3.5 NEUROPROTECTIVE ACTIVITY

Oxidative stress-induced cell damage mediated by reactive oxygen species (ROS), mainly superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) exerts damaging effect on deoxyribonucleic acid (DNA), lipid and protein (Weecharangsan *et al.*, 2006). Fortunately, our bodies' natural anti-oxidant defenses such as glutathione peroxidase, catalase and superoxide dismutase are able to remove these free radicals (Aruoma *et al.*, 1994). However, the imbalance between anti-oxidant and free radicals in the body can cause degenerative diseases and neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, stroke and dementia (Halliwell and Grootveld, 1987). Thus, the anti-oxidants naturally occurring in a variety of plant species represent an alternative solution to scavenge the ROS. Nevertheless as for now, only a few plant species have been studied and shown to exhibit neuro-activity (Dajas *et al.*, 2003) such as Kava and *Ginkgo biloba* (Assemi, 2001; Bastianetto and Quirion, 2002; Lee *et al.*, 2002).

The NG108-15 cells are formed by Sendai virus-induced fusion of the mouse neuroblastoma clone N18TG-2 and rat glioma clone C6 BV-1. This neuroblastoma-glioma hybrid cell line has been commonly applied for neuronal model in electrophysiology and pharmacology research (Brown and Higashida, 1988; Schmitt and Meves, 1995; Weecharangsan et u, 2006). Nearly all sources of oxidative stress generated H_2O_2. The H_2O_2-induced DNA damage *via* fenton reaction in the presence of oxygen and transition metal ion (Yen *et al.*, 2000) and the toxicity were attributed to its high membrane permeability (Vajragupta *et al.*, 2003; Weecharangsan *et al.*, 2006). It acted not only as a precursor for oxidizing radicals such as hydroxyl radicals (Reiter *et al.*, 2001) but also affected various mechanisms such as perturbing intracellular calcium homeostasis (Hyslop

et al., 1986) and intracellular ATP (Hyslop *et al.*, 1988), inducing DNA damage (Barbouti *et al.*, 2002) as well as apoptosis of the cells (Chandra *et al.*, 2000).

In the present study, sample pretreatment at various concentrations of compounds were performed to evaluate their neuroprotective effect against H_2O_2-induced oxidative damage. Exposure of the NG108-15 cells to H_2O_2 leads to the reduction in cell viability significantly by 45.94 % ± 1.24 as compared to the control. The results showed that all the treated samples displayed a dose dependent manner in the neuroprotection model. The neuroprotective activity of the galloylated cyanogenic glucosides (**1**-**7**), gallotannins (**8**-**14**), ellagitannins, ellagic acid derivatives and aromatic compounds (**15**-**22**) are shown in Figures 3.5.1, 3.5.2 and 3.5.3, respectively. Catechin was selected as a positive control in the present study since it has been reported as a neuroprotective agent (Unno and Hoshino, 2007).

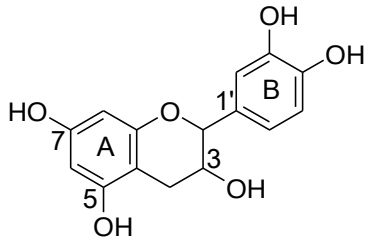
Thus far, there is yet no report on the study of neuroprotective activity for the galloylated cyanogenic glucosides. This study demonstrated that they protected NG108-15 cells in a concentration-dependant manner when the cells were exposed to H_2O_2. Nevertheless, as compared to catechin, these compounds showed less neuroprotection on the cell damage except for prunasin 2',3',4',6'-tetra-*O*-gallate (**7**). As shown in Figure 3.5.1, the percentage of cell viability decreases from prunasin-trigallate (**5**-**6**), prunasin-digallate (**2**-**4**) to prunasin-monogallate (**1**). Among the two prunasin-trigallate isomers, prunasin 2',3',6'-tri-*O*-gallate (**5**) possessed higher neuroprotective activity as compared to prunasin 3',4',6'-tri-*O*-gallate (**6**). On the other hand, prunasin 2',6'-di-*O*-gallate (**2**) has lower neuroprotective activity as compared to the other two isomers of prunasin-digallate (**3**-**4**). The mono-galloylated cyanogenic glucosides, prunasin 6'-*O*-gallate (**2**) showed lower neuroprotective effects against oxidative damage in the NG108-15 cells. However, it

significantly protected the cells from oxidative damage at the concentration of 50 µM and 100 µM.

Similarly, the gallotannins (**8**-**14**) also increased the neuroblastoma-glioma hybrid cell viability in a dose-dependent manner (Figure 3.5.2) which is in accordance to several reported neuroprotection studies (Choi *et al.*, 2002; Falsig *et al.*, 2004). The compound 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (**14**) and 1,2,3,6-tetra-*O*-galloyl-β-D-glucose (**13**) significantly inhibited the H_2O_2-induced neuron cells damage in a dose-dependent manner at concentrations of 6.25-100 µM. The inhibitory activity of 1,2,3,6-tetra-*O*-galloyl-β-D-glucose (**13**) was comparable to that of catechin. However, the neuroprotective activity of 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (**14**) was more potent than that of catechin. This compound has also been reported to not only increase the cellular resistance to H_2O_2 but also highly protected neuronal cells from H_2O_2-induction damage *via* induction of HO-1 gene expression (Choi *et al.*, 2002). As shown in Figure 3.5.2, the neuroprotective activity decreased when the number of galloyl esterification with glucose moieties was reduced, from trigalloyl-glucose (**10**-**12**) to 3,6-di-*O*-galloyl-D-glucose (**9**) and 6-*O*-galloyl-D-glucose (**8**). Compounds 3,4,6-tri-*O*-galloyl-D-glucose (**12**), 1,2,3-tri-*O*-galloyl-β-D-glucose (**10**) and 1,4,6-tri-*O*-galloyl-β-D-glucose (**11**) presented noticeable differences in neuroprotective activity although they are the isomers of trigalloyl-glucose.

Ellagitannins are hydrolysable tannins that contained the hexahydroxydiphenoyl (HHDP) group. These compounds (**15**-**18**) also showed remarkable inhibition on the H_2O_2-induced oxidative damage in NG108-15 cells as compared to catechin (Figure 3.5.3). They highly protected the neuron cells at concentrations ranging from 6.25-100 µM in dose-dependent manner. The sequence of high to low neuroprotective activity of ellagitannins was in order of pterocarinin C (**17**), praecoxin B (**16**), casuarinin (**18**) and 6-*O*-galloyl-2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose (**15**). These compounds (**15**-**18**) have at least a

hexahydroxydiphenoyl (HHDP) group whereas casuarinin (**18**) has two HHDP groups. The three galloyl groups in pterocarinin C (**17**), two galloyl groups in praecoxin B (**16**) and one galloyl group in 6-*O*-galloyl-2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose (**15**) also contributed to the neuroprotective activity. Casuarinin (**18**) has an open ring glucose together with two HHDP and a galloyl group which showed more protectivity on NG108-15 cells as compared to 6-*O*-galloyl-2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose (**15**). This result indicated that the number of galloyl esterification might influence the neuroprotective activity. The gallic acid (**21**) possessed similar level with 3'-*O*-methyl-3,4-methylenedioxyellagic acid 4'-*O*-β-D-glucopyranoside (**19**) and 3,3',4-tri-*O*-methylellagic acid 4'-*O*-β-D-glucopyranoside (**20**) against H_2O_2-induced oxidative damage in NG108-15 cells. Among the ellagic acid derivatives (**19**-**20**), 3,3',4-tri-*O*-methylellagic acid 4'-*O*-β-D-glucopyranoside (**19**) showed considerably higher neuroprotective activity (Figure 3.5.3). At the concentration of 100 μM, the compounds showing comparable neuroprotective activity with the positive control were in the sequence of **14** > **7** > **17** > **16** > **13** > **18** > **10** > **5** > **15** > **11** > **6** > catechin. Among them, three belonged to the galloylated cyanogenic glucosides, four belonged to the gallotannins and the remaining four compounds were ellagitannins.



Catechin

Catechin is also related to the family of flavonoids and the sub-group flavan-3-ol. The anti-oxidant potential of catechin was mainly attributed to the OH substitution at 3' and 4' which provided the peroxyl radical absorbing activity (Cao *et al.*, 1997) while the

hydroxyl group in position 3 has little influence in anti-oxidant due to its spatial position (Teixeira *et al.*, 2005). Other features in flavonoids such as 3',4',5'-triOH in ring B and 5,6-diOH 5,8-diOH, 6,7-diOH, 7,8-diOH, 5,6,7-triOH, 5,6,8-triOH, 5,7,8-triOH, 6,7,8-triOH and 5,6,7,8-tetraOH in ring A were also expected to impart the scavenging activity (Seyoum *et al.*, 2006). The oxidation potential was influenced by the position and numbers of hydroxyl group which played a role as hydrogen donating hydroxyl in radical neutralisation (Teixeira *et al.*, 2005; Seyoum *et al.*, 2006). The catechol B-ring particularly involved in electron transfer from the phenolate was also important and is most significant in scavenging ROS (Heim *et al.*, 2002; Dueñas *et al.*, 2010). The study of bioavailability in rats showed that the main catechin metabolites were the glucuronidated derivatives (Manach *et al.*, 1999), whereas methylated catechin metabolites were found mainly in the liver but much lesser in plasma and urine (Silberberg *et al.*, 2005). The hydrolysis of β-glycosidic bonds in the flavonoids glycosides only occurred in the colon by microorganisms and the aglycones were believed to be able to pass through the gut wall (Hollman and Katan, 1999). However, the mechanism for transporting the aglycones across the gut wall was still lacking (Hollman and Katan, 1999). The *O*-methylation of hydroxyl in the metabolism of catechin by enzyme catechol *O*-transferase in catechol B-ring resulted in a decrease of the anti-oxidant activity (Cao *et al.*, 1997; Dueñas *et al.*, 2010). Nevertheless, these metabolites retained significant radical scavenging activity which acted as better electron and hydrogen donors at pH 7.4 (Dueñas *et al.*, 2010). Their anti-oxidant activity was also strongly dependent on the pH of the medium which increased at greater pH values (Dueñas *et al.*, 2010). Thus, a potent neuroprotective compound is not only depended on anti-oxidant activity but also capable to provide proper hydrophobicity that allows the permeability of membrane cell. Apart from the hydrophobicity, the number, pattern and position of hydroxyl, presence of unsaturated 2-3 bond in conjunction with 4-

oxo, the number, position and structure of sugar and steric effect of the compounds (Heim *et al.*, 2002; Seyoum *et al.*, 2006; Rastija and Medić-Šarić, 2009), other factors such as phenolic O-H bond dissociation enthalpy (OH-BDE), energy-eigenvalue of the highest occupied molecular orbital (E_{HOMO}) and ionization potential (IP) were also important in determining the scavenging activity of anti-oxidant (Van Acker *et al.*, 1993; Lu *et al.*, 2006).

