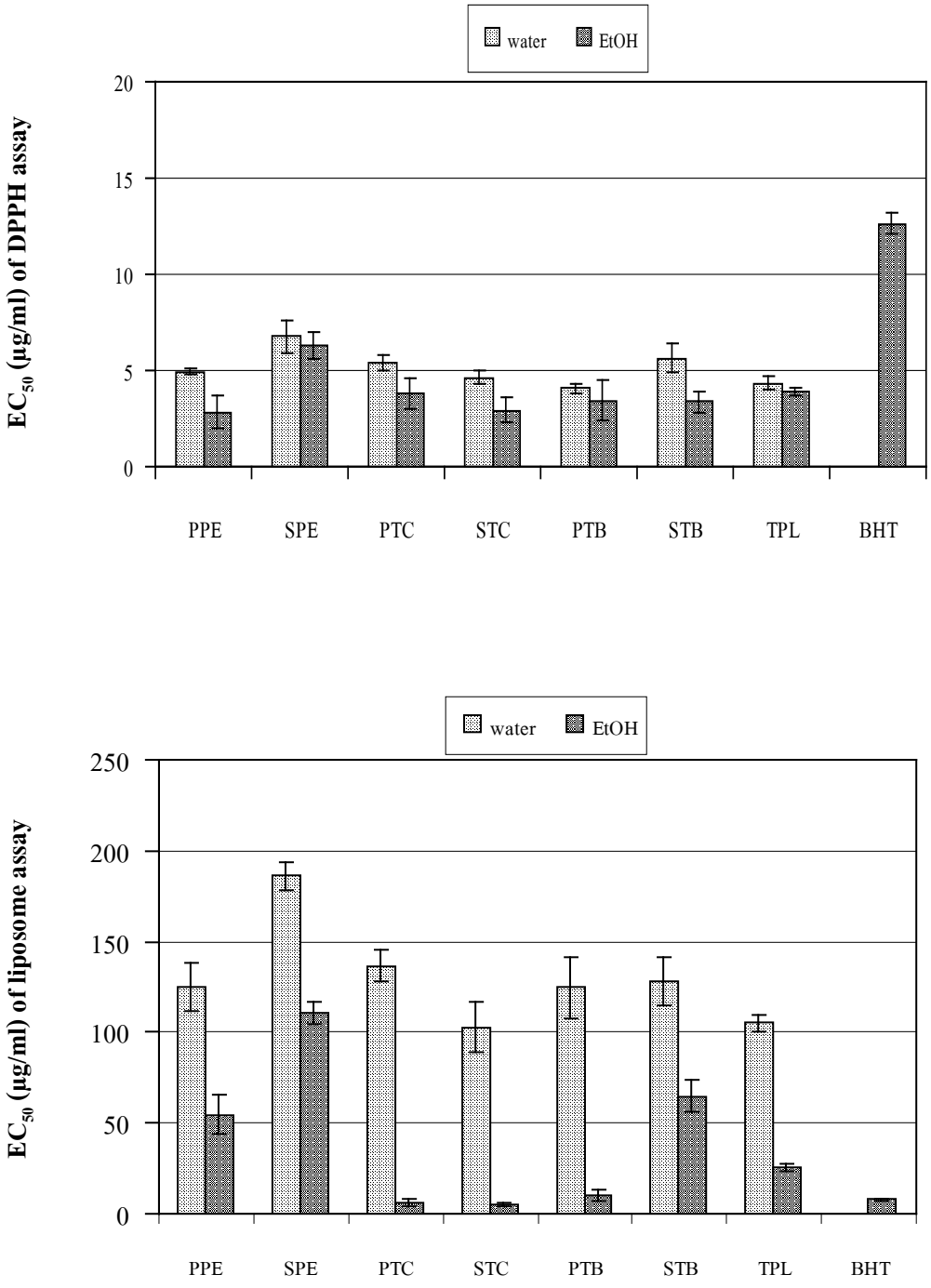


Figure 3-1 Comparative histogram of antioxidant activity of Triphala formula and its three ingredients on DPPH radical scavenging and lipid peroxidation assay, used BHT as positive control for DPPH assay (EC_{50} of BHT = 12.65 µg/ml) and propyl gallate ($1x10^{-4}$M) is positive control for lipid peroxidation assay (%inhibition at this concentration =80-82%)

3.1.3 Total phenolic contents

The contents of total phenolic compounds of the water and ethanolic extracts of the plants and their formula were determined by Folin-Ciocalteu's assay as described in section 2.5. The results are shown in Table 3-4.

Table 3-4 Gallic acid equivalents (GAE) of plant extracts and its formula on Folin-Ciocalteu's assay (n=3)

Plant Species	Part	Water extracts (Mean±SEM)	Ethanolic extracts (Mean±SEM)
Phyllanthus emblica Linn.	Pulp	73.527 ± 0.38	85.100 ± 5.22
	Seed	70.870 ± 0.92	62.956 ± 1.26
Terminalia chebula Retz.	Pulp	49.580 ± 1.42	33.557 ± 4.12
	Seed	68.368 ± 0.39	50.139 ± 1.13
Terminalia bellerica Gaertn.	Pulp	114.833 ± 13.84	89.480 ± 3.56
	Seed	89.737 ± 0.94	60.668 ± 1.51
Triphala		88.570 ± 10.97	49.667 ± 4.30

n = number of independent experiment which was performed in 3 replicates.

The results showed that the water extract of pulp of *T. bellerica*. contained the highest total phenolic contents as 114.833 ± 13.84 mg/g GAE. Interestingly, the water and ethanolic extracts showed high content of total phenolic compounds with the value in the range of 49.580 - 114.833 and 33.557 - 89.480 mg/g GAE, respectively. The total phenolic contents of the water extracts (except the pulp and seed of *P. emblica*) was found to be higher than that of the ethanolic extracts.

These results related with the previous report. It was found that the fruits of *T. bellerica*, *T. chebula* and *P. emblica* showed high total phenolic contents (72.0 – 167.2 mg/g). The fruit of *T. bellerica* was a rich source of gallic acid and ellagic acid and the fruit of *T. chebula*

had gallic acid (Bajpai *et al*., 2005). Gallic acid was found to be a major compound in the ethyl acetate extract of *P. emblica* (Kumaran and Karunakaran, 2006). The phenolic compounds presented in the aqueous extract of the fruits of *E. officinalis*, *T. chebula*, *T. bellerica* and Triphala formula were determined and expressed in terms of gallic acid equivalent and their contents were found to vary from 33% to 44% (Naik *et al*., 2005(a)). Gallic acid or gallic acid derivatives are major active ingredients in the 75% methanol extracts of *T. chebula*, *T. bellerica* and *P. emblica*. *T. bellerica* also contained ellagic acid as a major ingredient (Chase, 2002). Gallic acid is the major phenolic compounds in the most potent acetone extracts of Triphala (Kaur *et al*., 2005). L-malic acid 2-*O*-gallate, mucic acid 2-*O*-gallate, 1-*O*-galloyl-ß-D-glucose, corilagin and chebulagic acid were found to be the major phenolic constituents of fruit juice of *P. emblica* (Zhang *et al*., 2004; Zhang *et al*., 2001).

3.1.4 Cytotoxic activity

The cytotoxic activity of the water and ethanolic extracts of the plants and Triphala formula were evaluated by the sulphorhodamine B (SRB) assay as described in section 2.6. The results are shown in Tables 3-5 and 3-6.

Table 3-5 Percent survival of cancerous cells (Mean ± SEM) (breast cancer cell line = MCF-7, cervical cancer cell line = Hela and prostate cancer cell line = PC3) treated with extract concentration 50 µg/ml exposure time 72 hr (n = 6)

Plant Species	Part	Extracts	Code	Cell line		
				MCF-7	Hela	PC3
Phyllanthus emblica Linn.	Pulp	Water	PPE1	87.617 ± 3.17	70.598 ± 2.42	5.883 ± 0.31
		EtOH	PPE2	5.171 ± 0.22	15.949 ± 0.77	1.410 ± 0.11
	Seed	Water	SPE1	101.408 ± 1.53	80.022 ± 1.91	110.819 ± 1.32
		EtOH	SPE2	95.284 ± 1.67	69.486 ± 0.66	69.151 ± 0.94
Terminalia chebula Retz.	Pulp	Water	PTC1	103.055 ± 3.21	102.218 ± 3.02	111.547 ± 1.29
		EtOH	PTC2	87.886 ± 1.16	65.738 ± 1.00	115.918 ± 0.89
	Seed	Water	STC1	59.176 ± 2.92	52.084 ± 2.67	3.426 ± 0.37
		EtOH	STC2	84.672 ± 1.25	83.828 ± 0.53	108.196 ± 1.10
Terminalia bellerica Gaertn.	Pulp	Water	PTB1	37.500 ± 1.03	17.298 ± 0.42	2.486 ± 0.18
		EtOH	PTB2	8.901 ± 0.74	36.389 ± 0.75	1.969 ± 0.10
	Seed	Water	STB1	65.344 ± 1.75	77.458 ± 1.35	103.033 ± 1.54
		EtOH	STB2	88.007 ± 2.29	51.647 ± 0.86	53.883 ± 1.41

Table 3-5 (Continued)

Plant Species	Part	Extracts	Code	Cell line		
				MCF-7	**Hela**	**PC3**
Triphala		Water	TPL1	70.966 ± 1.21	47.575 ± 0.82	6.307 ± 0.11
		EtOH	TPL2	63.792 ± 1.56	8.082 ± 0.66	6.594 ± 0.15

n = number of independent experiment which was performed in 6 replicates.

Table 3-6 Cytotoxicity activity (IC_{50} µg/ml ± SEM) of plant extracts against three types of cancer cell (MCF-7, Hela and PC3) and one type of normal cells (MRC5) at exposure time 72 hr (n=4)

Plant Species	Part	Extracts	Cell line			
			MRC5	MCF-7	Hela	PC3
Phyllanthus emblica Linn.	Pulp	Water	> 50	> 50	30.136 ± 5.33	34.618 ± 2.69
		EtOH	64.070 ± 1.36	29.863 ± 0.89	32.366 ± 0.507	37.192 ± 1.10
	Seed	Water	> 100	> 50	> 50	> 50
		EtOH	> 100	> 50	54.781 ± 1.54	> 50
Terminalia chebula Retz.	Pulp	Water	> 100	> 100	>100	> 50
		EtOH	> 100	> 50	48.752 ± 2.98	> 50
	Seed	Water	> 100	> 50	39.023 ± 4.17	34.744 ± 1.66
		EtOH	> 100	> 50	54.443 ± 5.53	> 50

Table 3-6 (Continued)

Plant Species	Part	Extracts	Cell line			
			MRC5	**MCF-7**	**Hela**	**PC3**
Terminalia bellerica Gaertn.	Pulp	Water	> 100	38.650 ± 0.52	40.897 ± 5.14	33.785 ± 0.84
		EtOH	> 100	31.895 ± 2.18	35.368 ± 1.73	37.082 ± 0.81
	Seed	Water	> 100	> 50	> 50	> 50
		EtOH	> 100	> 50	50.013 ± 2.09	> 50
Triphala		Water	> 100	> 50	42.211 ± 3.02	38.479 ± 3.08
		EtOH	> 50	> 50	35.820 ± 0.76	> 50

n = Number of independent experiment which was performed in 4 replicates.