Our results indicated that as the number of galloyl esterification is increased in a compound, the neuroprotective activity is also increased accordingly (Yokozawa *et al.*, 1998). The *o*-dihydroxyl group is capable of donating hydrogen or electron and reducing iron (Tian *et al.*, 2009). However, the configuration and position of galloyl esterification would also affect the neuroprotective activity although these compounds have the same number of galloyl unit, possibly due to the spatial hindrance of the molecules which prevented the free radicals or iron reaction (Tian *et al.*, 2009). It was suggested that the scavenging effects of tannins were generally dependent on the molecular size and the number of hydroxyl groups with ortho-dihydroxyl (catechol) and ortho-trihydroxyl (pyrogalloyl) structure in the molecule (Yokozawa *et al.*, 1998; Yoshida *et al.*, 2000; Seyoum *et al.*, 2006). The anti-oxidant activities of hydrolysable tannins were also affected by their oxidation level (Okuda *et al.*, 2000). The twenty compounds isolated from the leaves of *P. rotundifolia* showed potential as neuroprotective agents. However, the absorption, pharmacokinetics, bio-transformation and bioavailabiliy of these type of compounds have not been well studied. Further investigation is required to ascertain the neuroprotective ability of these compounds.

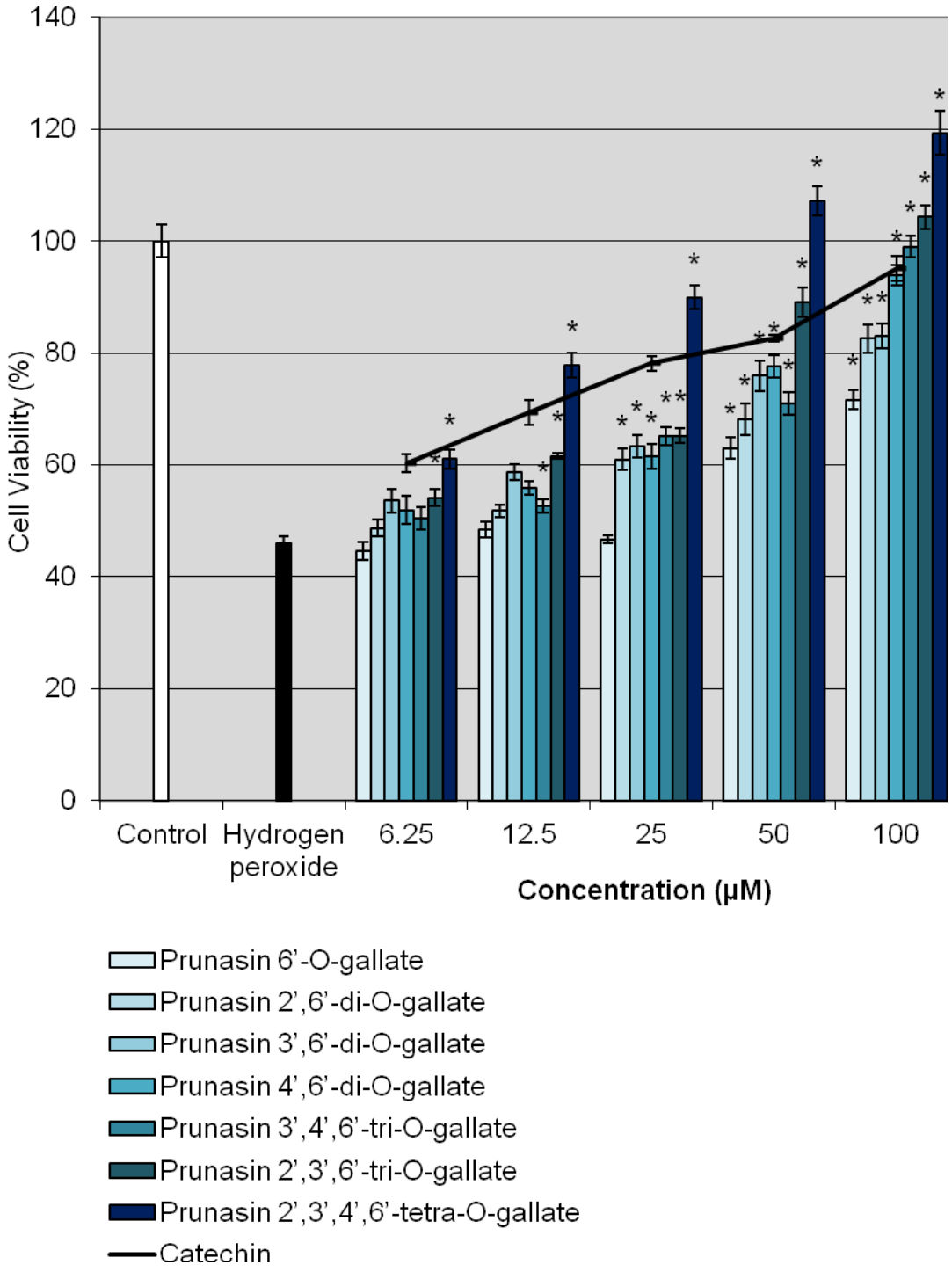


Figure 3.5.1: Neuroprotective activity of galloylated cyanogenic glucosides (**1-7**) in bar graph. * $P < 0.01$ was considered to be significant.

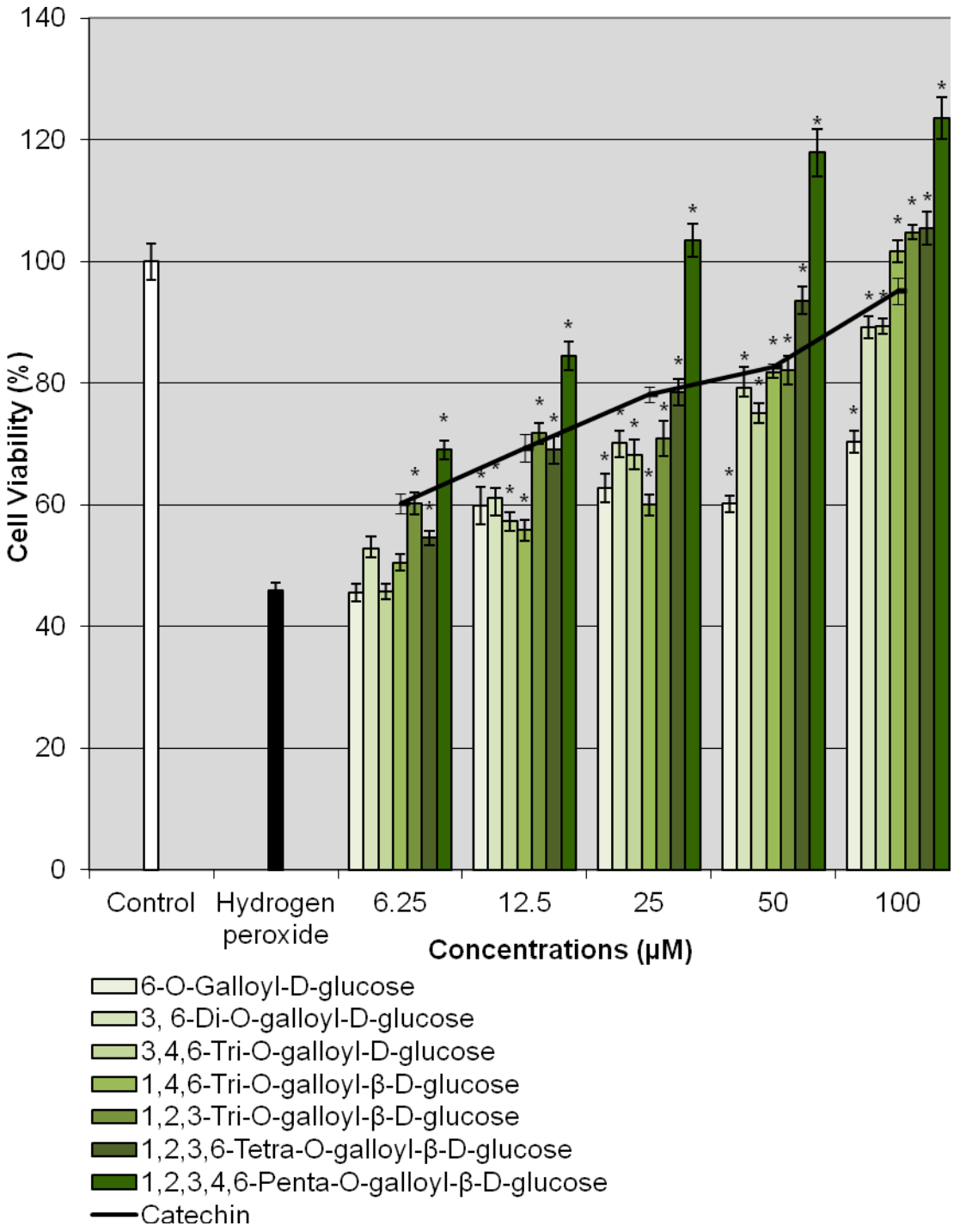


Figure 3.5.2: Neuroprotective activity of gallotannins (**8-14**) in bar graph. * $P < 0.01$ was considered to be significant.

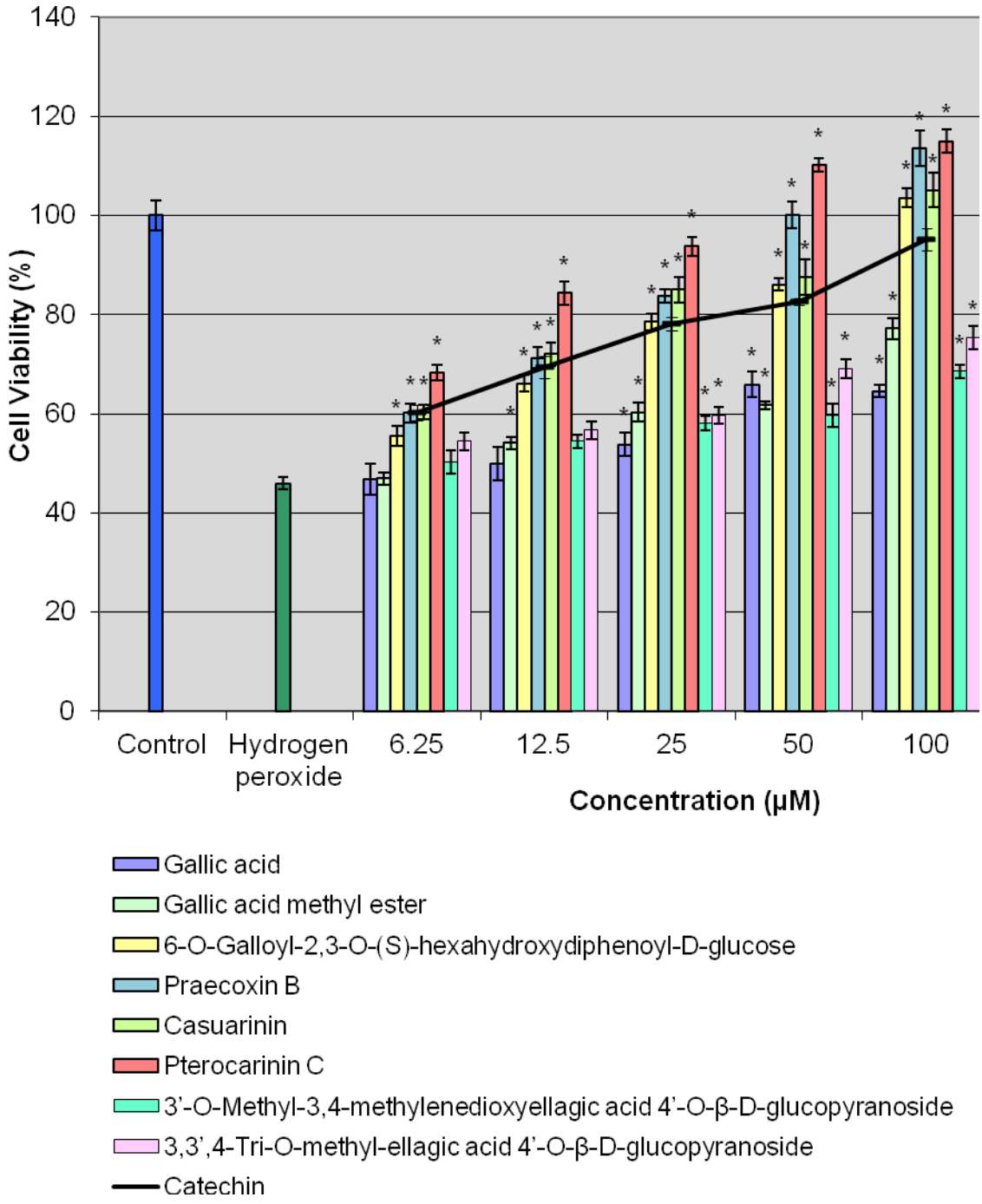


Figure 3.5.3: Neuroprotective activity of ellagitannins (**15-18**), ellagic acid derivatives (**19-20**), gallic acid (**21**) and gallic acid methyl ester (**22**). * $P < 0.01$ was considered to be significant.

3.6 *IN VITRO* CYTOTOXICITY AGAINST CANCER CELL LINES

3.6.1 CaSki Cell Line

The CaSki cell line (cervical epidermoid carcinoma) carries the HPV-16 virus which is a major causative agent of cervical cancer. The *in vitro* cytotoxicity on CaSki cell line was evaluated for the 22 compounds at different concentrations ranging from 0-100 µM. The galloylated cyanogenic glucosides (**1**-**7**) did not exhibit significant inhibition against the CaSki cell line (Figure 3.6.1.1) with only prunasin 3',4',6'-tri-*O*-gallate (**6**) showing lower percentage of cell viability. Similar results were also observed in the gallotannins (**8**-**14**) (Figure 3.6.1.2) where the highest inhibitory effect on CaSki cell line was shown by 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (**14**). Nonetheless, all the galloylated cyanogenic glucosides and gallotannins demonstrated their IC_{50} values of more than 100 µM. Thus rendering them not considered as potent anti-cancer agents for cervical cancer. The ellagitannins (**15**-**18**) demonstrated the *in vitro* cytotoxicity of CaSki cell line only moderately (Figures 3.6.1.3) while 3,3',4-tri-*O*-methylellagic acid 4'-*O*-β-D-glucopyranoside (**20**), 3'-*O*-methyl-3,4-methylenedioxyellagic acid 4'-*O*-β-D-glucopyranoside (**19**) and gallic acid (**21**) showed better inhibition on CaSki cell line among all the compounds.

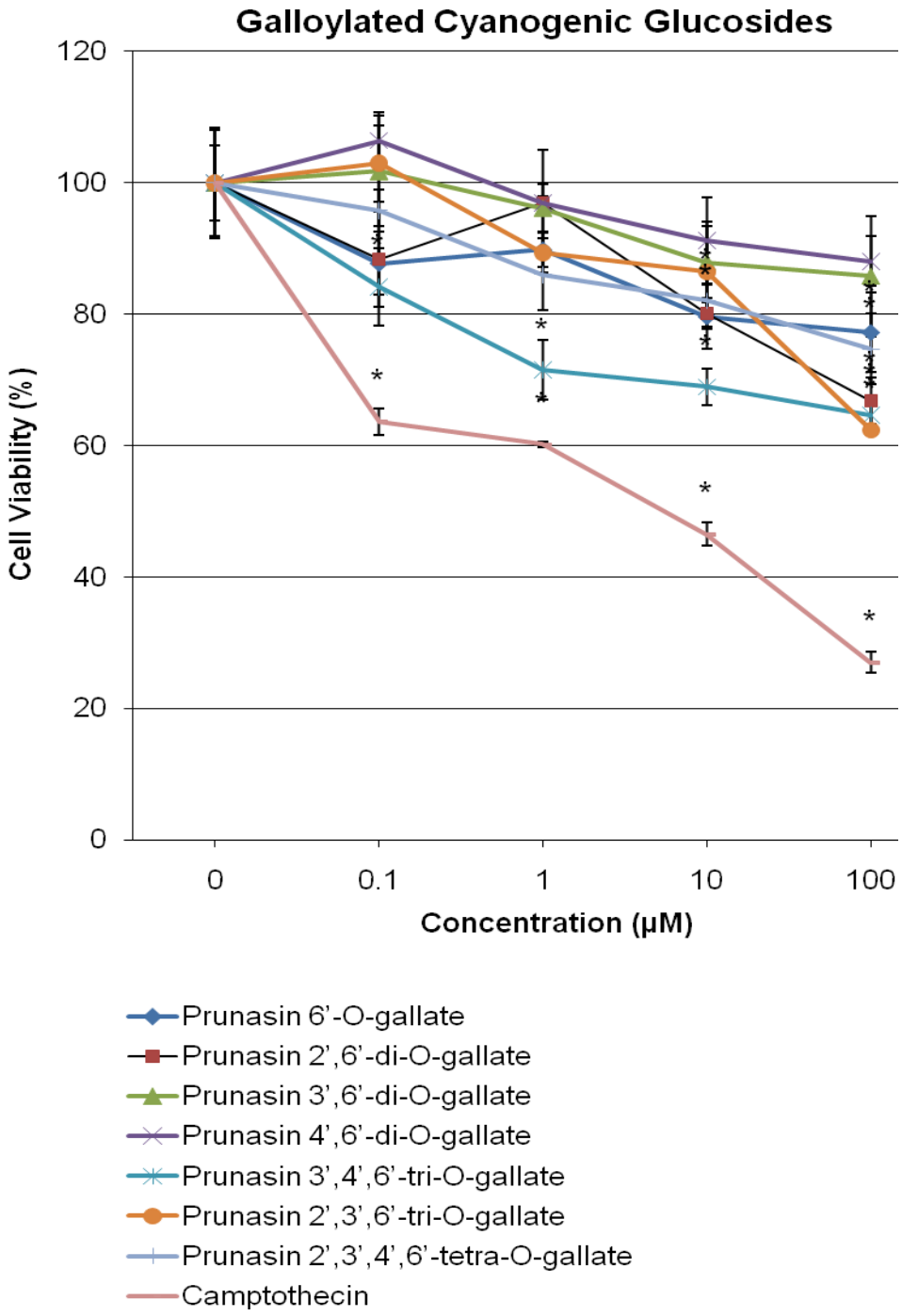


Figure 3.6.1.1: *In vitro* cytotoxicity on CaSki cell line of galloylated cyanogenic glucosides (**1**-**7**). * $P < 0.05$ was considered to be significant.