IC_{50} (µg/ml) of SRB assay

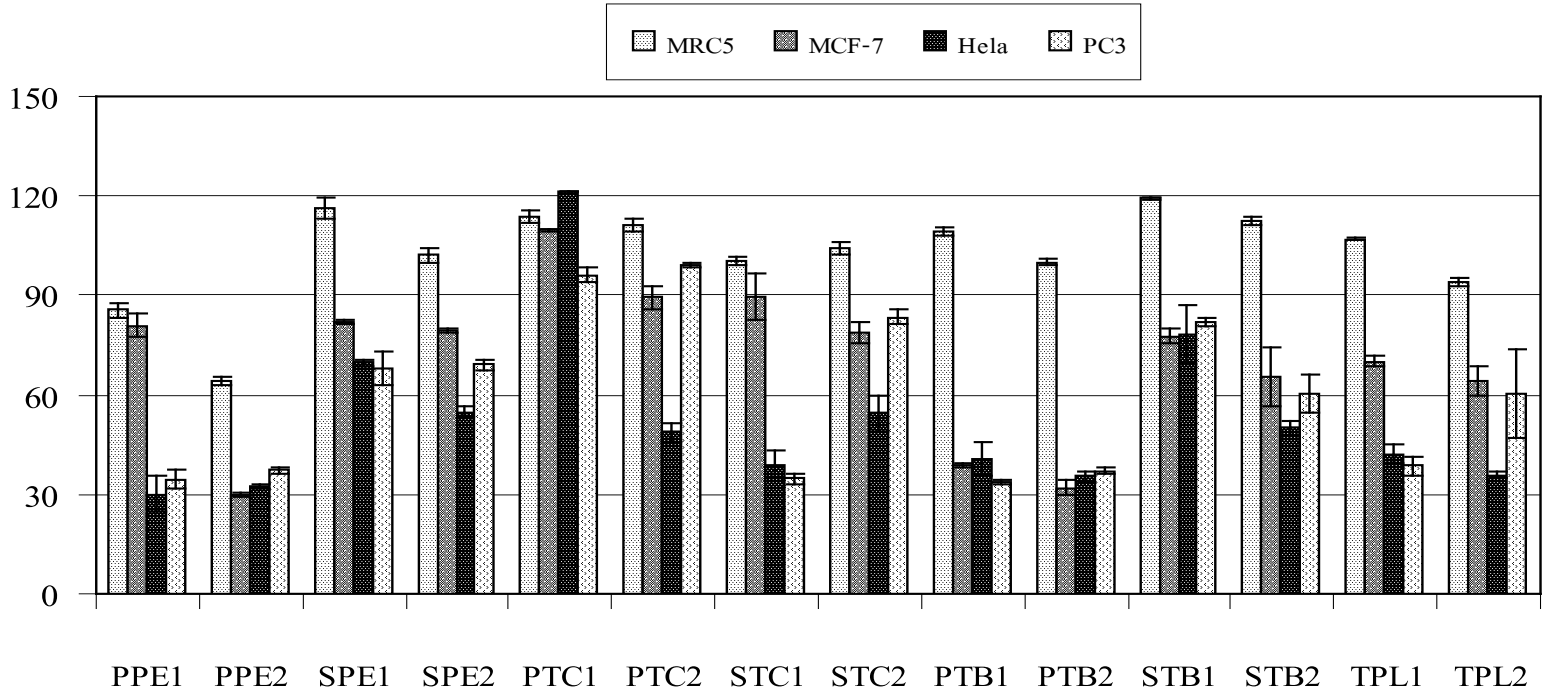


Figure 3-2 Histogram comparing IC_{50} (µg/ml) of three plants and Triphala formula on cell lines (n=4) exposure time 72 hr using student t-test from Prism to compare the significant difference between normal cell (MRC5) and each cancer cell (MCF-7, Hela and PC3)

Table 3-5 Percent survival of cancerous cells (Mean ± SEM) (breast cancer cell line = MCF-7, cervical cancer cell line = Hela and prostate cancer cell line = PC3) treated with extract concentration 50 µg/ml exposure time 72 hr (n = 6)

Plant Species	Part	Extracts	Code	Cell line		
				MCF-7	Hela	PC3
Phyllanthus emblica Linn.	Pulp	Water	PPE1	87.617 ± 3.17	70.598 ± 2.42	5.883 ± 0.31
		EtOH	PPE2	5.171 ± 0.22	15.949 ± 0.77	1.410 ± 0.11
	Seed	Water	SPE1	101.408 ± 1.53	80.022 ± 1.91	110.819 ± 1.32
		EtOH	SPE2	95.284 ± 1.67	69.486 ± 0.66	69.151 ± 0.94
Terminalia chebula Retz.	Pulp	Water	PTC1	103.055 ± 3.21	102.218 ± 3.02	111.547 ± 1.29
		EtOH	PTC2	87.886 ± 1.16	65.738 ± 1.00	115.918 ± 0.89
	Seed	Water	STC1	59.176 ± 2.92	52.084 ± 2.67	3.426 ± 0.37
		EtOH	STC2	84.672 ± 1.25	83.828 ± 0.53	108.196 ± 1.10
Terminalia bellerica Gaertn.	Pulp	Water	PTB1	37.500 ± 1.03	17.298 ± 0.42	2.486 ± 0.18
		EtOH	PTB2	8.901 ± 0.74	36.389 ± 0.75	1.969 ± 0.10
	Seed	Water	STB1	65.344 ± 1.75	77.458 ± 1.35	103.033 ± 1.54
		EtOH	STB2	88.007 ± 2.29	51.647 ± 0.86	53.883 ± 1.41

Table 3-5 (Continued)

Plant Species	Part	Extracts	Code	Cell line		
				MCF-7	**Hela**	**PC3**
Triphala		Water	TPL1	70.966 ± 1.21	47.575 ± 0.82	6.307 ± 0.11
		EtOH	TPL2	63.792 ± 1.56	8.082 ± 0.66	6.594 ± 0.15

n = number of independent experiment which was performed in 6 replicates.

Table 3-6 Cytotoxicity activity (IC_{50} µg/ml ± SEM) of plant extracts against three types of cancer cell (MCF-7, Hela and PC3) and one type of normal cells (MRC5) at exposure time 72 hr (n=4)

Plant Species	Part	Extracts	Cell line			
			MRC5	MCF-7	Hela	PC3
Phyllanthus emblica Linn.	Pulp	Water	> 50	> 50	30.136 ± 5.33	34.618 ± 2.69
		EtOH	64.070 ± 1.36	29.863 ± 0.89	32.366 ± 0.507	37.192 ± 1.10
	Seed	Water	> 100	> 50	> 50	> 50
		EtOH	> 100	> 50	54.781 ± 1.54	> 50
Terminalia chebula Retz.	Pulp	Water	> 100	> 100	>100	> 50
		EtOH	> 100	> 50	48.752 ± 2.98	> 50
	Seed	Water	> 100	> 50	39.023 ± 4.17	34.744 ± 1.66
		EtOH	> 100	> 50	54.443 ± 5.53	> 50

Table 3-6 (Continued)

Plant Species	Part	Extracts	Cell line			
			MRC5	**MCF-7**	**Hela**	**PC3**
Terminalia bellerica Gaertn.	Pulp	Water	> 100	38.650 ± 0.52	40.897 ± 5.14	33.785 ± 0.84
		EtOH	> 100	31.895 ± 2.18	35.368 ± 1.73	37.082 ± 0.81
	Seed	Water	> 100	> 50	> 50	> 50
		EtOH	> 100	> 50	50.013 ± 2.09	> 50
Triphala		Water	> 100	> 50	42.211 ± 3.02	38.479 ± 3.08
		EtOH	> 50	> 50	35.820 ± 0.76	> 50

n = Number of independent experiment which was performed in 4 replicates.

IC_{50} (µg/ml) of SRB assay

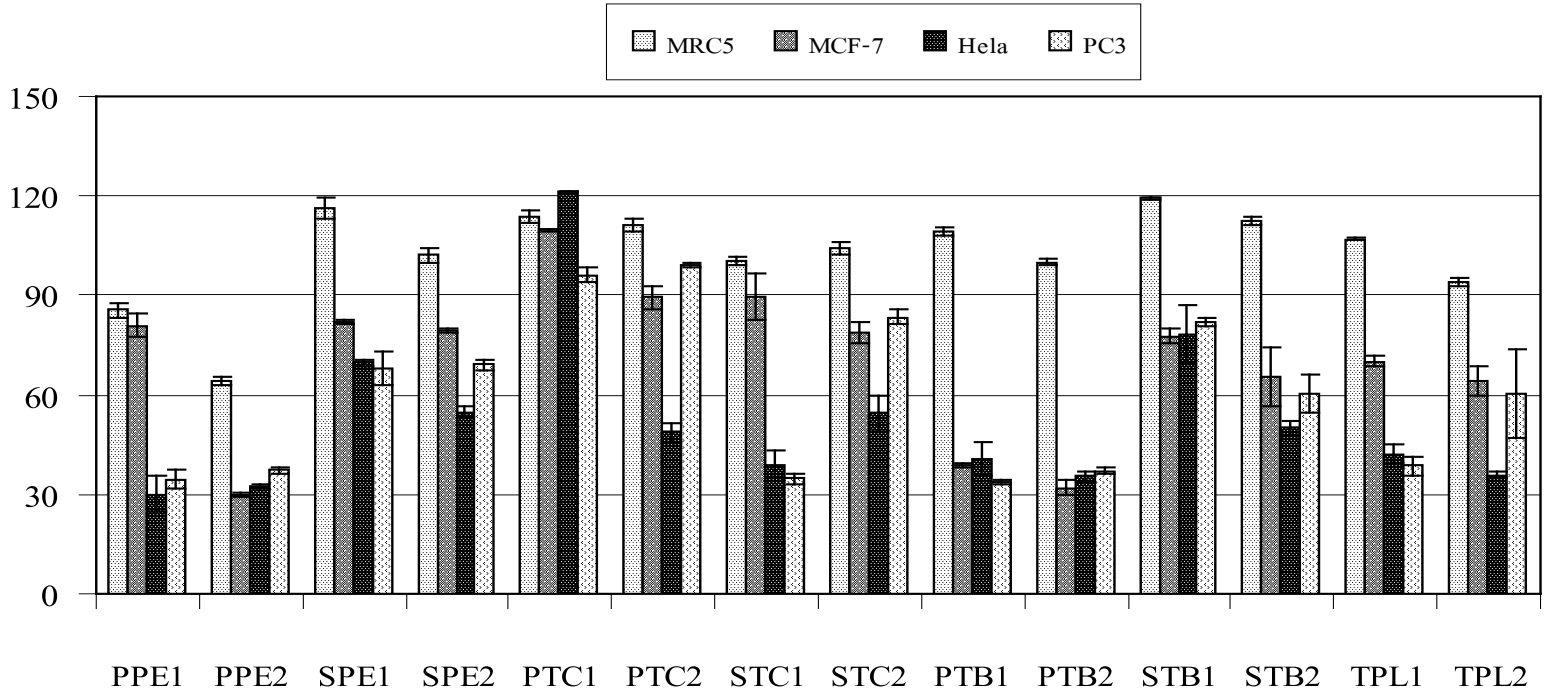


Figure 3-2 Histogram comparing IC_{50} (µg/ml) of three plants and Triphala formula on cell lines (n=4) exposure time 72 hr using student t-test from Prism to compare the significant difference between normal cell (MRC5) and each cancer cell (MCF-7, Hela and PC3)

The results of cytotoxicity evaluation of all plant extracts and Triphala formula at 50 µg/ml concentration and exposure time 72 hours are shown in Table 3-5. This data showed that the ethanolic extract of the pulp of *P. emblica* exhibited high cytotoxic activity against all types of human cancer cell lines with the percentage of survival on MCF-7, Hela and PC3 of 5.171 ± 0.22%, 15.949 ± 0.77% and 1.410 ± 0.11%, respectively. The ethanolic extract of the pulp of *T. bellerica* exhibited high cytotoxic activity against MCF-7 and PC3 cancer cell lines with the percentage of survival of 8.901 ± 0.74% and 1.969 ± 0.10%, respectively. The water extract of the pulp of *T. bellerica* exhibited high cytotoxic activity against Hela and PC3 cancer cell lines with the percentage of survival of 17.298 ± 0.42 and 2.486 ± 0.18, respectively, whereas those values for the ethanolic extract of Triphala formula were 8.082 ± 0.66 and 6.594 ± 0.15, respectively. The water extract of the seed of *T. chebula* and the water extract of Triphala formula showed high cytotoxic activity only against PC3 where the percentage survival of the cancer cell line for 50 µg/ml concentration at exposure time 72 hours were 3.426 ± 0.37 and 6.307 ± 0.11, respectively. Among them, the ethanolic extract of the pulp of *P. emblica* contained the most cytotoxic effect against MCF-7 (% survival = 5.171 ± 0.22) and PC3 (% survival = 1.410 ± 0.11) cancer cell lines and the ethanolic extract of Triphala formula possessed the most cytotoxic against Hela cancer cell line (%survival = 8.082 ± 0.66).