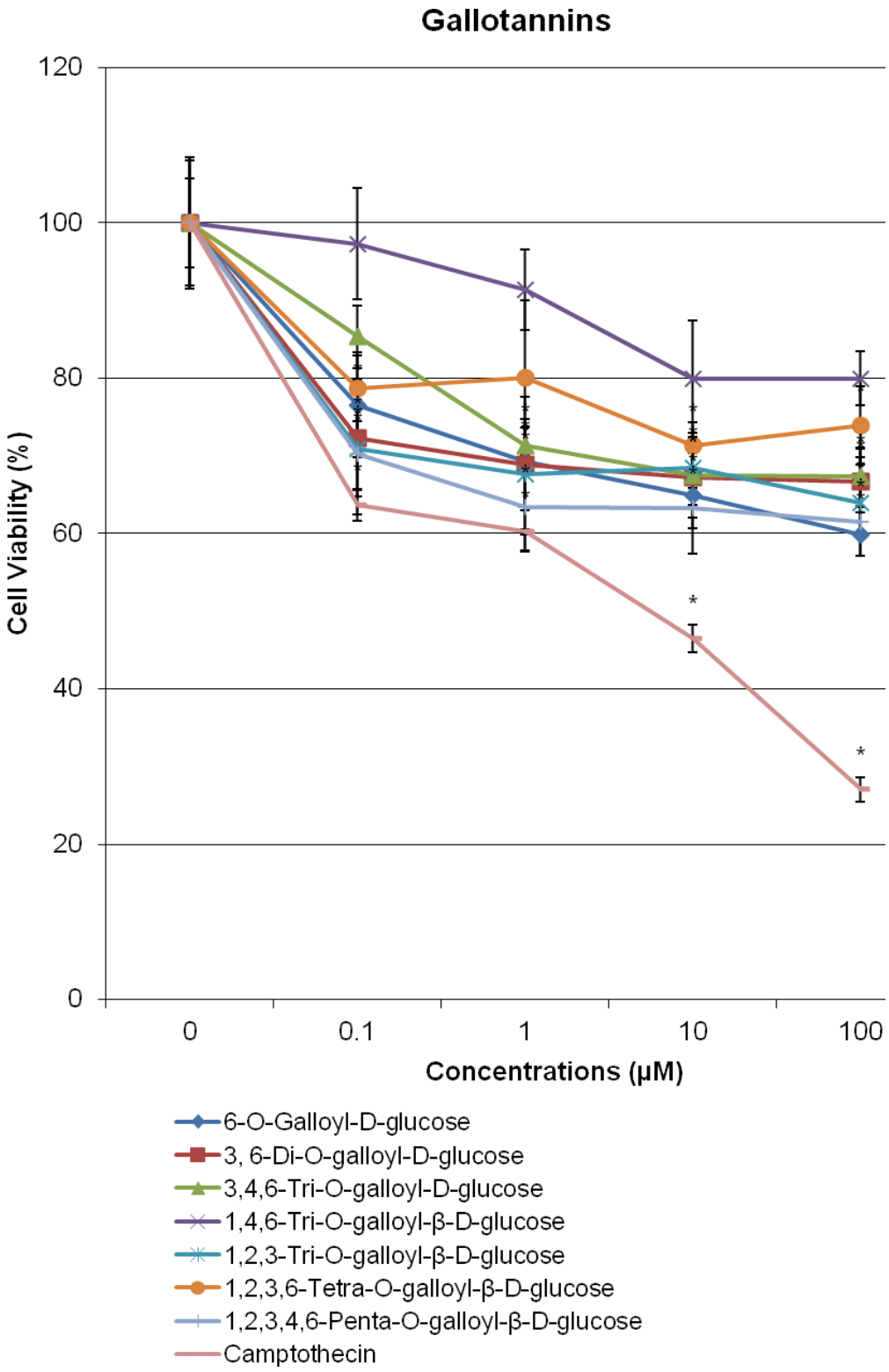


Figure 3.6.1.2: *In vitro* cytotoxicity on CaSki cell line of gallotannins (**8-14**). * $P < 0.05$ was considered to be significant.

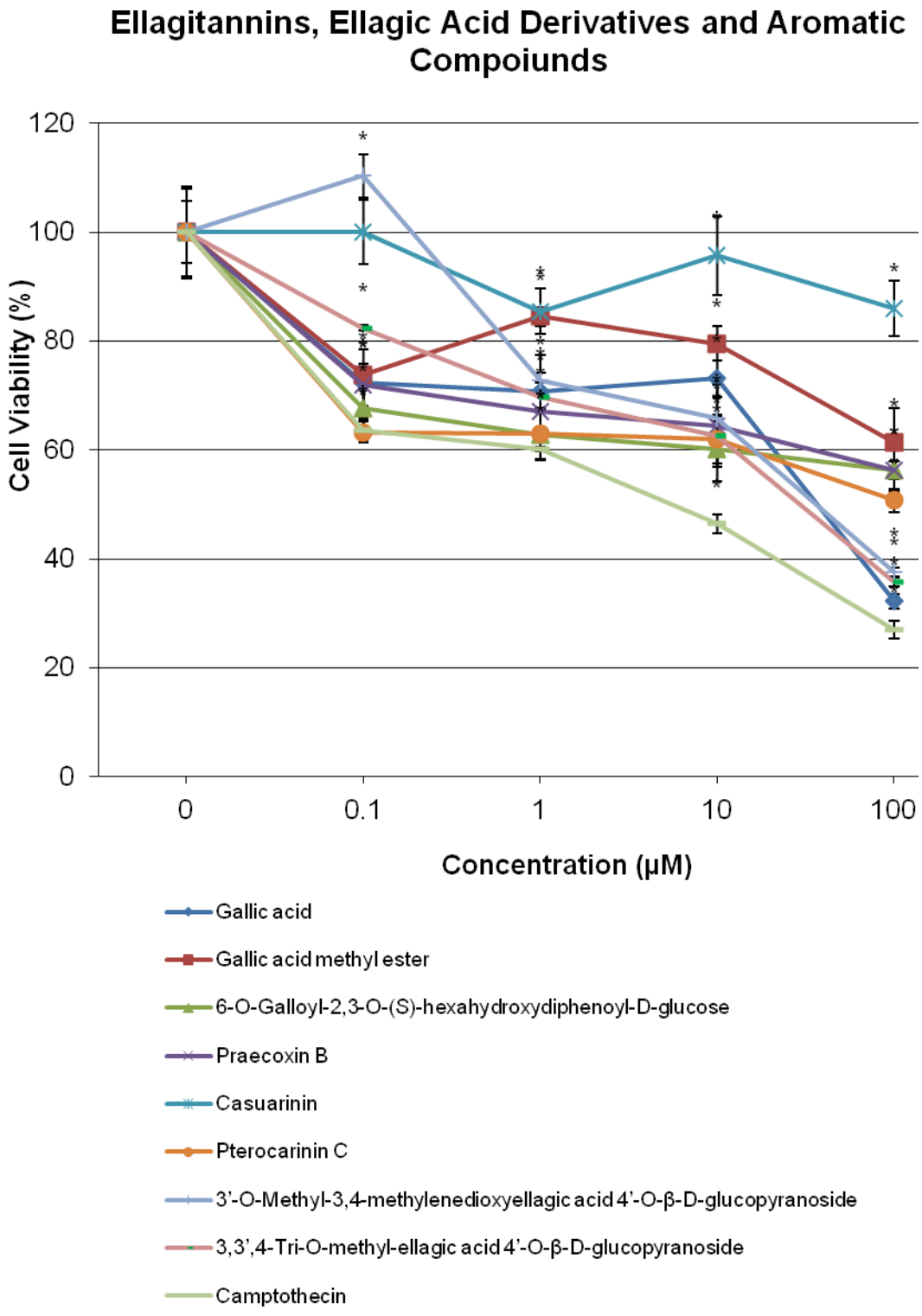


Figure 3.6.1.3: *In vitro* cytotoxicity on CaSki cell line of ellagitannins (**15-18**), ellagic acid derivatives (**19-20**) and aromatic compounds (**21-22**). **P* < 0.05 was considered to be significant.

3.6.2 HCT 116 Cell Line

Colon cancer is the third most common cancer in the world (Jemal *et al.*, 2007). The results showed that all the compounds exhibited more than 50 % inhibition against HCT 116 cell line (human colon carcinoma) through dose-dependent reduction of cell growth except prunasin 6'-*O*-gallate (**1**), 6-*O*-galloyl-D-glucose (**8**), 1,2,3-tri-*O*-galloyl-β-D-glucose (**10**) and gallic acid methyl ester (**22**). Most of the compounds inhibited the HCT 116 cell line drastically at the concentration of 10-100 µM and they included galloylated cyanogenic glucosides, ellagitannins, gallotannins, ellagic acid derivatives and aromatic acid (Figure 3.6.2.1-3.6.2.3). However, exception was observed in gallotannins such as 6-*O*-galloyl-D-glucose (**8**) and 1,2,3-tri-*O*-galloyl-β-D-glucose (**10**) which showed very low inhibition on HCT 116 cell even at the highest concentration used (Figure 3.6.2.2). Similar trend of inhibition was observed for 3,6-di-*O*-galloyl-D-glucose (**7**), 1,4,6-tri-*O*-galloyl-β-D-glucose (**11**), 3,4,6-tri-*O*-galloyl-D-glucose (**12**), 1,2,3,6-tetra-*O*-galloyl-β-D-glucose (**13**) and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (**14**) at concentration of 0-100 µM. Among the compounds, 3,3',4-tri-*O*-methylellagic acid 4'-*O*-β-D-glucopyranoside (**20**) and 3'-*O*-methyl-3,4-methylenedioxyellagic acid 4'-*O*-β-D-glucopyranoside (**19**) showed the highest inhibition on HCT 116 cell line (Figure 3.6.2.3).

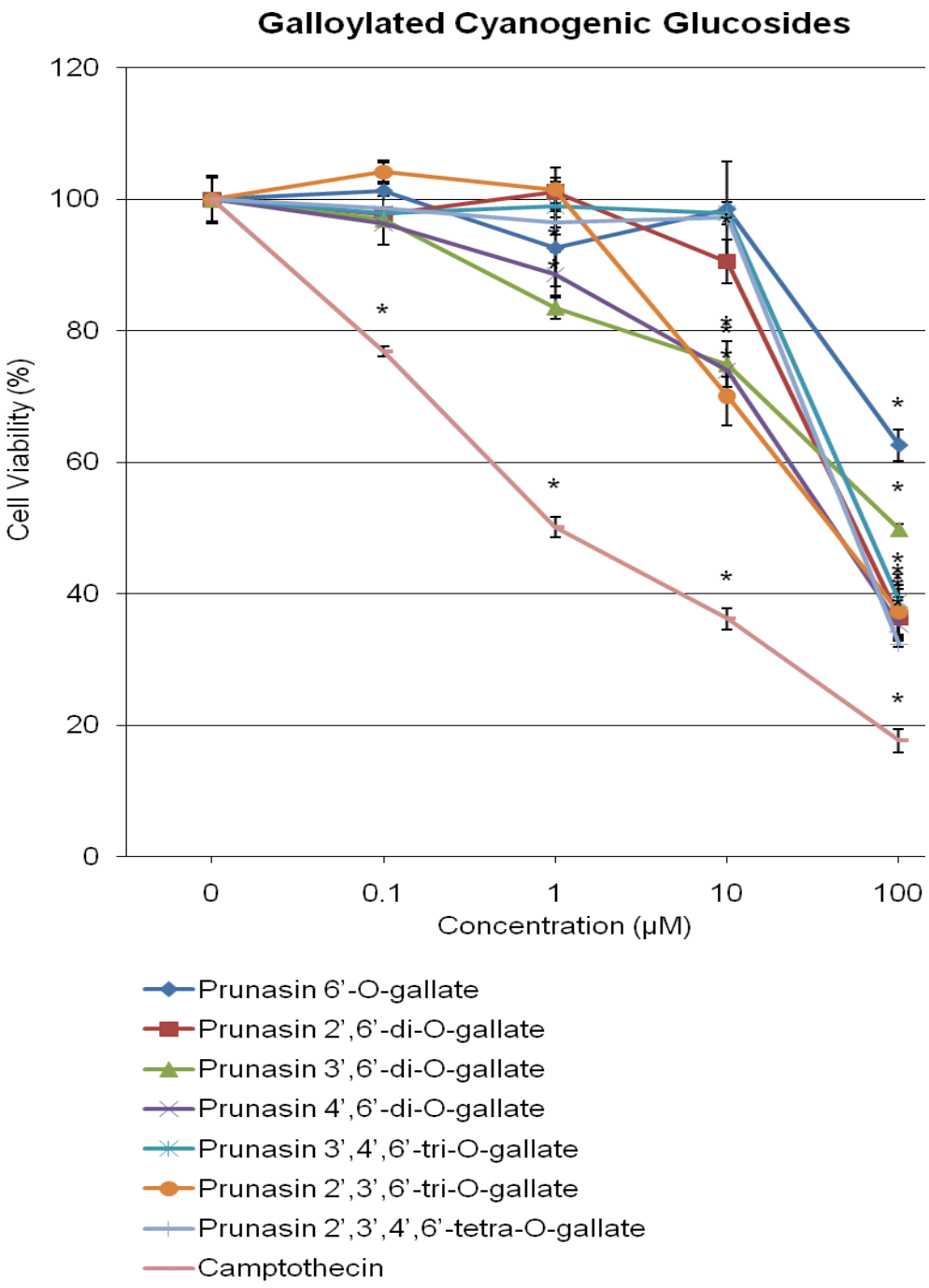


Figure 3.6.2.1: *In vitro* cytotoxicity on HCT 116 cell line of galloylated cyanogenic glucosides (**1-7**). * $P < 0.05$ was considered to be significant.

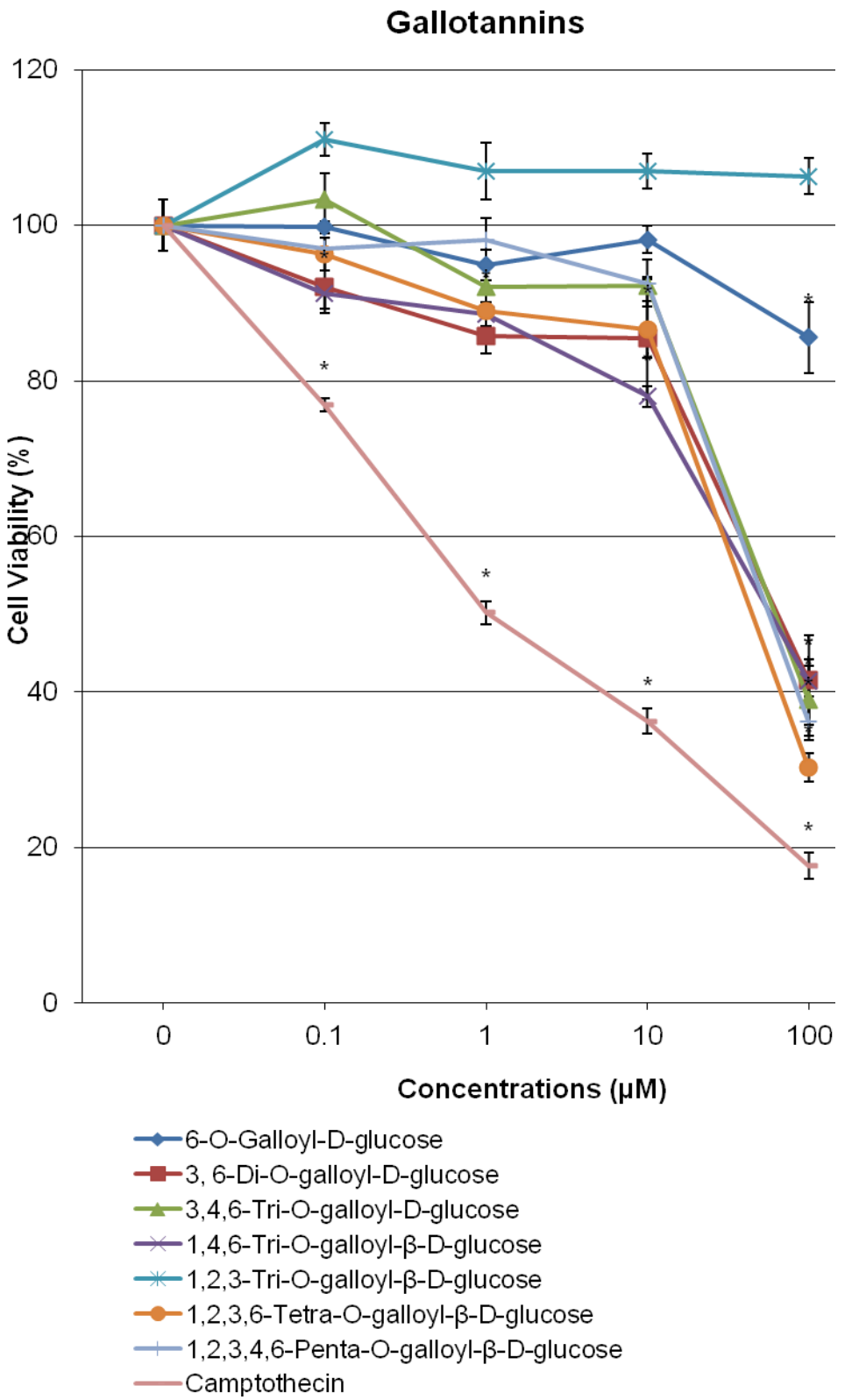


Figure 3.6.2.2: *In vitro* cytotoxicity on HCT 116 cell line of gallotannins (**8**-**14**). * $P < 0.05$ was considered to be significant.

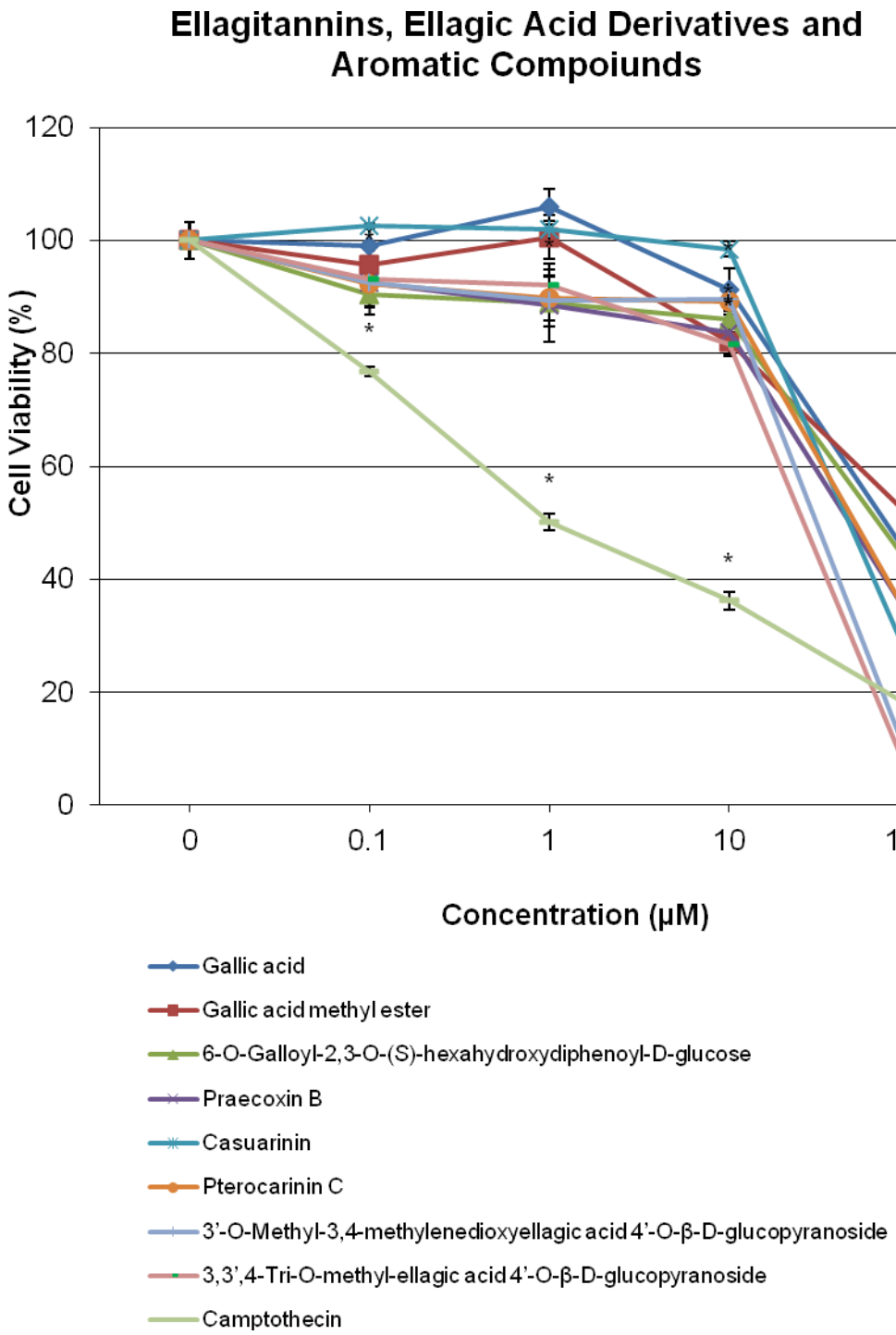


Figure 3.6.2.3: *In vitro* cytotoxicity on HCT 116 cell line of ellagitannins (**15-18**), ellagic acid derivatives (**19-20**) and aromatic compounds (**21-22**). **P* < 0.05 was considered to be significant.