Studies of making dilutions of all extracts are shown in Table 3-6. This data showed that the water and ethanolic extracts of all plants exhibited no cytotoxic activity against normal cells. The water and ethanolic extract of all plants and Triphala formula showed less cytotoxic activity against all types of cancer cell lines followed by the American National Cancer Institue (NCI) (IC_{50} < 20 µg/ml for crude extract) (Boyd,1997). The data showed that the IC_{50} value of the ethanolic extracts of the pulp of *P. emblica* showed the highest cytotoxic activity against MCF-7 cancer cell line and the water and ethanolic extracts of the pulp of *T. bellerica* showed the second most effective activity against MCF-7 cancer cell line. The IC_{50} value of the ethanolic extract of the pulp of *P. emblica* was 29.863 ± 0.89 µg/ml and the IC_{50} value of the water and ethanolic extract of the pulp of *T. bellerica* were 38.650 ± 0.52 and 31.895 ± 2.18 µg/ml, respectively. The water extract of the pulp of *P. emblica* showed the highest cytotoxic activity against Hela cancer cell line (IC_{50} = 30.136 ± 5.33 µg/ml) and the ethanolic extract of the pulp of *P. emblica* showed the second most effective activity against Hela cancer cell line (IC_{50} = 32.366

± 0.51 µg/ml). In addition, the water extract of the pulp of *T. bellerica* showed the highest cytotoxic activity against PC3 cancer cell line (IC_{50} = 33.785 ± 0.84 µg/ml) and the water extracts of the pulp of *P. emblica* the seed of *T. chebula* showed the second most effective activity against PC3 cancer cell line (IC_{50} = 34.618 ± 2.69 and 34.744 ± 1.66 µg/ml, respectively). They exhibited specific activity against Hela and PC3 cancer cell lines higher than MCF-7 cancer cell lines but less active with normal cell line. P-value was extremely significant ($p<0.0001$) calculated by student t-test from Prism program. From this result, it was concluded that the ethanolic extract of the pulp of *P. emblica* showed cytotoxicity against MCF-7, Hela and PC3 cancer cell lines and deserved for the plant extracts or their active ingredients which can kill cancer cells but less harmful to normal cells. So this result related with the objectives of cancer chemotherapy which can kill cancer cells with as little damage as possible to normal cells and should be selectively active (Halliwell and Gatteridge, 1988).

These results also related with the previous investigation, which reported that the water extract of *E. officinalis* fruits inhibited tumor incidences on two-stage process of skin carcinogenesis in Swiss albino mice, induced by 7,12-dimethyabenz(a)anthrecene (100 µg/100 µl acetone) (Sancheti *et al*., 2005). Aqueous extract of *E. officinalis* was found to be cytotoxic to L 929 cells in a dose dependent manner. Concentration needed for 50% inhibition was found to be 16.5 µg/ml. Animals treated with 1.25 g/kg body wt. of *E. officinalis* extract increased life span of tumour bearing animals (20%). The extract significantly reduced the solid tumours in mice induced by DLA (Daltons lymphoma ascites) cells, and also inhibited cell cycle regulating enzymes cdc 25 phosphatase in a dose dependent manner. Concentration needed for 50% inhibition of cdc 25 phosphatase was found to be 5 µg/ml and that needed for inhibition of cdc2 kinase was found to be more than 100 µg/ml (Jeena *et al*., 2001). The 10% aqueous extract of *E. officinalis* significantly inhibited hepatocarcinogenesis induced by *N*-nitrosodiethylamine (NDEA) in a dose dependent manner. The anticarcinogenic activity of the extract was evaluated by its effect on tumour incidence, levels of carcinogen metabolizing enzymes, levels of liver cancer markers and liver injury markers (Jeena *et al*., 1999). Surprisingly, there are no report that the ethanolic extract of *E. officinalis* against cancer cell especially breast cancer, and this study found that the method of production its extract for against breast cancer should use the ethanolic extract. However it will continue study in animal model. A 70% methanol extract of *T. chebula*

fruit decreased cell viability, inhibited cell proliferation and induced cell death in a dose dependent manner on all cell lines studied including, human (MCF-7) and mouse (S115) breast cancer cell line, human osteosarcoma cell line (HOS-1), human prostate cancer cell line (PC3) and a non-tumorigenic, immortalized human prostate cell line (PNTIA). The extract induced apoptosis at lower concentrations, but at higher concentrations, the major mechanism of cell death was necrosis (Saleem *et al*., 2002). The 90% methanol extract of *T. chebula* inhibited the melanin production of mouse B16 melanoma cells by using MTT method (Jin *et al*., 2006). Acetone extract of Triphala showed a significant cytotoxic effect on two types of breast cancer cells, Shionogi 115 (S115) mouse breast cancer cell line and human breast cancer cell line (MCF-7) and two types of prostate cancer cell PC3 and DU-145. However there are no report of ethanolic extract of Triphala against breast and prostate cancer which this result exhibited the potential for production in manufacturing level, because ethanolic extract used in drink but acetone cann't drink. The aqueous extract of Triphala (250 µg/ml) possessed ability to induce cytotoxicity against human breast cancer cell line (MCF-7) (~ 40%toxicity) but less toxic against normal breast epithelial cell (MCF-10 F) (~ 13%toxicity). The differential effect of Triphala on normal and tumor cells seems to be related to its ability to evoke differential response in intracellular ROS generation. The differential response of normal and tumor cells to Triphala in vitro and the substantial regression of transplanted tumor in mice fed with Triphala points to its potential use as an anticancer drug for clinical treatment (Sandhya *et al*., 2006). The human MCF-7 breast cancer cell line with wild type p53 was more sensitive to the water extract of Triphala than the human T 47 D breast cancer cell lines, which is p53 negative. The water extract of Triphala induced loss of cell viability was determine by MTT assay with the IC_{50} values for MCF-7 was found to be ~ 8 µ g/ml. Moreover, Triphala induced significant apoptosis in this cell lines in a dose dependent manner. Triphala was also found to induce dose and time dependent increase in intracellular reactive oxygen species in this cell lines (Sandhya and Mishra, 2006).

The specific activity against cancer cell lines will be the first criterion for further investigation. So the ethanolic extract of the pulp of *P. emblica* should be selected for further study. However, the evaluation of the ingredients in this cancer formula found that nearly 50% of ingredients showed cytotoxic activity followed the previous criteria of NCI on 1990 (IC_{50} of the active plant extracts < 20 µg/ml) (Suffness and Pezzuto, 1991).

3.2 Antioxidant and cytotoxic activity of bioassay-guided fractionation

Results from the preliminary assays for free radical scavenging activity in section 2.4 and cytotoxic activity in section 2.7 of the water and ethanolic extracts of three plants and Triphala formula are shown in section 3.1. They give evidences of the presence of active constituents in the ethanolic extract of the pulp of *P. emblica*, therefore a separation of these active extracts was carried out as shown in section 2.7. These five fractions, including FA1, FA2,FA3, FA4 and FA5 were tested for antioxidant and cytotoxic activities using the methods described in section 2.4 and 2.6 (data shown in Table 3-2, 3-3 and 3-7 and Figure 3-1 and 3-2)

Table 3-7 IC_{50} (μg/ml) ± SEM of the fractions from *Phyllanthus emblica* Linn. separated by vacuum liquid chromatography scavenge DPPH radical and also against three types of cancer cell lines at exposure time 72 hr (n=2)

Fraction	%Yield	DPPH	Cell lines		
			MCF-7	Hela	PC3
FA1 (Hexane)	0.14	> 50	> 50	> 100	> 50
FA2 (Hexane:$CHCl_3$)	1.19	> 50	4.698 ± 0.30	2.260 ± 1.23	1.833 ± 0.11
FA3 ($CHCl_3$)	1.00	1.796 ± 0.15	6.497 ± 0.01	15.69 ± 2.73	4.878 ± 0.12
FA4 ($CHCl_3$:MeOH)	63.99	1.247 ± 0.32	22.16 ± 2.97	25.02 ± 0.89	27.63 ± 1.91
FA5 (MeOH)	4.27	7.701 ± 0.24	> 50	> 50	> 50

n = number of independent experiment which was performed in 2 replicates.

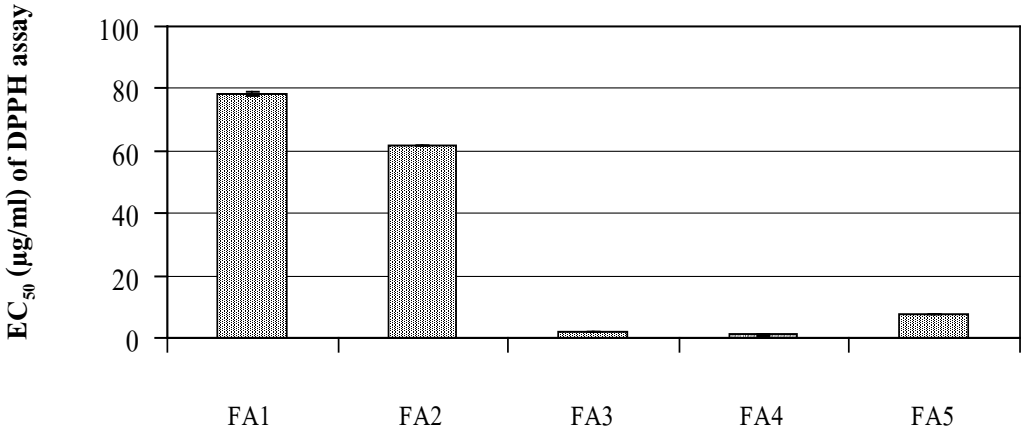


Figure 3-3 Histogram comparing EC_{50} (µg/ml) of each fraction of the ethanolic extract of *P. emblica* pulp on DPPH assay

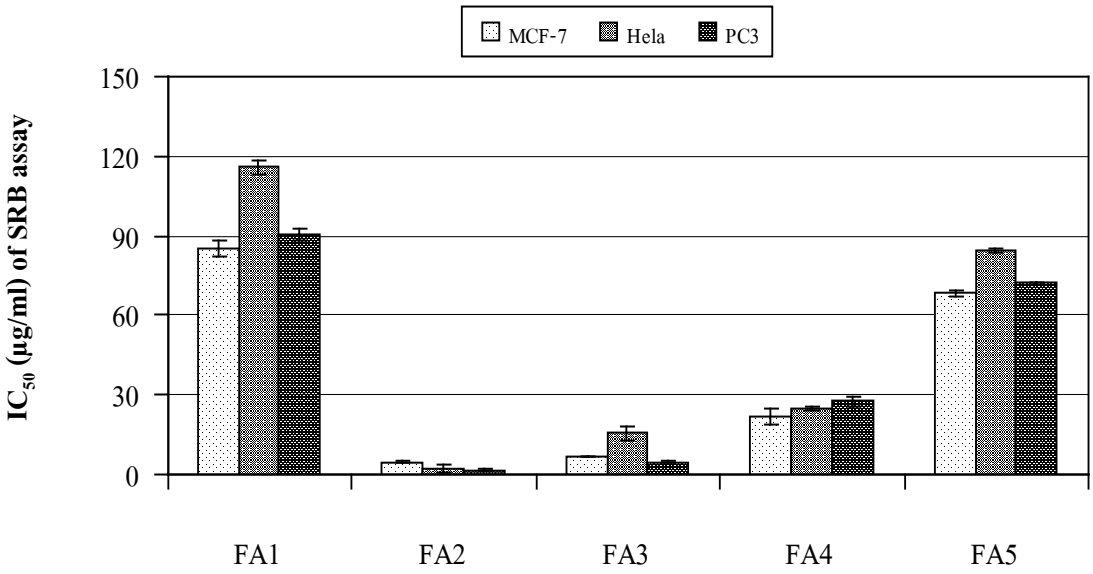


Figure 3-4 Histogram comparing IC_{50} (µg/ml) of each fraction the ethanolic extract of *P. emblica* pulp against three cancer cell lines at exposure time 72 hr

FA3, FA4 and FA5 showed high antioxidative effect with DPPH assay. FA3 and FA4 showed higher antioxidant activity than the crude extract of the pulp of *P. emblica* (Table 3-2). Moreover, FA2, FA3 and FA4 showed high cytotoxicity against the human breast cancer cell lines (MCF-7), the human cervical cancer cell lines (Hela) and the human prostate cancer cell lines (PC3) than the crude extract of the pulp of *P. emblica* (Table 3-5 and 3-6). Interesting, FA2 showed high and specific cytotoxicity against three types human cancer cell lines. However, following the results (Tables 3-2, 3-5 and 3-6) FA2, FA3 and FA4 were explicitly chosen for separation to find active compounds because of their high antioxidant and cytotoxicity.

3.3 Analysis of chemical composition and structure determination of the isolated compounds

3.3.1 Structure elucidation of the isolated compounds

Results from the bioassay-guided fractionation for antioxidant and cytotoxic activity were shown in section 3.2. Thus, the separation of the active extracts was carried out as shown in section 2.8 to give the pure compounds as follows.

3.3.1.1 C2

C2 (ß-sitosterol): $C_{29}H_{50}O$ (1.70 mg, 0.0022%w/w); white needle crystal solids. C2 was the compound isolated from the ethanolic extract of the pulp of *P. emblica*, obtained as white needle crystal solids. Analysis of chemical shifts, integration and spin coupling patterns from ^{1}H NMR data indicate that C2 was a sterol. The ^{1}H NMR spectrum are shown in Table 3-8 and Figure 3-7. The TLC analysis of this compound was compared with authentic sample ß-sitosterol (Sigma). It was strongly supported that this compound is ß-sitosterol. The structure was shown below.

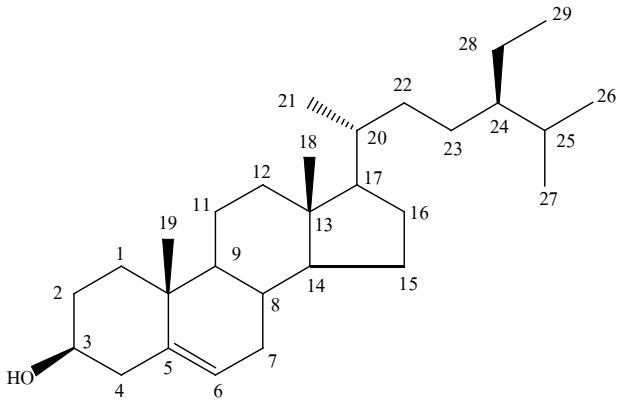


Figure 3-5 Structure of ß-sitosterol

3.3.1.2 C3

C3 (ß-sitosterol-3-*O*-ß-D-glucopyranoside): $C_{35}H_{60}O_6$ (3.8 mg, 0.019%w/w); white amorphous solids. C3 was compound isolated from the ethanolic extract of the pulp of *P. emblica* fruit, obtained as white solids and the ^{1}H NMR spectrum shown in Table 3-8 and Figure 3-8. The ^{1}H NMR of C3 was similar to C2. The difference was ^{1}H NMR of C3 showed more signals at δ between 3.28-4.40. This signal could be signal of major moiety and signal δ 4.40 (J = 7.5 Hz) should be signal of anomeric proton. Analysis of sugar part from splitting pattern compared with publish paper this sugar could be glucose (Agrawal, 1985; Shujiro *et al*., 1978 and Blonquist and Wesserman, 1972). ^{1}H NMR and TLC analysis of this compound compared with an authentic sample from Itharat (Itharat., 2003). It was strongly supported that this compound is ß-sitosterol-3-*O*-ß-D-glucopyranoside. The structure showed below.

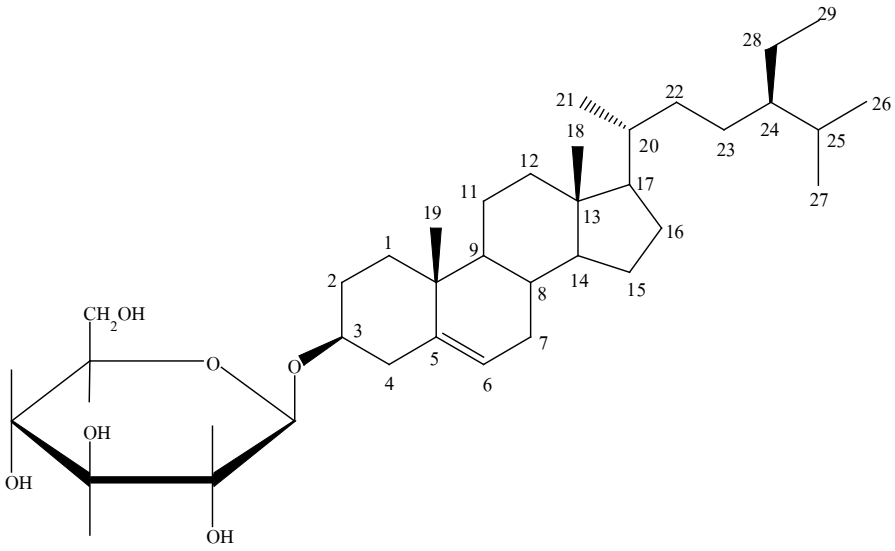


Figure 3-6 Structure of ß-sitosterol-3-*O*-ß-D- glucopyranoside

Table 3-8 NMR spectral data (500 MHz for ^{1}H) of **C2 (ß-sitosterol)** in $CDCl_3$ and **C3 (ß-sitosterol-3-*O*-ß-D- glucopyranoside)** in $CDCl_3$:CD3OD

Carbon position	**Chemical shift (δ) of ^{1}H (mult, *J* in Hz)**			
	C2	**ß-sitosterol**	**C3**	**ß-sitosterol-3-*O*-ß-D-glucopyranoside**
3	3.56 (m)	3.43 (m)	3.43 (m)	3.58 (tt, 5, 11.5)
6	5.36 (br d)	5.33 (dt)	5.40 (br d)	5.37 (br d)
18	0.69 (s)	0.66 (s)	0.69 (s)	0.68 (s)
19	1.01 (s)	0.97 (s)	1.05 (s)	1.01 (s)
21	0.92 (d, 6.6)	0.91 (d, 6.5)	0.95 (d, 6.6)	0.92 (d, 6.5)
26	0.81 (d, 6.8)	0.80 (d, 6.8)	0.82 (d, 7.0)	0.81 (d, 7.0)
27	0.83 (d, 7.3)	0.80 (d, 6.8)	0.84 (d, 7.5)	0.83 (d, 7.5)
29	0.84 (d, 7.6)	0.83 (t, 6.5)	0.85 (t, 7.5)	0.85 (t, 7.5)
Glucose-1	-		4.40 (d, 7.5)	4.42 (d, 7.0)
2	-		3.55-3.62 (m)	3.45-3.47 (dd, 7.0,9.0)
3	-		3.45-3.48 (m)	3.38-3.41 (tt, 3.0,3.0)
4	-		3.36-3.40 (m)	3.29-3.32 (o)
5	-		3.75-3.88 (m)	3.75-3.86 (dt, 9.0, 6.0)
6	-		3.28 (m)	3.28 (o)

Note: ß-sitosterol in $CDCl_3$ from Ali *et al.*, 2002; ß-sitosterol-3-*O*-ß-D-glucopyranoside in $CDCl_3$:CD_3OD from Itharat, 2002; o signals unresolved due to overlapping

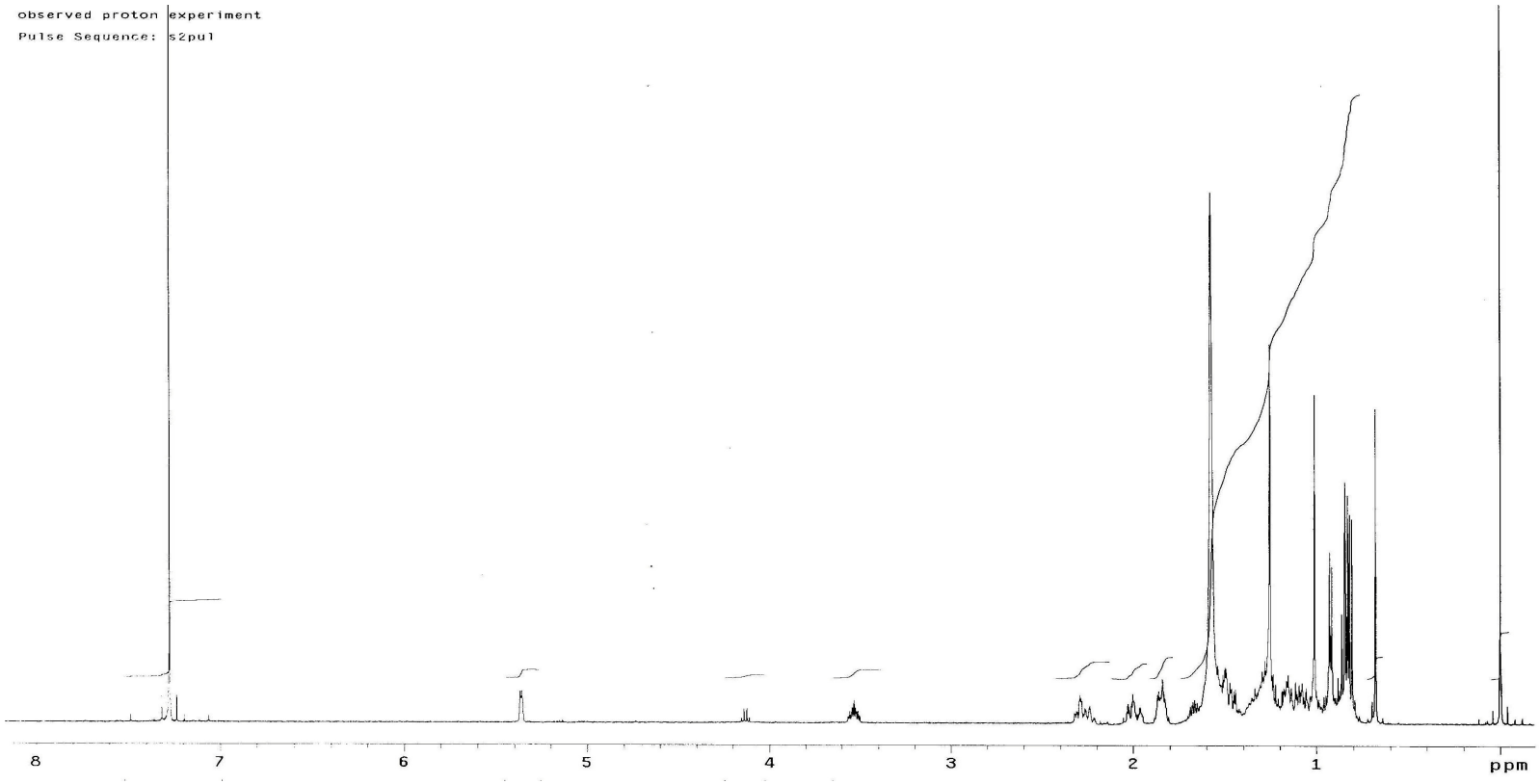


Figure 3-7 ^{1}H NMR spectrum of ß-sitosterol in $CDCl_3$

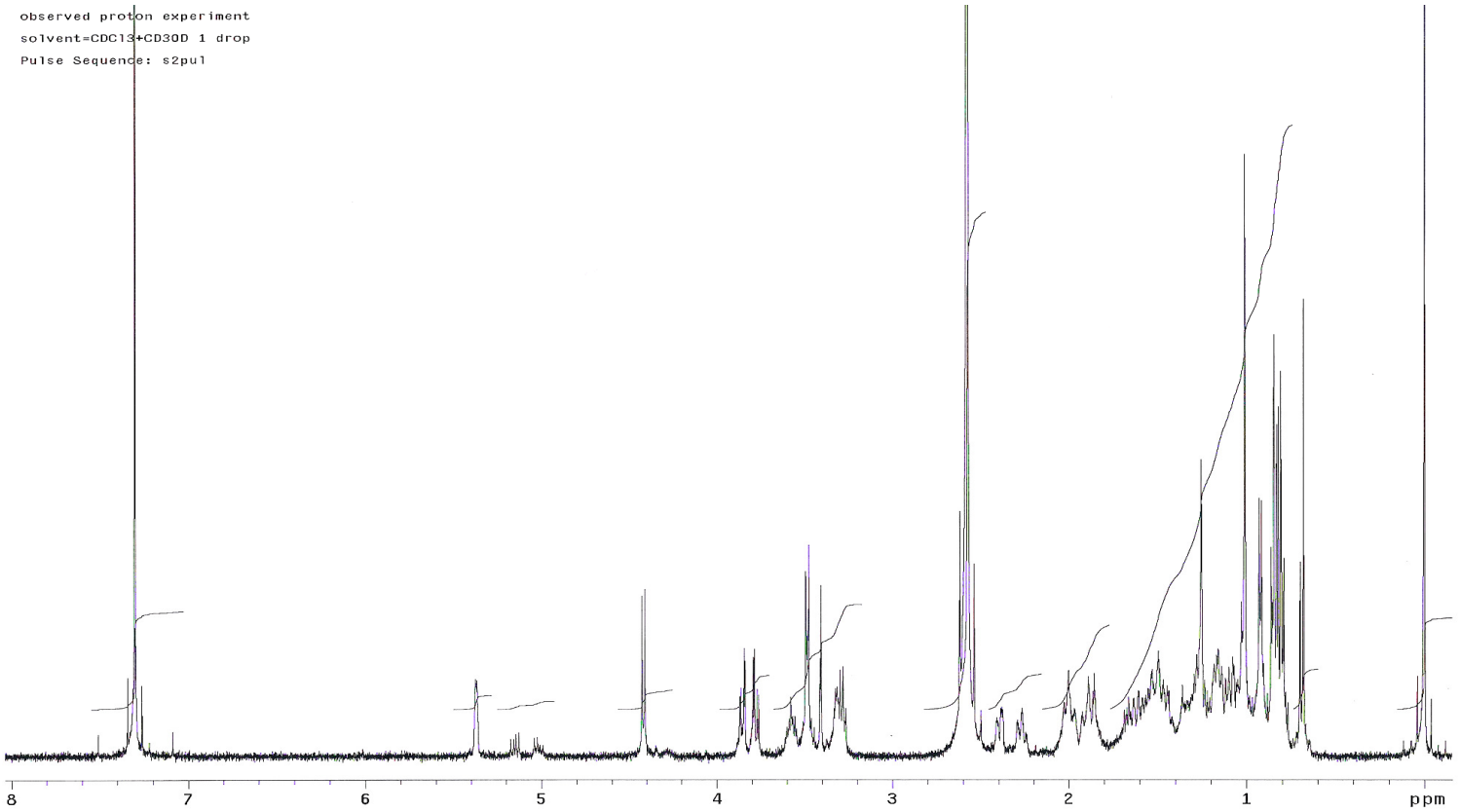


Figure 3-8 ^{1}H NMR spectrum of ß-sitosterol-3-*O*-ß-D-glucopyranoside in $CDCl_3$:CD_3OD