3.6.3 MCF 7 Cell Line

MCF-7 (Michigan Cancer Foundation-7) is a breast carcinoma cell line established by Soule *et al.* in 1973 at the Institute of Detroit. In the *in vitro* cytotoxicity on MCF-7 cell line, the galloylated cyanogenic glucosides showed considerably higher inhibitory activity than gallotannins (Figure 3.6.3.1). Meanwhile, prunasin 4',6'-di-*O*-gallate (**4**) and prunasin 3',4',6'-tri-*O*-gallate (**6**) exhibited higher inhibition as compared to the other galloylated cyanogenic glucosides. The higher inhibitory effect in the former may be due to the esterification of galloyls at position 4' and 6'. In contrast, additional esterification of galloyl at position 3' might decrease the inhibitory activity and this may explain the reduction of inhibitory activity for prunasin 2',3',4',6'-tetra-*O*-gallate (**7**). Thus, galloyl esterification at position 4' and 6' in the cyanogenic glusosides might contribute to better inhibitory activity on the MCF 7 cell line. The gallotannins (**8**-**14**) did not show remarkable inhibition against MCF 7 cell line as presented in Figure 3.6.3.2 where the cell viability remained more than 60 % even at a high concentration of 100 µM. However, 3'-*O*-methyl-3,4-methylenedioxyellagic acid 4'-*O*-β-D-glucopyranoside (**19**), 3,3',4-tri-*O*-methylellagic acid 4'-*O*-β-D-glucopyranoside (**20**) and and gallic acid (**21**) posed significant inhibition on MCF 7 cell line at concentrations of 10-100 µM (Figure 3.6.3.3). It was also observed that the inhibitory activity on MCF 7 cell line was higher for 3'-*O*-methyl-3,4-methylenedioxyellagic acid 4'-*O*-β-D-glucopyranoside (**19**) than that of 3,3',4-tri-*O*-methylellagic acid 4'-*O*-β-D-glucopyranoside (**20**), conversely to their inhibition on CaSki and HCT 116 cell lines.

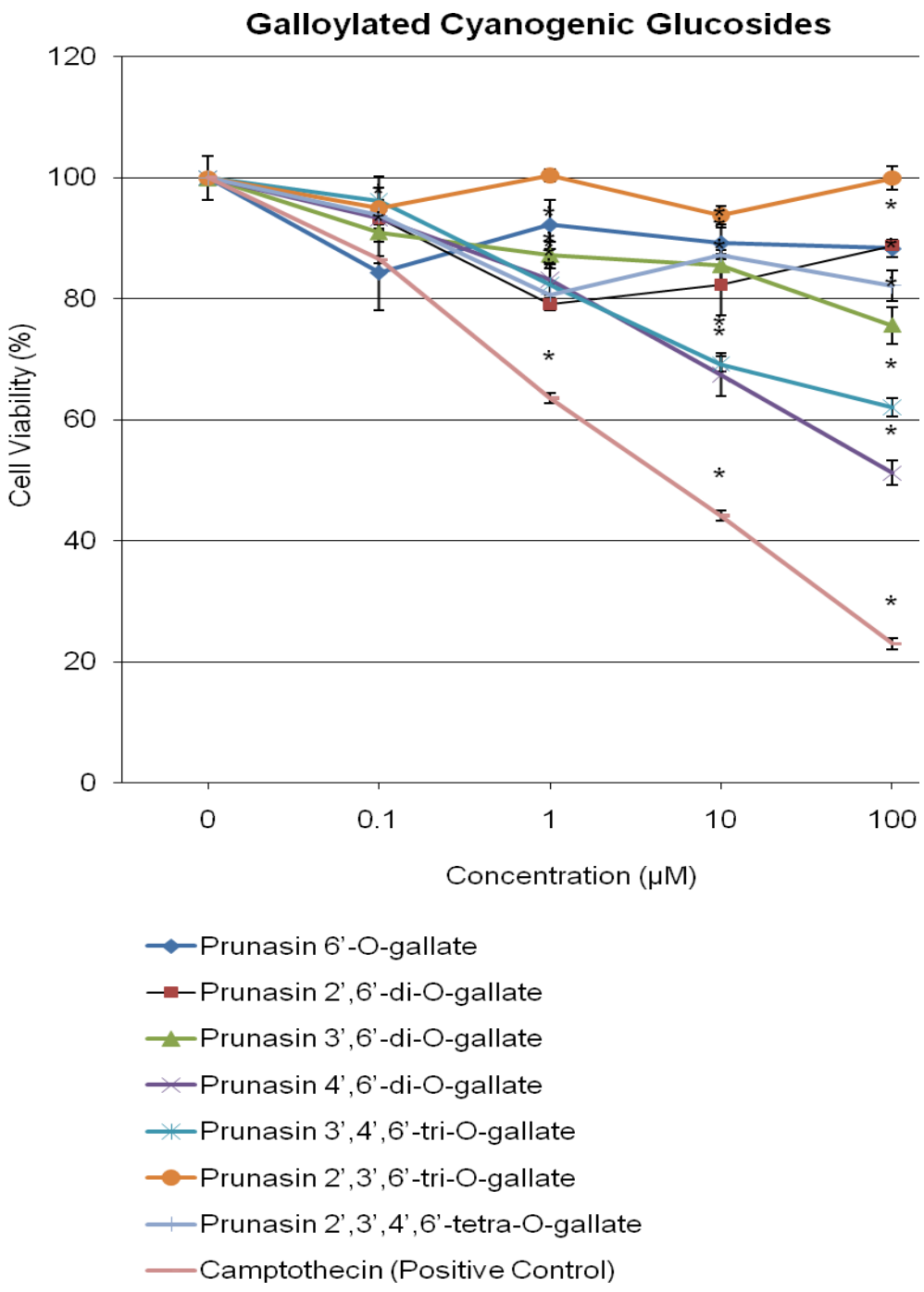


Figure 3.6.3.1: *In vitro* cytotoxicity on MCF 7 cell line of galloylated cyanogenic glucosides (**1-7**). * $P < 0.05$ was considered to be significant.

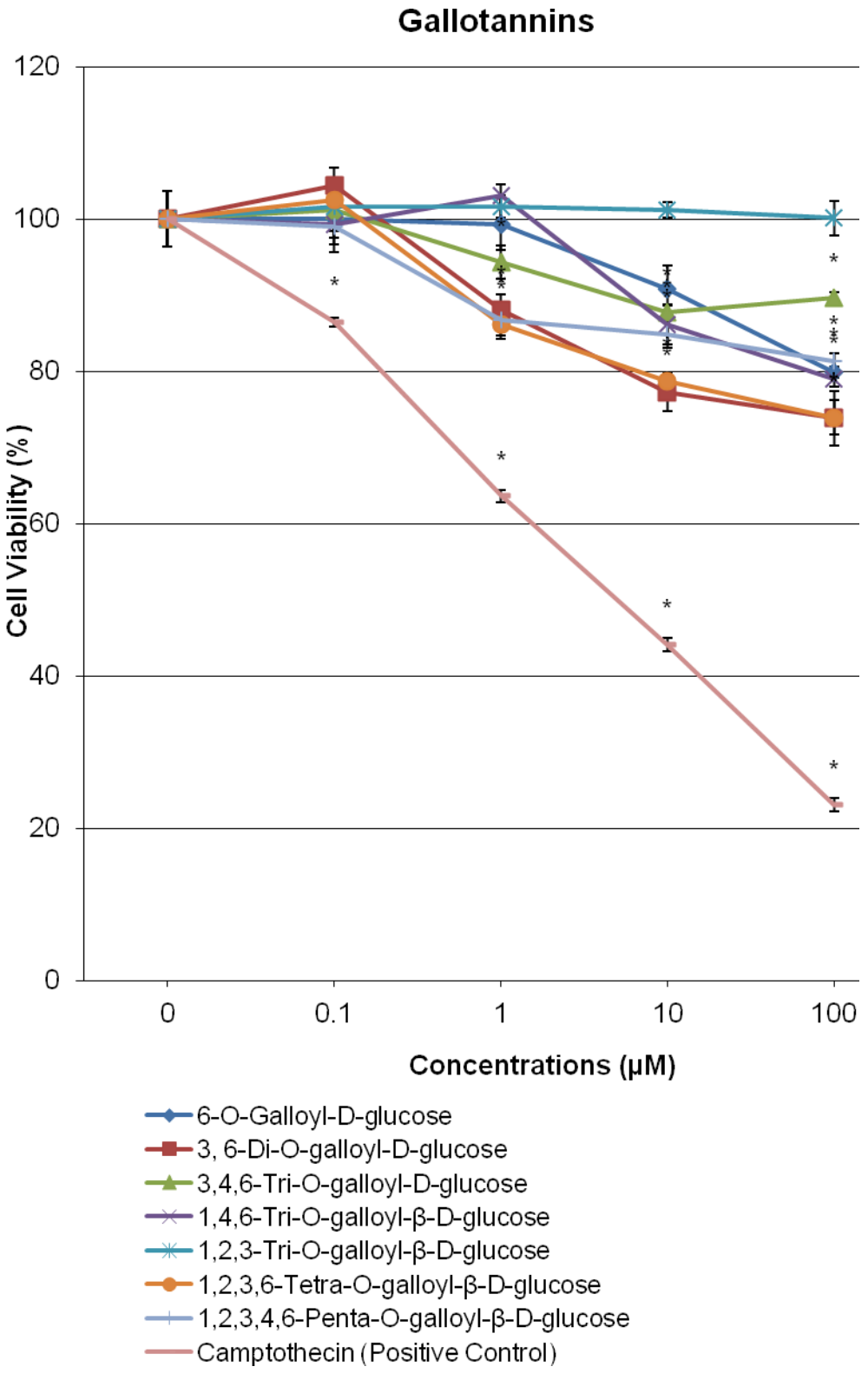


Figure 3.6.3.2: *In vitro* cytotoxicity on MCF 7 cell line of gallotannins (**8**-**14**). * $P < 0.05$ was considered to be significant.

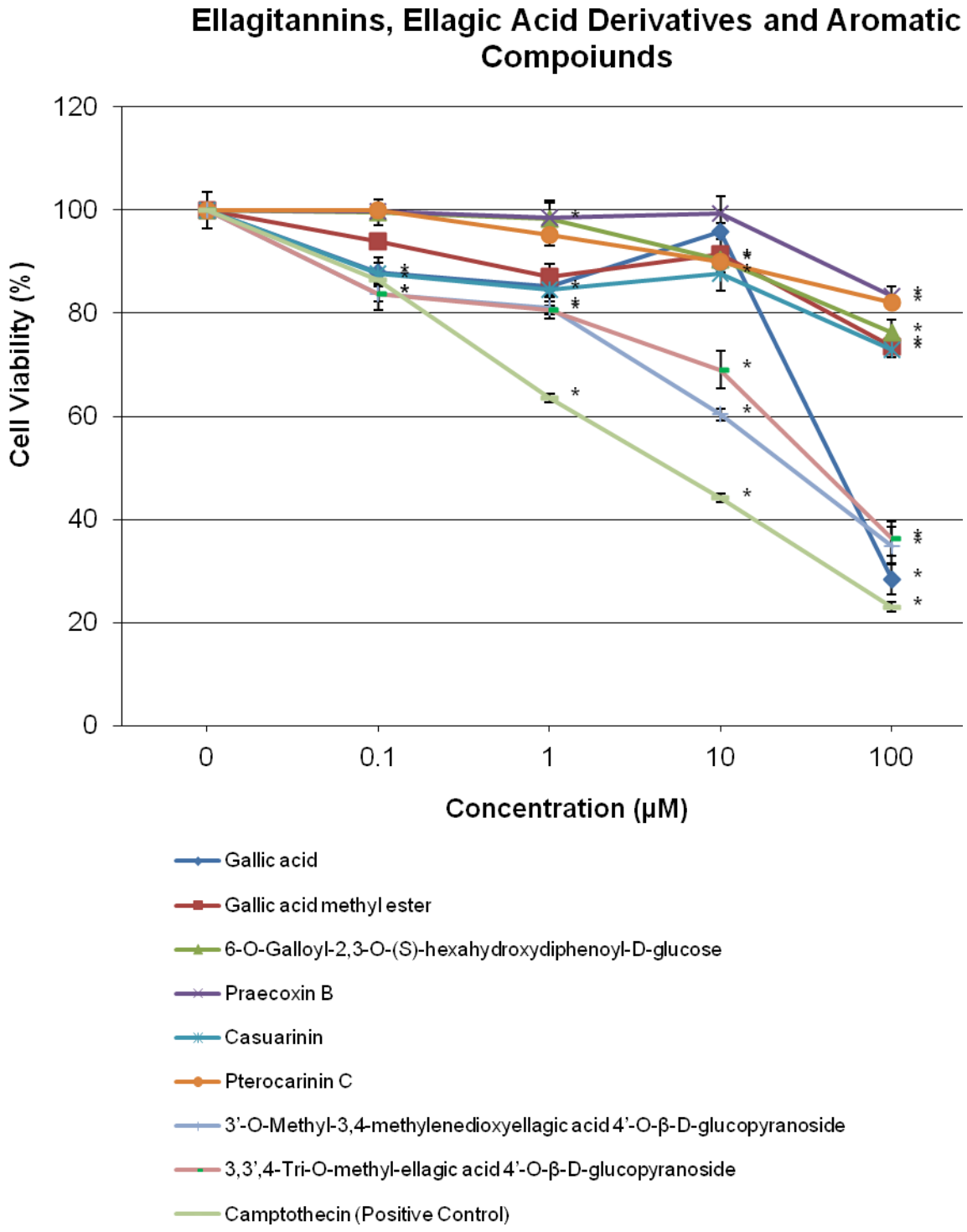


Figure 3.6.3.3: *In vitro* cytotoxicity on MCF 7 cell line of ellagitannins (**15-18**), ellagic acid derivatives (**19-20**) and aromatic compounds (**21-22**). **P* < 0.05 was considered to be significant.

3.6.4 *In vitro* Cytotoxic IC_{50} Values against Cancer Cell Lines

The IC_{50} values of compounds (**1**-**22**) isolated from the leaves of *P. rotundifolia* and *P. praetermissa* on CaSki cell line (cervical epidermoid carcinoma), HCT 116 cell line (colon carcinoma) and MCF 7 cell line (breast carcinoma) are given in Table 3.6.4.1. In the experiments, camptothecin (**23**) was used as the positive control to compare the *in vitro* cytotoxic activity. Cell viability of less than 50 % was considered as having a positive growth inhibition whereas more than 50 % was considered as having a negative growth inhibition. The galloylated cyanogenic glucosides (**1**-**7**) and hydrolysable tannins (**8**-**18**) did not show significant inhibition on the proliferation of CaSki and MCF 7 cell lines which indicated that these compounds have no cytotoxic effect at the concentrations used. However, 3'-*O*-methyl-3,4-methylenedioxyellagic acid 4'-*O*-β-D-glucopyranoside (**19**), 3,3',4-tri-*O*-methylellagic acid 4'-*O*-β-D-glucopyranoside (**20**) as well as gallic acid (**21**) displayed comparatively lower IC_{50} values and exhibited significant inhibition on the three cell lines. The DNA damaging activity of 3'-*O*-methyl-3,4-methylenedioxyellagic acid 4'-*O*-β-D-glucopyranoside (**19**) and 3,3',4-tri-*O*-methylellagic acid 4'-*O*-β-D-glucopyranoside (**20**) has been studied by Xu *et al.* in 2003. The *in vitro* anti-proliferative, apoptotic and anti-oxidant activities of ellagic acid have been extensively studied (Losso *et al.*, 2004; Seeram *et al.*, 2005; Larrosa *et al.*, 2006; Fjaeraa and Nånberg, 2009; Strati et al, 2009) as compared to its derivatives (Ito *et al.*, 2002). Ellagic acid reduced the cancer cell viability by decreasing the ATP levels of the cells (Losso *et al.*, 2004). However the apoptosis induction of ellagic acid derivatives remains unclear. The gallic acid also been shown to exhibit suppression on the growth of DU145 prostate cancer cells (Chen *et al.*, 2009). Several studies have proposed that a combination of ellagitannins gave better inhibitory activity than a single compound alone in the cell culture (Losso *et al.*, 2004; Seeram *et al.*, 2005; Heber, 2008). In the study, casuarinin (**18**) did not considerably inhibit CaSki and

MCF7 cell lines. However, the study by Yang *et al.* in 2000 has reported that it significantly inhibited human promyelocytic leukemia cell line (HL-60) and demonstrated less cytotoxicity on human adenocarcinoma cell line (SK-HEP-1), normal human lymphocytes and Chang liver cells.

Table 3.6.4.1: *In vitro* cytotoxic IC_{50} values on CaSki, HCT 116 and MCF 7 cell lines of compounds isolated from the leaves of *P. rotundifolia* and *P. praetermissa*.

		IC_{50} (µM)		
No.	**Compounds**	**CaSki**	**HCT 116**	**MCF 7**
1	Prunasin 6'-*O*-gallate	>100	>100	>100
2	Prunasin 2',6'-di-*O*-gallate	>100	77.32	>100
3	Prunasin 3',6'-di-*O*-gallate	>100	99.21	>100
4	Prunasin 4',6'-di-*O*-gallate	>100	65.73	>100
5	Prunasin 2',3',6'-tri-*O*-gallate	>100	65.05	>100
6	Prunasin 3',4',6'-tri-*O*-gallate	>100	83.13	>100
7	Prunasin 2',3',4',6'-tetra-*O*-gallate	>100	75.52	>100
8	6-*O*-Galloyl-D-glucose	>100	>100	>100
9	3,6-Di-*O*-galloyl-D-glucose	>100	82.65	>100
10	1,2,3-Tri-*O*-galloyl-β-D-glucose	>100	>100	>100
11	1,4,6-Tri-*O*-galloyl-β-D-glucose	>100	78.93	>100
12	3,4,6-Tri-*O*-galloyl-D-glucose	>100	81.39	>100
13	1,2,3,6-Tetra-*O*-galloyl-β-D-glucose	>100	68.54	>100
14	1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose	>100	77.97	>100
15	6-*O*-Galloyl-2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose	>100	84.79	>100
16	Praecoxin B	>100	70.27	>100
17	Pterocarinin C	>100	72.89	>100
18	Casuarinin	>100	69.94	>100
19	3'-*O*-Methyl-3,4-methylenedioxyellagic acid 4'-*O*-β-D-glucopyranoside	60.39	53.54	46.61
20	3,3',4-Tri-*O*-methylellagic acid 4'-*O*-β-D-glucopyranoside	52.28	47.14	62.30
21	Gallic acid	60.92	88.43	71.22
22	Gallic acid methyl ester	>100	>100	>100
23	Camptothecin (Positive control)	7.71	1.12	7.30