3.3.1.3 C4

C4 (5-hydroxymethylfurfural): $C_6H_6O_3$ (14.0 mg, 0.068%w/w); yellow crystal; EIMS (low resolution) *m/z* (% relative intensity) 126 (M^+, 56), 109 (11), 97 (100), 84 (24), 81 (7), 69 (28).

C4 was the major compound of the ethanolic extract of the pulp of *P. emblica*, obtained as yellow crystal and showed protonated molecular ion peak in EI mass spectrum at *m/z* 126, corresponding with a molecular formula of $C_6H_6O_3$ (MW = 126). ^1H and ^{13}C NMR spectra of C4 were showed in Figure 3-10 and 3-11. Analysis of chemical shifts, integration and spin coupling patterns of these functional groups indicate that C4 was a furaldehyde. In the ^{13}C NMR (Table 3-9), six carbon signals was observed with signal of carbonyl function at δ 177.6. The ^1H NMR showed aldehyde proton at δ 9.62 which was shown by sharp and strong signals and other important atom for ^1H NMR and ^{13}C NMR are shown in Table 3-9. It was strongly supported that this compound is 5-hydroxymethylfurfural. The structure showed below.

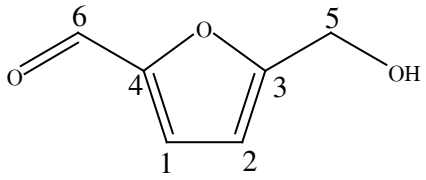


Figure 3-9 Structure of 5-hydroxymethylfurfural

Table 3-9 NMR spectral data (500 MHz for ^{1}H and 125 MHz for ^{13}C) of C4 (5-hydroxymethylfurfural) in $CDCl_3$

Carbon position	**δ_H (mult., *J* in Hz) (5-hydroxymethylfurfural)**	**δ_C (5-hydroxymethylfurfural)**
1	7.24 (d, 3.67)	122.7
2	6.54 (d, 3.2)	110.0
3	-	160.4
4	-	152.4
5-CH_2OH	4.74 (s)	57.7
6-COH	9.62 (s)	177.6