3.7 INHIBITION OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA)

Twenty two compounds were evaluated for their inhibitory potential against *Staphylococcal* isolates. In the experiment, ATCC 33591 (standard MRSA), ATCC 25923 (standard MSSA) as well as two clinical MRSA isolates: N441 and U949 were used to determine the minimum inhibitory concentration (MIC) values. The MIC values of these galloylated cyanogenic glucosides (**1**-**7**), gallotannins (**8**-**14**), ellagitannins (**15**-**18**), ellagic acid derivatives (**19**-**20**) and phenolic acid (**21**-**22**) are shown in Table 3.7.1. According to El-Deeb *et al.* (2003) and Stavri *et al.* (2007), MIC value >200 µg/mL was considered as weak anti-bacterial agent. The result showed that prunasin 2',3',4',6'-tetra-*O*-gallate (**7**) has remarkable inhibition on isolates ATCC 33591 and ATCC 25923 at 125 µg/mL and on local isolates N441 and U949 at 250 µg/mL as compared to the other compounds. These prunasin based cyanogenic glucosides (**1**-**7**) have not been reported for their anti-microbial potentials. Similar MIC values were observed for compounds **13**, **14**, **17** and **18** against several of the test isolates. Both 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (**14**) and pterocarinin C (**17**) inhibited isolate ATCC 33591, N441, U949 and ATCC 25923 at 250 µg/mL. At the concentration of 250 µg/mL, 1,2,3,6-tetra-*O*-galloyl-β-D-glucose (**13**) inhibited isolates ATCC 33591 and ATCC 25923. Casuarinin (**18**) inhibited *Staphylococcal* isolates ATCC 33591 at 250 µg/mL and isolates N441, U949 and ATCC 25923 at 500 µg/mL concentration. Although the anti-microbial activity of these compounds were not notable but ellagitannins have been claimed for synergistic effects with antibiotics against antibiotic-resistant bacteria (Okuda and Ito, 2011). The inhibitory activity of compounds **7**, **13**, **14**, **17** and **18** could be associated to the esterified linkage between sugar and galloyl groups. The highly galloylated compounds have higher inhibitory effect against MRSA as

compared to compounds with fewer galloyl groups. Gallic acid (**21**) is a phenolic acid which exerted inhibitory activity on ATCC 33591, N441, U949 and ATCC 25923 at 500 µg/mL. Gallic acid methyl ester (**22**) was formed by substitution of carboxyl group in gallic acid (**21**). Although both compounds have similar chemical structure, gallic acid methyl ester (**22**) exhibited higher MIC value than gallic acid (**21**). This might be due to polarity effect in anti-MRSA activity. The remaining 16 compounds showed the MIC values of > 500 µg/mL for the ATCC 33591, N441, U949 and ATCC 25923. Most of the compounds except prunasin 2',3',4',6'-tetra-*O*-gallate (**7**) exhibited moderate effect on anti-MRSA activity. This prunasin 2',3',4',6'-tetra-*O*-gallate (**7**) has the potential as anti-MRSA agent but its structural-activity relationship (SAR) remained unclear. Further investigation such as cytotoxicity and bioavailability would be crucial and necessary in order to enhance and optimize the anti-MRSA activity.

Table 3.7.1: MIC values of compounds against *Staphylococcal* isolates.

No.	Compounds	MIC (µg/mL)			
		ATCC 33591	**ATCC 25923**	**N441**	**U949**
1	Prunasin 6'-*O*-gallate	>500	>500	>500	>500
2	Prunasin 2',6'-di-*O*-gallate	>500	>500	>500	>500
3	Prunasin 3',6'-di-*O*-gallate	>500	>500	>500	>500
4	Prunasin 4',6'-di-*O*-gallate	>500	>500	>500	>500
5	Prunasin 2',3',6'-tri-*O*-gallate	>500	>500	>500	>500
6	Prunasin 3',4',6'-tri-*O*-gallate	>500	>500	>500	>500
7	Prunasin 2',3',4',6'-tetra-*O*-gallate	125	125	250	250
8	6-*O*-Galloyl-D-glucose	>500	>500	>500	>500
9	3, 6-Di-*O*-galloyl-D-glucose	>500	>500	>500	>500
10	1,2,3-Tri-*O*-galloyl-β-D-glucose	>500	>500	>500	>500
11	1,4,6-Tri-*O*-galloyl-β-D-glucose	>500	>500	>500	>500
12	3,4,6-Tri-*O*-galloyl-D-glucose	>500	>500	>500	>500
13	1,2,3,6-Tetra-*O*-galloyl-β-D-glucose	250	250	>500	>500
14	1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose	250	250	250	250
15	6-*O*-Galloyl-2,3-*O*-(*S*)-hexahydroxydiphenoyl-D-glucose	>500	>500	>500	>500
16	Praecoxin B	>500	>500	>500	>500
17	Pterocarinin C	250	250	250	250
18	Casuarinin	250	500	500	500
19	3'-*O*-Methyl-3,4-methylenedioxyellagic acid 4'-*O*-β-D-glucopyranoside	>500	>500	>500	>500
20	3,3',4-tri-*O*-Methylellagic acid 4'-*O*-β-D-glucopyranoside	>500	>500	>500	>500
21	Gallic acid	500	500	500	500
22	Gallic acid methyl ester	>500	>500	>500	>500

3.8 CONCLUSIONS

The chemical constituents from the leaves of *P. rotundifolia* and *P. praetermissa* were characterised using Liquid Chromatography Mass Spectrometry (LC-MS), Fourier Transform Infrared (FTIR) spectroscopy and High Performance Liquid Chromatography (HPLC). A total of 22 compounds were isolated from the two *Phyllagathis* species and their identities were confirmed by NMR experiments as well as the MS^n analysis. The fragmentation schemes obtained for the galloylated cyanogenic glucosides, gallotannins, ellagitannins and ellagic acid derivatives would contribute to the MS^n information which is useful for their identification or enable identification of similar metabolites in complex samples for a more complete fingerprinting of plant materials.

Apart from that, the multi-steps IR macro-fingerprinting consisting of 1D-IR, second derivative spectra and 2D-correlation IR provided a more recent, rapid and non-destructive tool for identification and discrimination of *P. rotundifolia* and *P. praetermissa* collected from different localities. The 2D-correlation IR analysis provided additional information on their similarities and dissimilarities under thermal perturbation. The IR spectra combined with PCA also afforded an alternative approach for herbal quality control.

Chromatographic analysis of plant extracts has been routinely utilised in the quality control of raw materials as well as in the pharmaceutical and natural product industries. In this study, the HPLC fingerprints coupled with PCA were able to characterise *P. rotundifolia* and *P. praetermissa* more effectively as well as to assess the intra-species variation with respect to location. The HPLC chromatographic profiles of *P. rotundifolia* collected from Sungai Batang and Takar Melor were quite different from that of the Pasoh

Forest Reserve despite of the same species. The chemical analysis of both species showed the presence of hydrolysable tannins and ellagic acid derivatives. Two major compounds, 3'-*O*-methyl-3,4-methylenedioxyellagic acid 4'-*O*-β-D-glucopyranoside (peak **15**) and 3,3',4-tri-*O*-methylellagic acid 4'-*O*-β-D-glucopyranoside (peak **16**) were consistently present in the chemical profiles. Comparatively, these compounds appeared at relatively lower concentration in *P. praetermissa*. The differences in chemical profiles might be contributed by factors such as variation in soil and climate, harvesting time, maturation process and storage time of the plant material. The LC-MS analysis provided MS^n data that are useful in the identification of the unknown chromatographic peaks without going through tedious isolation step. The results of this study have demonstrated the usefulness of the spectroscopic and chromatographic fingerprints as the latest approach that could be accepted in the quality control and authentication of herbal materials.

The biological activities of the 22 compounds were evaluated with respect to their neuroprotectivity, *in vitro* cytotoxicity on CaSki (cervical epidermoid carcinoma), HCT 116 (colon carcinoma) and MCF 7 (breast carcinoma) and inhibition of methicillin-resistant *Staphylococcus aureus* (MRSA). Overall the galloylated cyanogenic glucosides, gallotannins, ellagitannins and ellagic acid derivatives significantly protected the NG108-15 cells against H_2O_2-induced cell damage. High neuroprotective activities were exhibited by 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (**14**), pterocarinin C (**17**) and prunasin 2',3',4',6'-tetra-*O*-gallate (**7**). However, all the compounds were found to inhibit HCT 116 cell line only slightly while no significant inhibition was noted on CaSki cell line and MCF 7 cell line. Generally, the ellagic acid derivatives (**19** and **20**) as well as gallic acid (**21**) demonstrated lower IC_{50} values as compared to other compounds indicating higher inhibitory effect on CaSki, HCT 116 and MCF 7 cell lines. For the anti-MRSA activity, prunasin 2',3',4',6'-tetra-*O*-gallate (**7**) has the lowest MIC values followed by 1,2,3,4,6-

penta-*O*-galloyl-β-D-glucose (**14**) and pterocarinin C (**17**). Thus, the prunasin 2',3',4',6'-tetra-*O*-gallate (**7**) may be further explored as potential anti-microbial agent.

In conclusion, the combination and interpretation of scientific data from various chemical and biological analyses is a more comprehensive approach to characterize the plants used in the traditional medicines. The exploitation of chemometrics in the analysis of huge number of data greatly improved the feasibility and efficiency of data interpretation. The results of the biological activity evaluation on the various groups of compounds have brought forth the relationship between the chemical structures and biological activities which are directly associated with their medicinal properties. Further investigation and exploration is necessary to clarify the underlying mechanisms of these naturally occurring bioactive compounds.