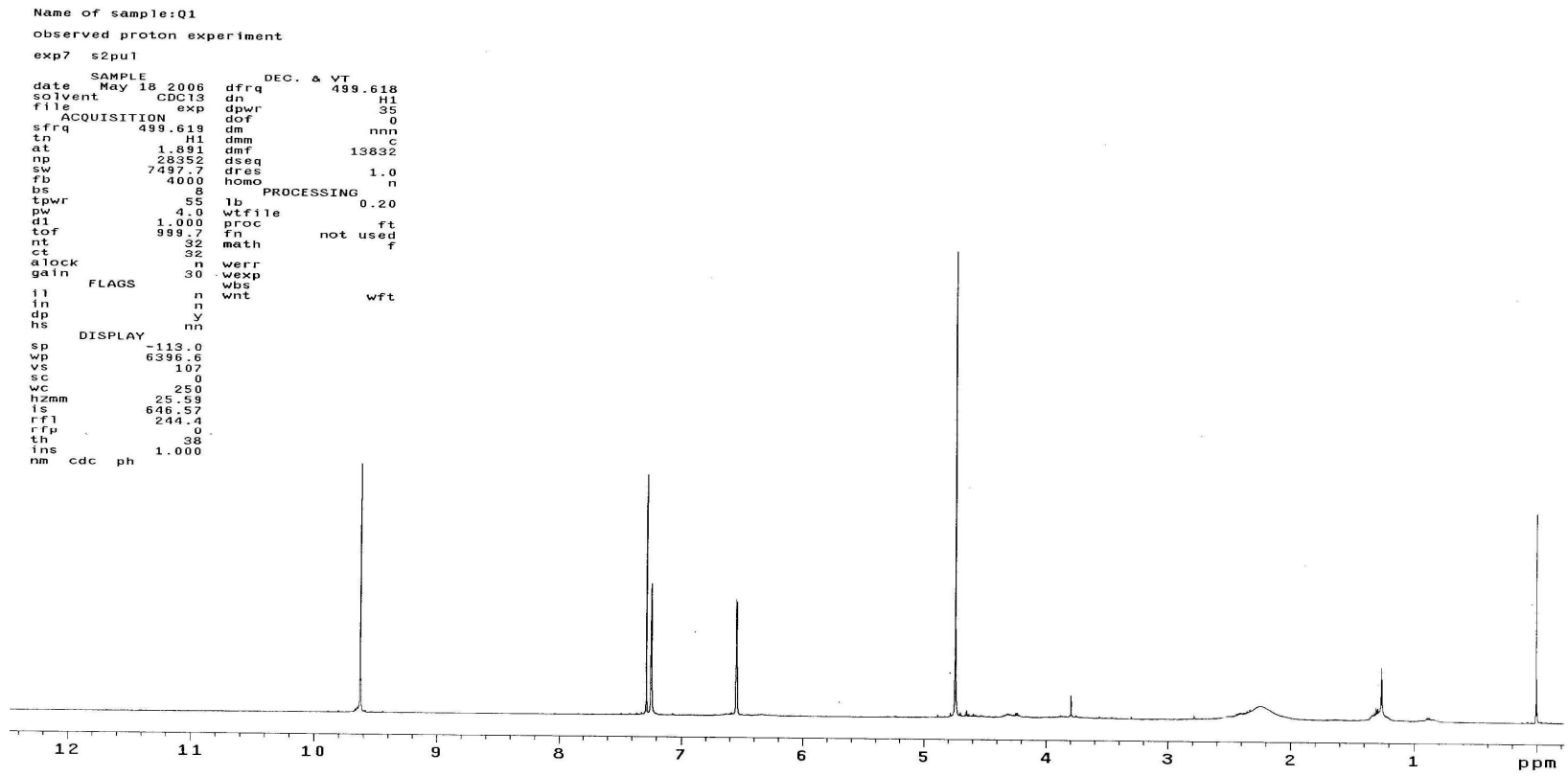


Figure 3-10 ^{1}H NMR spectrum of 5-hydroxymethylfurfural in $CDCl_3$

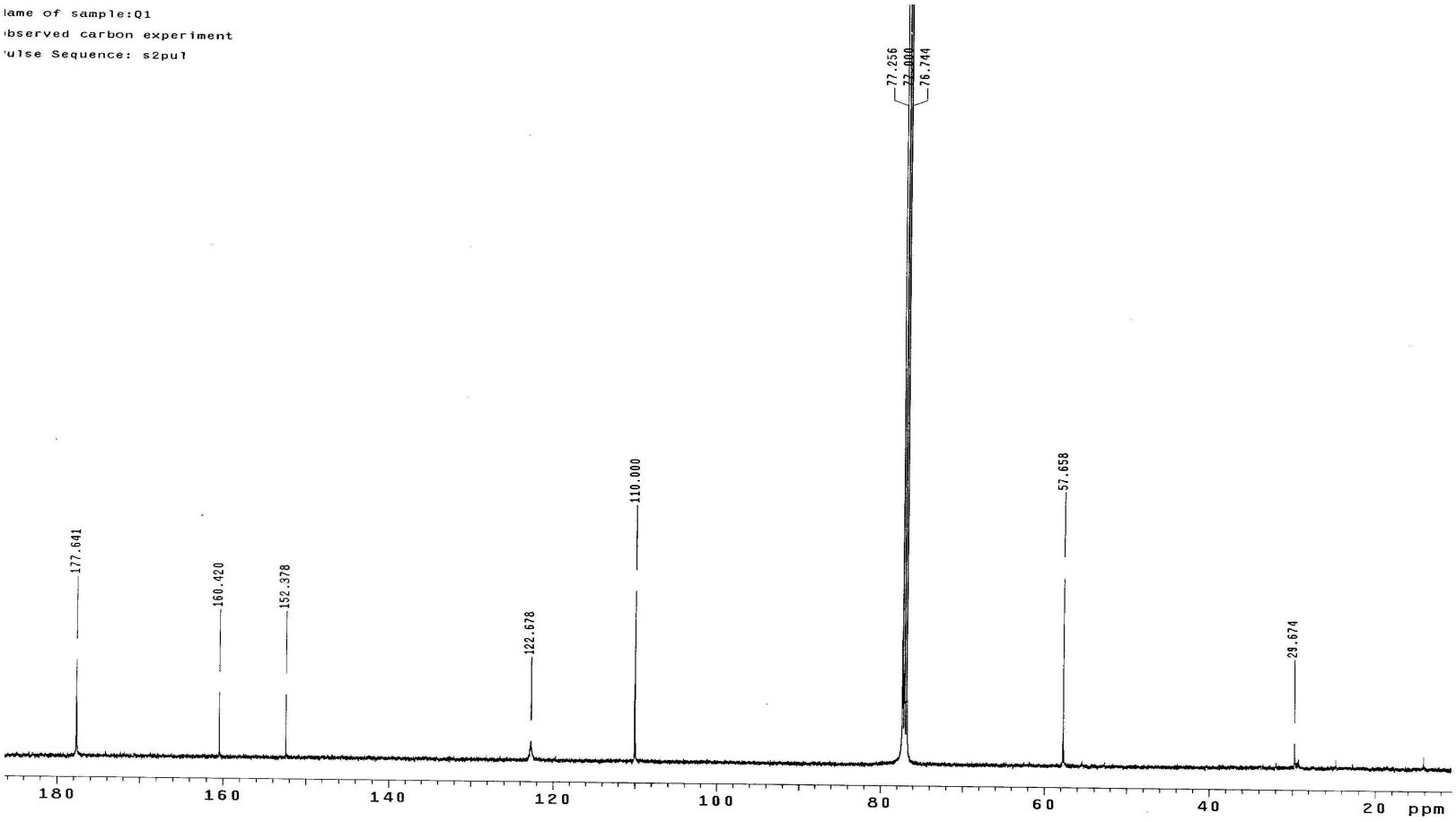


Figure 3-11 ^{13}C NMR spectrum of 5-hydroxymethylfurfural in $CDCl_3$

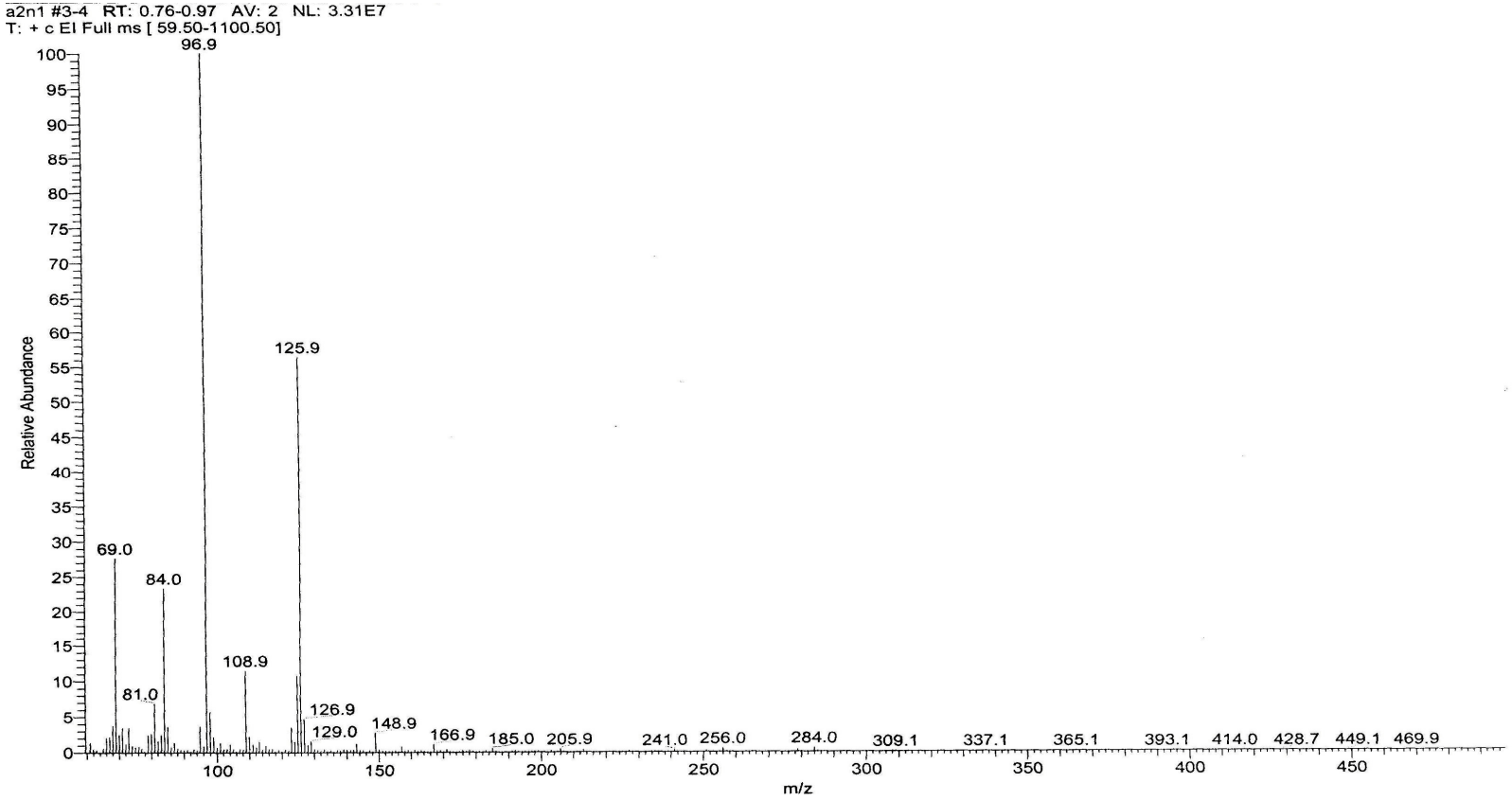


Figure 3-12 EIMS spectrum of 5-hydroxymethylfurfural

3.3.1.4 C5

C5 (Gallic acid): $C_7H_6O_5$ (23.90 mg, 0.11%w/w (Chapter 2.8.3.2) and 726.8 mg, 3.57%w/w (Chapter 2.8.3.2)); white crystals; EIMS *m/z* (% relative intensity) 171 (M^+, 94), 153 (41), 134 (10), 127 (15), 125 (11), 80 (11), 78 (7).

C5 was the major compound isolated from the ethanolic extract of the pulp of *P. emblica*, obtained as white crystals and showed protonated molecular ion peak in EI mass spectrum at *m/z* 171, corresponding with a molecular formula of $C_7H_6O_5$ (MW = 170).

^1H and ^{13}C NMR spectra of C5 showed in figure 3-14 and 3-15. Analysis of chemical shifts, integration and spin coupling patterns of these functional groups indicate that C5 was a benzoic acid (Table 3-10). In the ^{13}C NMR (Figure 3-16), five carbon signals was observed with signal of carboxylic group at δ 170.5. The ^1H NMR (Figure 3-15) showed two protons at δ 7.05 which were shown by sharp and strong signals at high field and other important atoms for ^1H NMR and ^{13}C NMR are shown in Table 3-10. Analysis of major part from spitting pattern compared with publish paper (Lu and Foo, 1999) are shown in Table 3-10. It was strongly supported that this compound is gallic acid. The structure showed below.

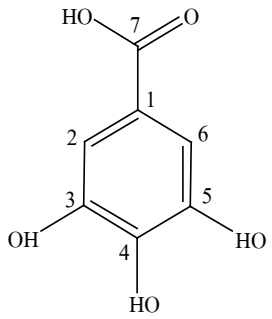


Figure 3-13 Structure of gallic acid

Table 3-10 NMR spectral data (500 MHz for ^{1}H and 125 MHz for ^{13}C) of C5 gallic acid in CD_3OD

Carbon position	δ_H (mult., *J* in Hz) (C5)	δ_H (mult., *J* in Hz) (Gallic acid)	δ_C (C5)	δ_C (Gallic acid)
1	-	-	122.1	121.38
2,6	7.05 (s)	7.10 (s)	110.3	110.52
3,5	-	-	146.4	144.94
4	-	-	139.5	135.93
7	-	-	170.5	170.76

Note: Gallic acid in D_2O from Lu and Foo, 1999

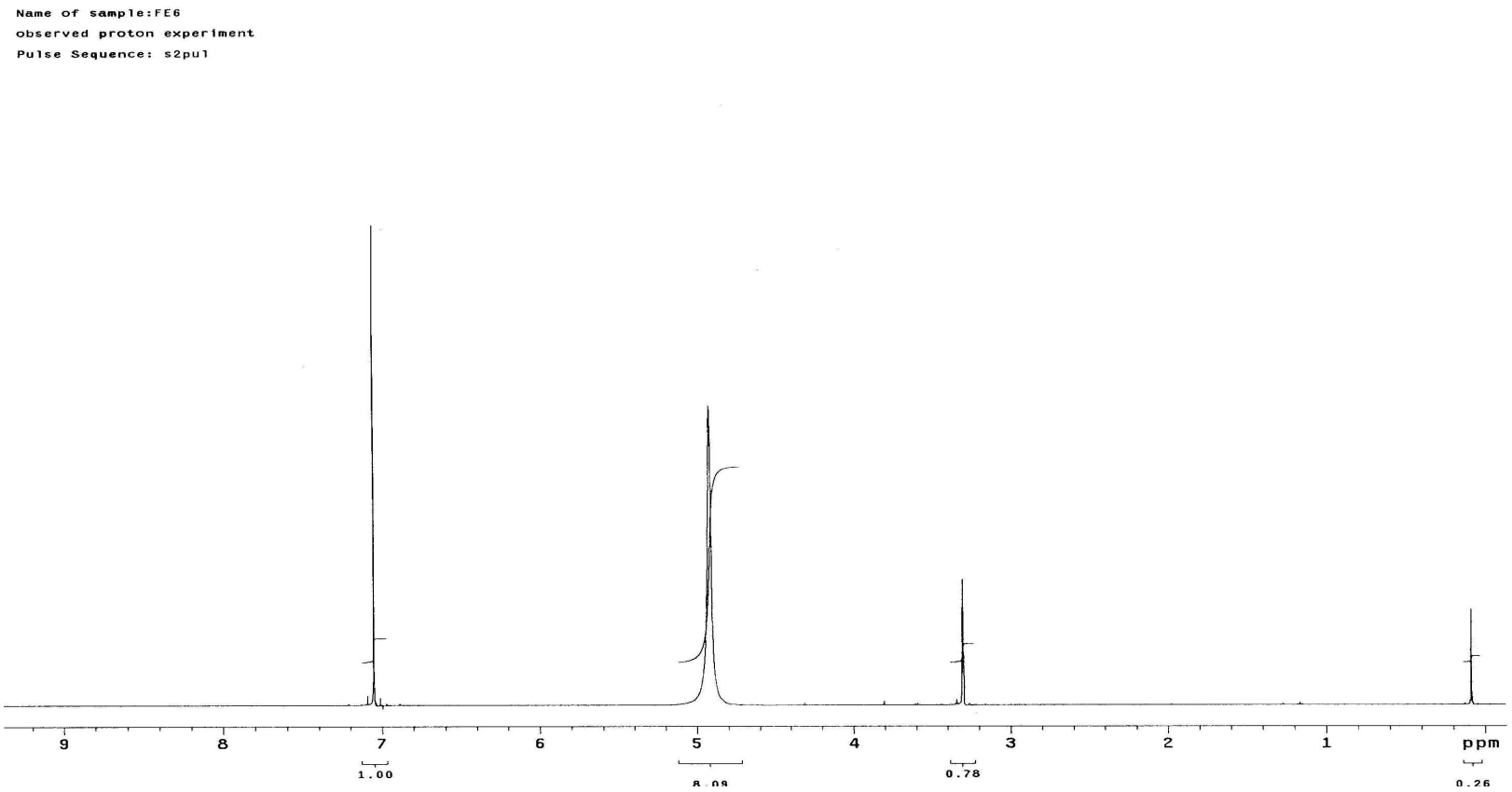


Figure 3-14 ^{1}H NMR spectrum of gallic acid in CD_3OD

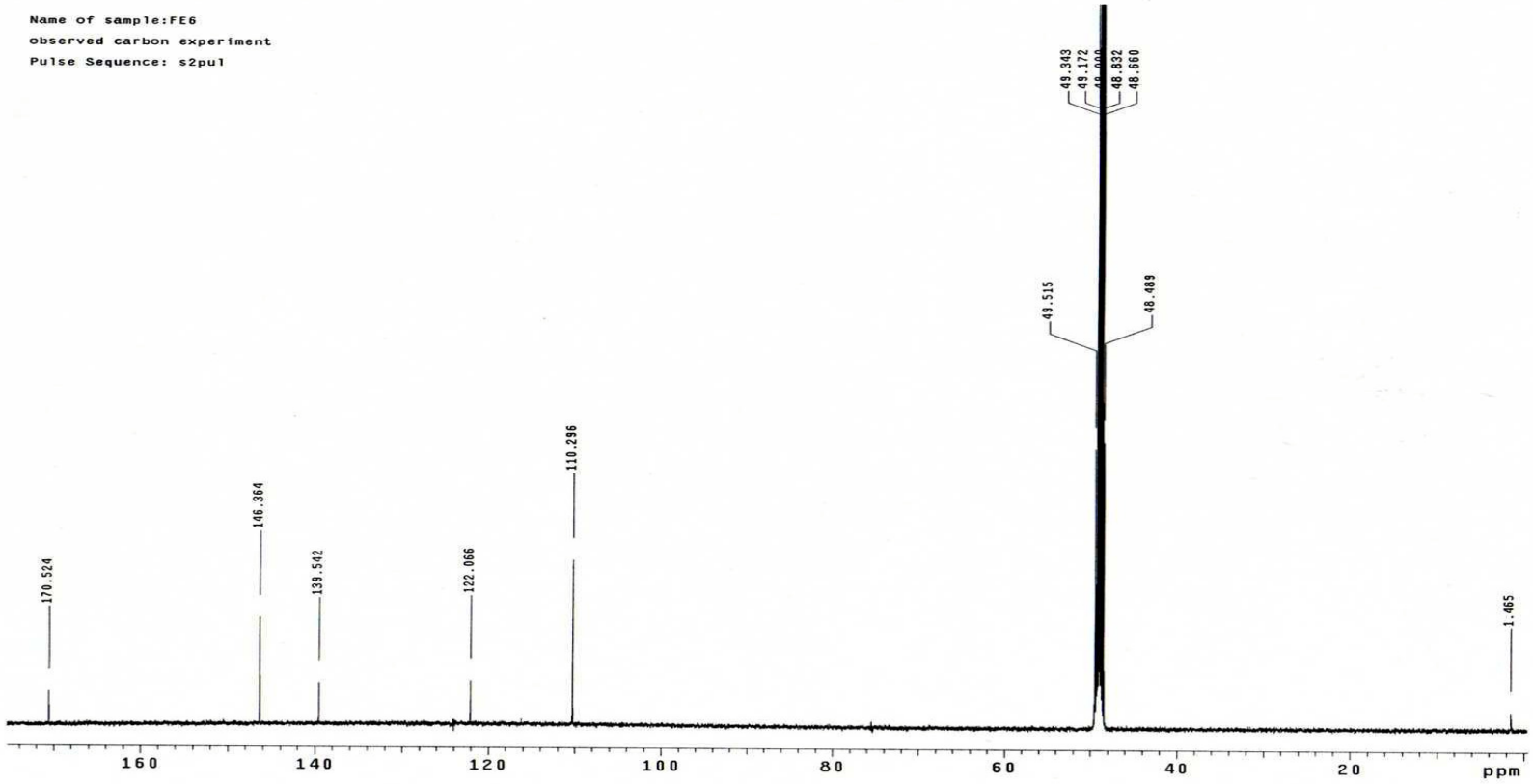


Figure 3-15 ^{13}C NMR spectrum of gallic acid in CD_3OD

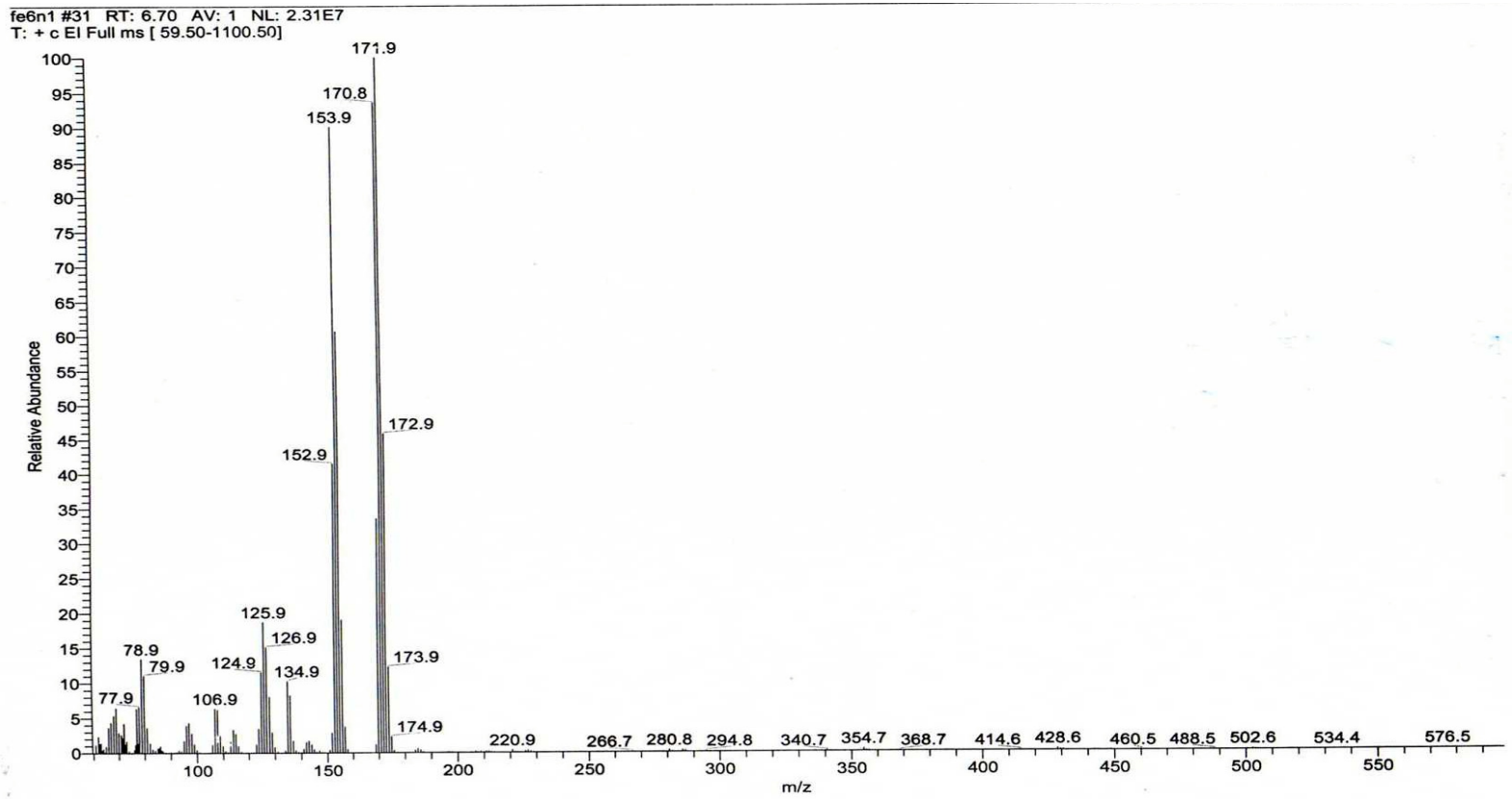
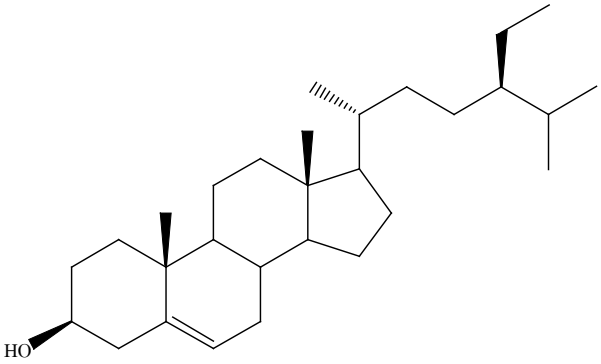


Figure 3-16 EIMS spectrum of gallic acid

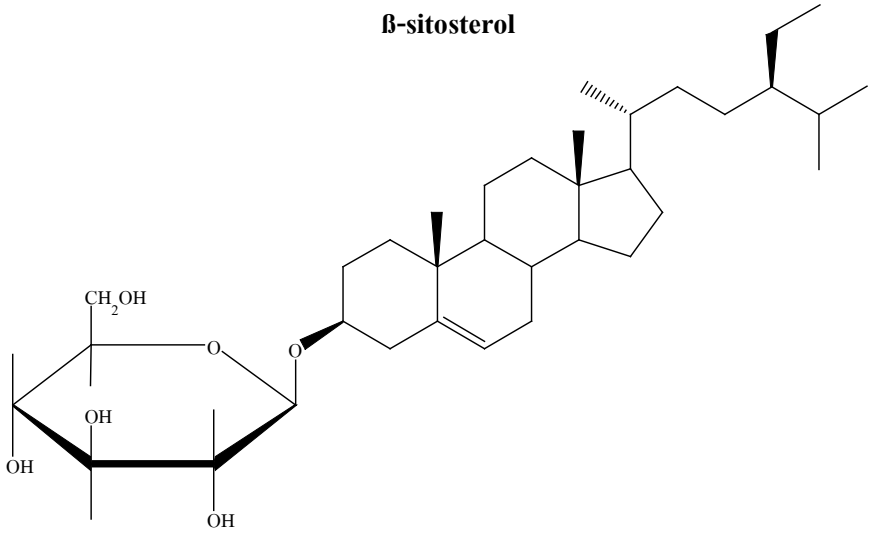
3.4 Discussion on phytochemical investigation

The ethanolic extracts of the pulp of *P. emblica* were separated by column chromatography using an isocratic solvents: hexane, chloroform and methanol. Four compounds were isolated. All pure compounds were detected by application of the general spraying reagent anisaldehyde in sulphuric acid, giving different colours after heating. C2, C3 and C5 were white colour amorphous and C4 was a yellow colour crystal. Only two compounds (C4 and C5) could be detected by UV 254 nm.

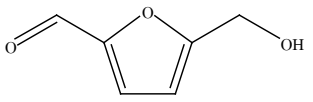
The four pure compounds could be divided into three chemical groups. They were sterols (ß-sitosterol and ß-sitosterol-3-*O*-ß-D-glucopyranoside), aldehyde (5-hydroxymethylfurfural) and phenolic derivative (gallic acid). The structures are shown in Figure 3-17. The investigation on chemical constituents of the pulp of *P. emblica* found that gallic acid, a major antioxidant compound, is the main compound and normally found in this species. ß-Sitosterol-glucoside and ß-Sitosterol were found in small amount and this these compounds are commonly sterols which were found in higher plants and have been found in many plant species. 5-Hydroxymethylfurfural (HMF) from the ethanolic extracts can be found as naturally of fruit liquors. Surprisingly there are no report of discovery HMF, ß-sitosterol-3-*O*-ß-D-glucopyranoside and ß-sitosterol in Triphala and *P. emblica*. The HMF as sugar derivative and gallic acid were major compound in pulp of *P. emblica*. The taste of this fruit as astringen followed by sweet occurred these two compounds.



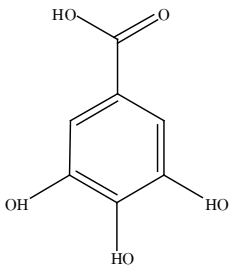
ß-sitosterol



ß-sitosterol-3-*O*-ß-D-glucopyranoside



5-hydroxymethylfurfural



gallic acid

Figure 3-17 The chemical structure of four compounds isolated from the ethanolic extracts of the pulp of *P. emblica* fruit

3.5 Activities of the isolated compounds

The ß-sitosterol, ß-sitosterol-3-*O*-ß-D-glucopyranoside, 5-hydroxymethyl-furfural and gallic acid isolated in this study, were assessed for antioxidant and cytotoxic activity against cancer cell lines (MCF-7, Hela and PC3) and normal cell lines (MRC5).

3.5.1 Antioxidant activity

Gallic acid which were isolated from the ethanolic extract of the pulp of *P. emblica* was found as antioxidant compounds for DPPH and lipid peroxidation assay (EC_{50} = 0..21 and 1.66 µg/ml respectively). These results related with the previous study which showed that gallic acid and methyl gallate showing strong NO scavenging activity (Kumaran and Karunakaran, 2006) and it also found that gallic acid was the major antioxidant components of *E. officinalis* (Kumar *et al*., 2006). ß-sitosterol, ß-sitosterol-3-*O*-ß-D-glucopyranoside and 5-hydroxymethylfurfural had no antioxidant activity (EC_{50} > 100 µg/ml) (Table 3-11).

Table 3-11 EC_{50} (µg/ml) of pure compounds on DPPH assay (n=3) and lipid peroxidation assay

Compounds	DPPH assay	Lipid peroxidation
ß-sitosterol	> 100	> 100
ß-sitosterol-3-*O*-ß-D- glucopyranoside	> 100	> 100
5-hydroxymethylfurfural	> 100	> 100
gallic acid	0.207 $\pm$ 0.01	1.66 $\pm$ 0.82

n = number of independent experiment which was performed in 3 replicates and NT = not test

3.5.2 Cytotoxic activity of the isolated compounds

The Results of pure compounds which were isolated from the ethanolic extract of the pulp of *P. emblica* showed no cytotoxic activity against cancer cell line (MCF-7), (Hela) and prostate cancer cell lines (PC3) and normal cell line (MRC5) (IC_{50} > 100 µg/ml) at exposure time 72 hours are shown on Table 3-12 and Figure 3-18. Vinblastine sulphate was used as the standard drug in the cytotoxicity assay. From the previous results indicate that all crude extracts showed less cytotoxicity against all three types of cancer cell lines (MCF-7, Hela and PC3) followed by the American National Cancer Institute (NCI) (IC_{50} < 20 µg/ml for the crude extracts) and less toxicity against MRC5 but the fractions from the pulp of *P. emblica* separated by the VLC found that FA2, FA3 and FA4 had cytotoxic activity against three type of cancer cell lines. Gallic acid showed less cytotoxic activity against all three types of cancer cell lines (MCF-7, Hela and PC3) and ß-sitosterol, ß-sitosterol-3-*O*-ß-D-glucopyranoside and 5-hydroxymethylfurfural showed less cytotoxicity against prostate cancer cell (PC3). Following the criteria for cytotoxic activity of a pure compound established by the American National Cancer Institute (NCI) should show the IC_{50} less than 4 µg/ml. The previous study reported the contrast results that L-malic acid 2-*O*-gallate, mucic acid 2-*O*-gallate and 1-*O*-galloyl-ß-D-glucose showed stronger inhibition against B16F10 (murine melanoma) cell growth than against HeLa (human uterine carcinoma) and MK-1 (human gastric adrenocarcinoma) cell growth by MTT method (Zhang *et al*., 2004). Moreover, gallic acid derivatives are know to cause apoptosis in tumour cell lines and to inhibit lymphocyte proliferation (Fiuza *et al*., 2004) and gallic acid which is a major polyphenol observed in Triphala also showed suppression of the growth of cancer cells in cytotoxic assays (Kaur *et al*., 2005).

Table 3-12 IC_{50} (μM) value of compounds tested against breast, cervical and prostate cancer cell lines (MCF-7, Hela and PC3) and normal cell lines (MRC5) (mean±SEM) exposure time 72 hrs (n=3)

Compounds	IC_{50} (μg/ml)			
	MRC5	**MCF-7**	**Hela**	**PC3**
ß-sitosterol-3-*O*-ß-D-glucopyranoside	> 100	> 100	> 100	80.27 ± 1.05
5-hydroxymethylfurfural	> 100	> 100	> 100	> 100
ß-sitosterol	> 100	> 100	> 100	76.87 ± 0.91
gallic acid	> 100	40.753 ± 0.30	69.115 ± 0.31	51.405 ± 0.56

n = number of independent experiment which was performed in 3 replicates.

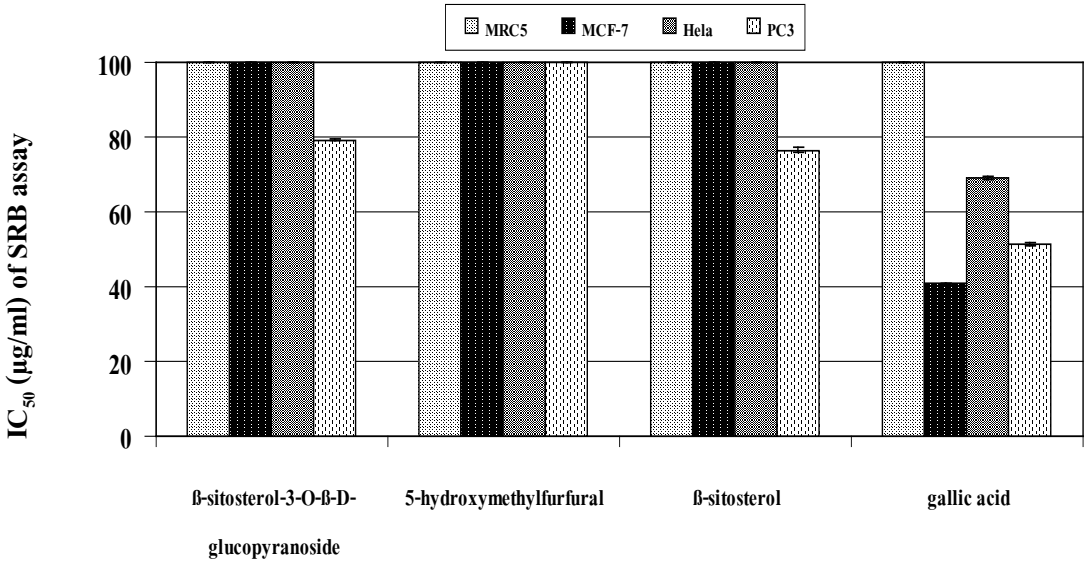


Figure 3-18 IC_{50} values (μM) and SEM of cytotoxic compounds isolated from the ethanolic extracts of *P. emblica* against breast, cervical and prostate cancer cell lines (MCF-7, Hela and PC3) and normal cell lines (MRC5) at exposure time 72 hrs

3.6 Determination of gallic acid

3.6.1 Standardization of gallic acid

For quantitative determination, gallic acid was used as the marker substances evaluate the quantity of an active compound from the plant extracts. The content of gallic acid was determined in chapter 2.9. The retention time and the peak area were calculated as percentage for gallic acid. To determine the linearity equations and linear scope for the analysis, a series of mixed standard solutions ranged from 1.0-5.0 μg/ml were testes. The results are summarized in Table 3-13. The chromatogram of Triphala showed in Figure 3-21 and gallic acid was also indicated in its chromatogram.

3.6.2 Analysis of gallic acid content by HPLC

The contents of the gallic acid in each plant extracts and Triphala were determined as described in the chapter 2.9. The results are obtained within 5 min for the HPLC separation. The contents of gallic acid in the plant extracts are ranged from 0.62-6.00%w/w. During sample analysis, the UV absorbance of the targeted peak was compared with standard for confirmation. The assay indicated that triphala contained the highest percentage of gallic acid content. The result related with determination gallic acid content by Folin-Ciocalteu's assay. It was found that the water extract of Triphala showed higher gallic acid contents than the ethanolic extract. The gallic acid content from the ethanolic extract of *P. emblica* was higher than that of water extract. The result are showed in Table 3-14 and Figure 3-20.

Table 3-13 The regression equation for the plant extracts and Triphala formula

Compound	Regression equation	Correlation coefficient	Linear range (μg/ml)
Gallic acid	y = 61748x - 18209	0.9996	1.0-5.0

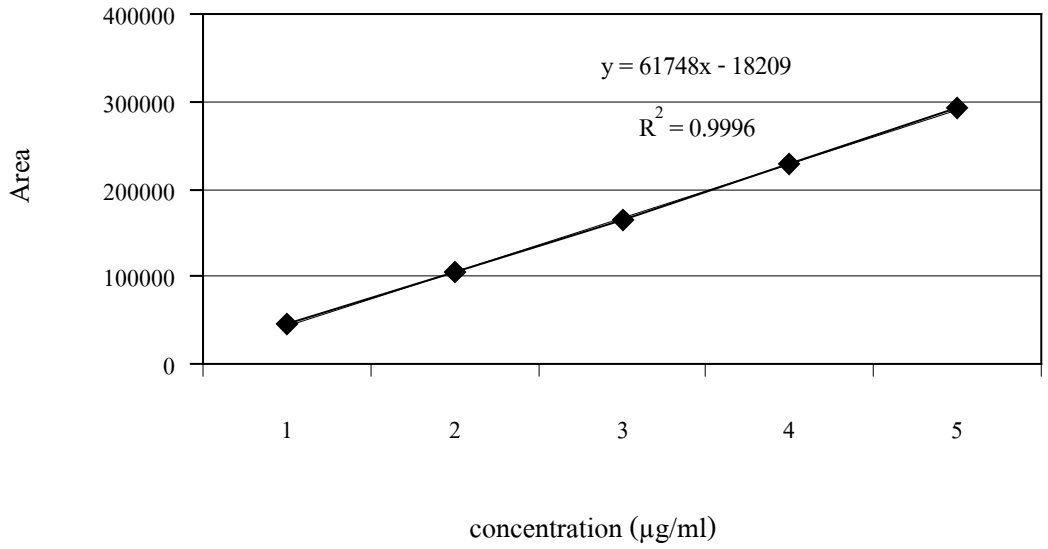


Figure 3-19 Standard curve of gallic acid, the y value is the peak area of analytes and the x value is the concentration of the analysis (μg/ml)

Table 3-14 Gallic acid contents of three plants and Triphala formula by HPLC analysis

Plant Species	Part	Water extracts % content (w/w)	Ethanolic extracts % content (w/w)
Phyllanthus emblica Linn.	Pulp	2.4406	3.7081
	Seed	3.7290	2.2571
Terminalia chebula Retz.	Pulp	0.7021	0.6220
	Seed	2.8344	2.5092
Terminalia bellerica Gaertn.	Pulp	1.2579	1.4698
	Seed	1.9978	1.7013
Triphala		5.9971	3.8300

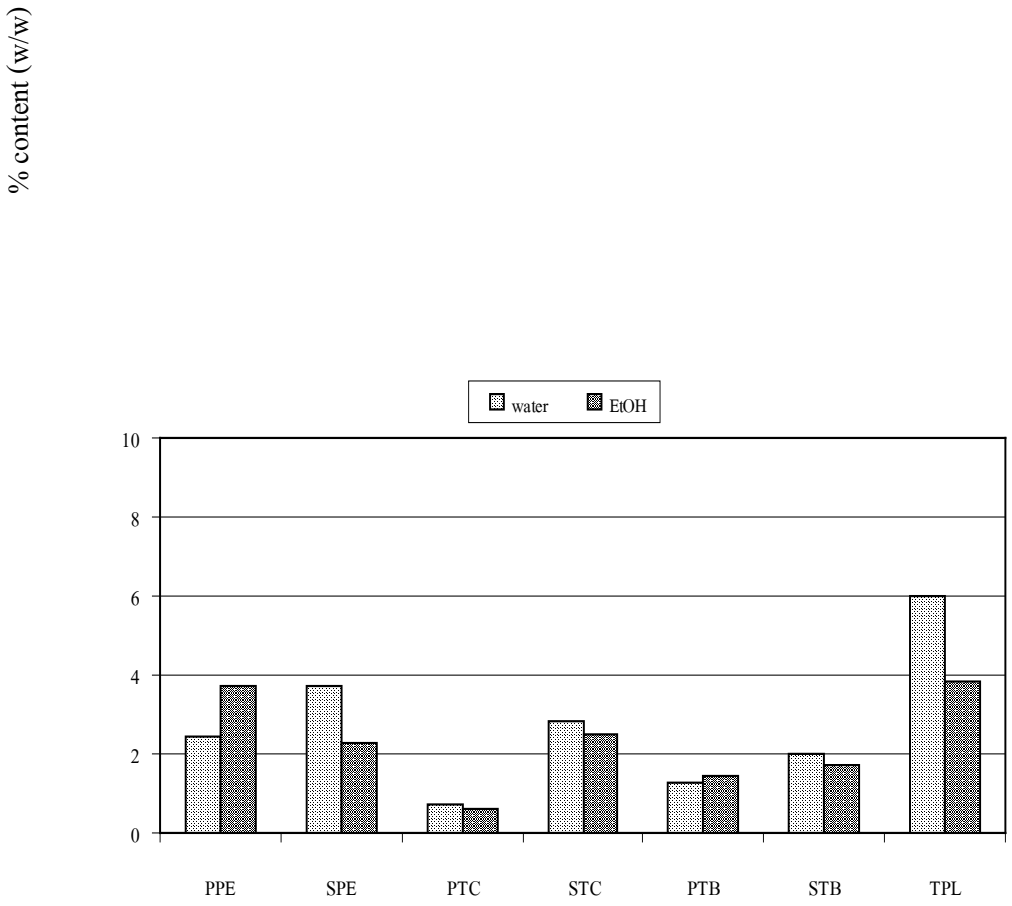
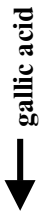


Figure 3-20 Percentage of gallic acid contents of three plants and Triphala formula



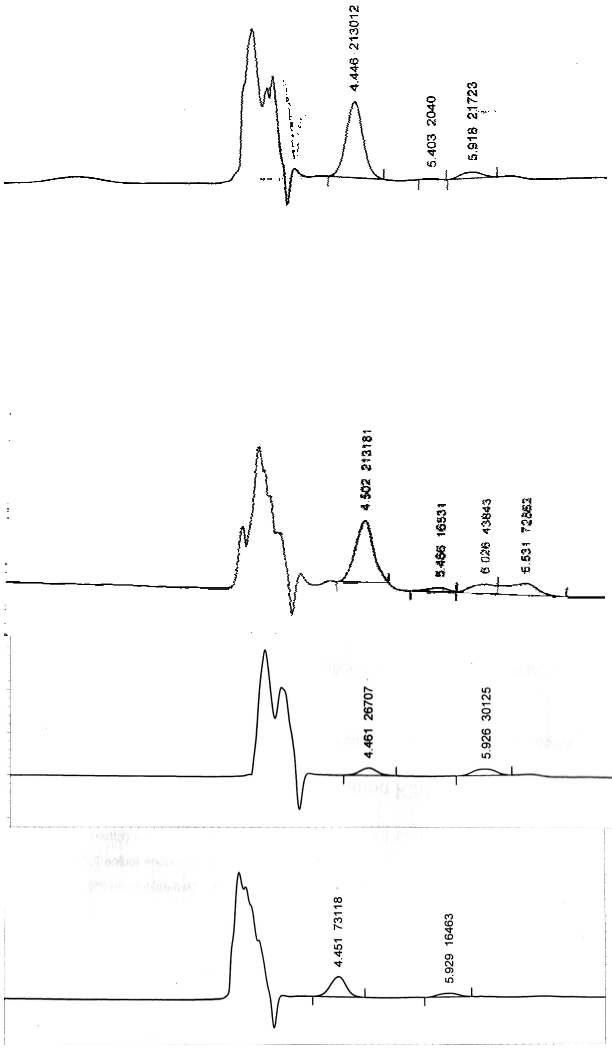


Figure 3-21 Typical chromatograms of gallic acid (a), Triphala (b), *P. emblica* (c), *T. chebula* (d) and *T. bellerica* (e) recored at 280 nm